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Study of the Potential Application of Lactic Acid Bacteria in the Control of Infection Caused by *Agrobacterium tumefaciens*

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Abstract

Inhibition of crown gall on test plants in case of co-inoculation with lactic acid bacteria (LAB) has been investigated. From nine LAB strains tested, eight reduced amount of galled carrot explants by 36.4-87.7% and decreased the intensity of disease manifestation. The antagonistic activity against *Agrobacterium tumefaciens*, *in vitro*, was due to the low pH of organic acids produced by LAB. However, in the same pH, different LAB cultures displayed various levels of inhibition *in vivo*. *Lactobacillus plantarum* ONU 12 with the best results in tumor inhibition on carrots, showed high antagonistic activity on surfaces of kalanchoe and grapevines. Depending on the method of inoculation, the culture of *L. plantarum* ONU 12 could protect from 72.7% to 100% of wounded kalanchoe tissues. Evaluation of number of surviving cuttings and amount of buds that grew indicated that co-inoculation with agrobacteria and LAB removed completely the negative influence of phytopathogen on grapevines and reduced the number of infected cuttings by approximately 80%. One-hour treatment with *L. plantarum* ONU 12 helped to decrease the number of infected plants by approximately 68%. The studied strain *L. plantarum* ONU 12 can be proposed for further evaluation of possibility of practical use in plant protection.

Keywords: Antagonism; Crown gall; *Lactobacillus; Enterococcus*

Introduction

Lactic acid bacteria (LAB) are widely used in many fields of human activity and especially in food preservation and in medicine. Some authors have proposed to apply lactobacilli for improvement of crop plant yields and for protection of agriculturally important species against certain phytopathogens. Visser et al. [1] reported antagonistic action of *Lactobacillus plantarum* L292 against *Pseudomonas syringae* observed *in vivo* on haricot beans resulting in significant reduction of disease symptoms. Inhibition of *Xanthomonas campestris* growth with *L. plantarum* strains was described by Trias et al. [2] and Dalirsaber Jalali et al. [3]. Other LAB such as *Enterococcus mundtii* suppressed the growth of *Erwinia carotovora*. Bacterial mixtures containing mostly lactobacilli were efficient against *Ralstonia solanacearum* [4].

Recent publications described also the antimycotic effects of LAB. These effects are often species- and strain-specific. For instance, certain strains of *Weissella cibaria, L. plantarum, Leuconostoc mesenteroides* and *Lactococcus lactis* reduced rot caused by *Penicillium expansum* in wounds of stored apples while other tested strains did not exhibit such activities [2]. El-Mabrok et al. [5] reported the inhibition of phytopathogenic fungi *Colletotrichum gloeosporioides* by strains of *L. plantarum*. Lactobacilli were used for decreasing disease symptoms or production of metabolites responsible for food intoxication caused by *Fusarium* [6], *Aspergillus flavus* [7], *Aspergillus ochraceus* [8], *Aspergillus niger* and *Penicillium expansum* [9].

The main metabolites from LAB found to be active against phytopathogens were organic acids and hydrogen peroxide; the microbial competition was also described [2,7,10].

Interesting results of aforementioned authors stimulated to study the possibilities of use of LAB against known phytopathogen *Agrobacterium tumefaciens* (*Rhizobium radiobacter* according to the nomenclature proposed by Young et al. [11], which can be devastating for the nurseries of stone fruits and ornamental plants. The symptoms of the caused disease (crown gall) are tumor formations on stems and roots of infected plants resulting in deficiency of nutrients and water supply (reviewed in Burr and Otten, [12]). Tissue proliferations induced by pathogenic agrobacteria also include cane gall caused by *A. rubi* (*R. rubi*) and hairy-root as the result of infection with *A. rhizogenes* (*R. rhizogenes*) [11,13]. Strains of *A. tumefaciens* biotype 3 were reclassified as a distinct species *A. vitis* (*R. vitis*) on the basis of their host (*Vitis spp.*) and their genetic peculiarities [11,14]. Besides by *A. vitis*, crown gall on grapevine in some cases can be caused by *A. tumefaciens* biotype 1 strains [15,16].

The pathogenesis of crown gall is unique and includes the transfer of the part of Ti-plasmid from *A. tumefaciens* into the chromosome of the plant [17]. As the result, plant cells start to produce an increased amount of hormones leading to uncontrolled tissue proliferation [18] and to synthesize the unusual compounds such as are opines – derivatives of sugars and special amino acids used by bacteria as nutritional sources [19,20].

Representatives of several bacterial genera have been used for efficient biocontrol of crown gall. Some avirulent agrobacteria produce highly specific bacteriocin agrocin as *A. rhizogenes* K84 does – the

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most widely used antagonistic strain against *A. tumefaciens* [21]. The genes responsible for agrocin synthesis and self-immunity are localized on a plasmid, which can be transferred into pathogenic strains and make them resistant [22]. To overcome biocontrol failure, a stable Tra$^-$ deletion mutant of K84 – the strain K1026 was constructed [23]. Biopreparations based on K84 have been traditionally used for many years [20,24]. Being highly effective in many dicotyledonous plants, *A. rhizogenes* K84 does not affect crown gall agents in grapevines [25]. One of the most active antagonists decreasing level of tumour formation in grape is *A. vitis* F2/5 [26-28]. Its inhibitory effect cannot be explained by agrocin production and competition for attachment sites but it is directly related to interactions with grapevine cells resulting in cambium necroses resembling hypersensitivity response [29-31]. *A. vitis* F2/5 does not affect the growth of pathogen population in plant tissue but inhibits the tissue transformation [32].

Several other efficient antagonists strains belonging to species *A. vitis* are known. *A. vitis* E26 effectively inhibited crown gall on peach and cherry caused by *A. tumefaciens* and on grapevine caused by *A. vitis* [33]. The antagonistic effect was explained by bacteriocin production [34,35].

A. radiobacter HLB-2 supressed grape crown gall by bacteriocin production and competing for attachment sites and nutrients [36,37]. *A. vitis* VAR03-1 has shown a significant reduction effect of gall formation in tomatoes, roses and grapevines [38,39]. Recently described novel antagonistic strain *A. vitis* ARK-1 reduced the tumor incidence in grapevine plants and stably survived on roots [40].

The other important antagonists against crown gall agents belong to *Pseudomonas* genus. Crown gall formation on grapevine and raspberry was efficiently inhibited with *P. aureofaciens* and *P. fluorescens* [41,42]. The similar clear antagonistic activity was revealed in *P. corrugate* isolated from grapevine xylem sap. Besides this bacterium, the inhibitory effect against *A. vitis* was demonstrated by strains of *Enterobacter agglomerans* and *Rahnella aquatilis* isolated from the same source [43]. It was revealed also that a specific antimicrobial compound with wide spectrum of action was responsible for the suppressive effect of *Rahnella aquatilis* [44]. Treatments with *Pseudomonas putida*, *Burkholderia phytofirmans* and *Azospirillum brazilense* strains producing 1-aminocyclopropane-1-carboxylate deaminase, which degrades the precursor of ethylene in plants, inhibited the tumor formation in tomatoes [45].

Bacilli also have been investigated as possible biocontrol agents against crown gall. *Bacillus subtilis* and *Bacillus* spp. reduced gall size in treated plants and densities of internal populations of *R. vitis* [42]. A biopreparation based on *B. subtilis* could significantly reduce disease incidence. Additional use of resistance inducers for plants was strongly recommended to decrease crown gall severity under the field conditions [46].

Trials of using LAB against *A. tumefaciens* described in literature have not been found, although this trend of biological control in plant protection is very attractive because of some important characteristics of these agents, such as their capability of inhibiting other microorganisms by competition and production of antimicrobial compounds such as organic acids and bacteriocins. Besides some LAB, such as *Lactobacillus plantarum*, are considered by the Food and Agriculture Organization (FAO) as GRAS (Generally Recognized As Safe) for the application in biopreservation systems.

Hence, the aim of this work was to test *in vivo* the antagonistic activity of some LAB strains isolated from various sources against *A. tumefaciens*.

Materials and Methods

Bacterial strains

The tested LAB strains originated from the collection of cultures of Microbiology, Virology and Biotechnology Chair of ONU, Odessa, Ukraine and from the collection of BIA-FIP laboratory, INRA, Nantes, France (Dr. S. Migaw and Dr. M. Barbosa). Pathogenic strain *A. tumefaciens* C58 was kindly provided by the collection of microorganisms of D.K. Zabolotny Institute of Microbiology and Virology (Dr. F.I. Tovkach), Kiev, Ukraine. All LAB strains were stored in MRS broth [47] and *A. tumefaciens* C58 in Luria Bertani (LB) broth [48] at -80°C with 20% glycerol. Strains *L. plantarum* ONU 87, ONU 206 and ONU 991 were isolated from dairy products and *L. plantarum* ONU 12, ONU 311, ONU 312, ONU 313 from grape berries collected in Ukraine; *Enterococcus faecium* C8 was isolated from Azerbaijan cheese, and *Enterococcus durans* 3y from Tunisian fish.

Inoculation of carrot explants

Carrots (*Daucus carota* L.) were purchased on local markets of France and Ukraine, washed with commercial "Javel" (sodium hypochlorite) solution, rinsed in tap water, immersed in ethanol, flamed, peeled and sliced in discs [49]. The disks were placed in sterile Petri dishes. *A. tumefaciens* was cultivated overnight in LB broth at 28°C, and the final concentration of cells reached up 2-8 x 10^9 CFU/mL. LAB cell suspensions were obtained by inoculation of each strain in MRS broth followed by incubation overnight at 37°C (final concentration 1-5 x 10^{10} CFU/mL). The inoculum was obtained by mixing the culture of *A. tumefaciens* with the LAB cultures in equal volumes (1/1). To inoculate the carrots, 100 µL of the mixture were applied on the basal surfaces of the disks. Besides mixtures "*A. tumefaciens* C58/LAB", agrobacterial culture mixed with sterile saline solution (0.85% NaCl, w/v) at a ratio 1:1 was applied on explants as a positive control, and LAB cultures – as negative controls. After 21 days, explants were observed for the presence of tumors and fermentation. Amount of galled explants was calculated and manifestation of crown gall symptoms was evaluated by the modified method of Ryder et al. [48] as follows: "++++" 100% cambial ring covered with tumors; "+++" 75% of cambial ring with tumors; "++" 50% of cambial ring with tumors; "+" less than 25% of cambial ring with tumors.

Production of antimicrobial metabolites

Cell Free Supernatant (CFS) instead of bacterial cultures was applied on carrot explants together with agrobacteria. LAB strains with the best results in the previous experiments (*L. plantarum* ONU 311, *L. plantarum* ONU 312, *L. plantarum* ONU 12, *E. faecium* C8 and *E. durans* 3y) were used. To obtain the CFS, LAB were grown in MRS broth at 37°C for 24 h, and cells were harvested by centrifugation (8000 g, 4°C, 10 min).

The production of antimicrobial compounds was also evaluated *in vitro* by agar-well diffusion assay according to Batdorj et al. [49] using CFS of the same LAB strains applied in the *in vivo* tests. CFS was obtained after incubation of LAB at 30°C and 37°C for 24 h in MRS broth. For the agar-well diffusion test, Brain Heart Infusion (BHI) soft agar (0.8%, w/v) or LB soft agar (0.8% agar, w/v) were inoculated with 10^6 CFU/mL of the indicator strain, *L. ivanovii* ATCC 19119 (as a classic test strain for the study of LAB bacteriocinogenic activity) or *A. tumefaciens* C58, respectively. The concentrations of bacterial

cultures were assesed spectrophotometrically and serial dilutions were carried out to obtain the needed concentrations of cells. 50 µL of CFS with initial acidic pH and pH adjusted to neutral with 1 N NaOH were poured into wells made on the surface of the plates containing each indicator strain. After 24 h of incubation at 37°C for *L. ivanovii* ATCC 19119 or at 28°C for *A. tumefaciens*, the presence of inhibition zones was observed [50].

Co-incubation of agrobacteria in a mixture with LAB suspensions with different initial pH (4.1-4.5 and 5.0-5.5) during one hour was carried out at 28°C. After the incubation period, bacterial mixtures were diluted ten-fold, plated on LB medium, incubated overnight and colonies of agrobacteria were counted.

Inoculation of *Kalanchoe daigremontiana* Mill

Five methods of inoculation were applied. (1) 50 µl of *L. plantarum* ONU 12 overnight culture were injected together with 50 µl of agrobacterial overnight culture into upper tissues of leaves by sterile syringe. *Agrobacterium tumefaciens* culture with equivalent volumes of sterile distillated water (SDW) were injected as positive controls. The culture of lactobacilli was applied as a negative control. (2) 100 µl of a mixture "*A. tumefaciens* C58/*L. plantarum* ONU 12" were spotted on one-cm wounds made on leaves. (3) Wounds were treated with agrobacteria and after 30 min with *L. plantarum* ONU 12 culture. (4) Scars on leaves were treated with LAB and after 30 min inoculated with the phytopathogen. (5) Roots and the aerial parts (crowns) of plants were wounded and dipped for one hour in agrobacterial culture (positive control), SDW, culture of *L. plantarum* ONU 12 (negative controls) and in the mixture "C58/ONU 12". Plants were cultivated under greenhouse conditions. Leaves were observed for crown gall symptoms on the 60th day after inoculation. Treated roots and crowns were observed after six months. Tumor tissues were excised and weighted.

Inoculation of grapevine cuttings

Cuttings of *Vitis vinifera* L. cv. Pinot noir, Vostorg, Moldova (in equal quantities each) cultivated in the south of Ukraine were gathered during March of 2011-2013. Cuttings with freshly cut basal parts soaked for one hour in agrobacterial culture were used as positive controls. Cuttings were also treated for one hour with *L. plantarum* ONU 12 culture and with the mixture "C58/ONU 12". One variant of the treatments was soaking for one hour in *L. plantarum* ONU 12 culture and after inoculation with *A. tumefaciens* C58 culture for 15 min.

As negative controls, cuttings soaked for one hour in tap water were brought to assay. Other negative controls were MRS (pH 4.1) and a mixture "MRS/LB" (1:1) with pH 5.5-6.0 indicating the effect of nutritional media with pH of subsequent bacterial cultures on grape cutting development.

Cuttings were planted in commercial pot soil with abundance of peat and cultivated under greenhouse conditions for 30 days. After, amount of surviving cuttings and number of buds that grew were evaluated as percentages from the total quantity of tested cuttings and buds. Green shoots on survived cuttings were measured, and mean lengths were calculated in each variant. Cuttings were tested for the presence of pathogens by a bacterial culture method followed by polymerase chain reaction (PCR) assay with the primers to *ipt* oncogene of pathogenic agrobacteria according to Haas et al. [51].

Statistical analyses

Carrots were inoculated in three independent experiments with 20-22 explants in each variant for bacterial cultures and CFS. For kalanchoe inoculation, a total of 90 plants of each variant was used in three independent experiments. 30-50 grape cuttings were treated in each variant in three independent experiments carried out during springs of 2011-2013. Agar-well diffusion assay was carried out in five repeats for each variant. CFU/mL in bacterial suspensions were evaluated by counting colonies grown in five repeats. The obtained results were presented in percentages and standard errors (SE) for qualitative attributes (number of infected plants and explants with necroses, amount of buds that grew) and in mean values with 95% confidence intervals (CI) for quantitative attributes (lengths of green shoots, amount of CFU/mL). Significant differences between the control and test samples were estimated in *t*-test ($p < 0.05$) and marked in the tables with data. Software "Microsoft Excel" was used for calculations and graphics.

Results

Tests on carrot explants

The best results for tumor growth inhibition on carrot discs co-inoculated with *A. tumefaciens* C58 and LAB cultures were obtained with 5 strains from 9 tested. *L. plantarum* ONU 12, *E. faecium* C8, *L. plantarum* ONU 312, *E. durans* 3y and *L. plantarum* ONU 311 suppressed completely tumor development on the majority of carrot discs, suggesting a high antagonistic activity of the mentioned lactobacilli and enterococci (Table 1).

The reduction in galled samples was observed in case of all tested LAB strains except *L. plantarum* ONU 991 (Figure 1).

The obtained results suggest that the antagonistic activity against *A. tumefaciens* within *L. plantarum* species is strain-specific.

Co-inoculation with LAB cultures shifted the level of crown gall manifestation to smaller area of galled tissue ("+" level compared with the positive control where "++" – "++++" levels were prevalent) (Table 2).

If after the treatments with active antagonistic strain *L. plantarum* ONU 12 some discs still appeared infected, less than 25% of cambial ring on disc were covered with tumors in such explants, while in case of non-active strain *L. plantarum* ONU 991 the range of tissue proliferation resembled that in the positive control.

The fermentation of carrot by some LAB strains occurred but it

Inoculum	Percentage of discs with tumors	Inoculum	Percentage of discs with tumors
A. tumefaciens C58 (positive control)	61.0 ± 6.0	*A. tumefaciens* C58 + *L. plantarum* ONU 311	16.4 ± 4.7^{d}
A. tumefaciens C58 + *L. plantarum* 991	54.5 ± 6.1	*A. tumefaciens* C58 + *E. durans* 3y	13.6 ± 4.2^{d}
A. tumefaciens C58 + *L. plantarum* ONU 313	38.8 ± 5.9^{d*}	*A. tumefaciens* C58 + *L. plantarum* ONU 312	10.7 ± 3.8^{d}
A. tumefaciens C58 + *L. plantarum* ONU 206	30.0 ± 5.9^{d}	*A. tumefaciens* C58 + *E. faecium* C8	9.1 ± 3.5^{d}
A. tumefaciens C58 + *L. plantarum* ONU 87	27.2 ± 5.4^{d}	*A. tumefaciens* C58 + *L. plantarum* ONU 12	7.5 ± 3.2^{d}

Note: ^{*d} - significant differences between values of the control and the test sample ($p<0.05$, t-test)

Table 1: Tumor formation on carrot explants after co-inoculation with A. tumefaciens C58 and LAB (%).

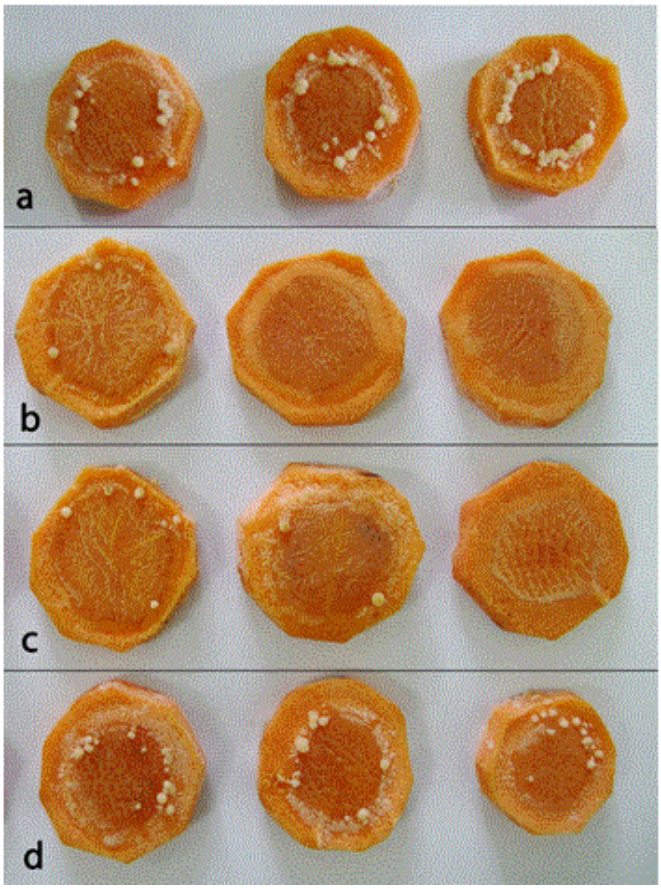

Figure 1: Carrot explants co-inoculated with various bacterial mixtures. a - carrots inoculated only with *A. tumefaciens* C58 (positive control); b - carrots co-inoculated with *A. tumefaciens* C58 and *L. plantarum* ONU 12; c - carrots co-inoculated with *A. tumefaciens* C58 and *L. plantarum* ONU 312; d - carrots co-inoculated with *A. tumefaciens* C58 and *L. plantarum* ONU 991.

Inoculum	+	++	+++	++++
A. tumefaciens C58 (positive control)	30.0	40.0	20.0	10.0
A. tumefaciens C58 + *L. plantarum* 991	38.3	27.7	11.1	22.2
A. tumefaciens C58 + *L. plantarum* ONU 313	84.6	7.7	0	7.7
A. tumefaciens C58 + *L. plantarum* ONU 206	77.8	16.7	0	5.5
A. tumefaciens C58 + *L. plantarum* ONU 87	83.3	5.5	11.1	0
A. tumefaciens C58 + *L. plantarum* ONU 311	60.0	20.0	20.0	0
A. tumefaciens C58 + *E. durans* 3y	55.5	22.2	11.1	11.1
A. tumefaciens C58 + *L. plantarum* ONU 312	85.7	14.3	0	0
A. tumefaciens C58 + *E. faecium* C8	66.6	33.3	0	0
A. tumefaciens C58 + *L. plantarum* ONU 12	100.0	0	0	0

Table 2: Manifestation of crown gall symptoms on carrot discs in presence of LAB (%).

did not attain more than 7.5 ± 3.2% of discs (*E. faecium* C8) and 6.1 ± 2.9% (*L. plantarum* ONU 991), and was not detected in case of *L. plantarum* ONU 12, *L. plantarum* ONU 312, *L. plantarum* ONU 87 and *L. plantarum* ONU 206. Thus, number of fermented discs reached only 4.5 ± 0.5% when *L. plantarum* ONU 313 was applied, 3.0 ± 0.4 in case of *Enterococcus durans* 3y, 1.6 ± 0.2% in case of *L. plantarum* ONU 311. Fermentative activity was less intense in case of the treatments of carrot explants with mixtures "*Agrobacterium*/LAB".

Production of antimicrobial metabolites

When CFS instead of cultures was applied, the results were similar to inoculation with alive LAB suspensions. Thus, treatments of explants with *A. tumefaciens* C58 in a mixture with CFS of *L. plantarum* ONU 12 caused crown gall symptoms only in 10.6 ± 3.6% of explants, in case of *E. faecium* C8 CFS – in 8.3 ± 4.8% of carrot discs, when treated with *L. plantarum* ONU 312 CFS – in 11.6 ± 4.0%, in case of *E. durans* 3y CFS – in 15.0 ± 4.6% of explants and in 18.3 ± 4.9% of discs treated with CFS of *L. plantarum* ONU 311.

In case of the *in vitro* tests of production of antimicrobial compounds, it was observed that none of the strains was able to inhibit *A. tumefaciens* C58 when the CFS pH 6.5 was used. Otherwise, when the acidic CFS was applied into the agar wells, clear zones of inhibition were observed, suggesting that the activity against the phytopathogen was due to the production of organic acids. The pH of the acidic CFS obtained after cultivation of the selected LAB at 30°C or 37°C varied between 4.1 and 4.7 for lactobacilli and 4.7 and 5.0 for enterococci. Incubation of agrobacteria in a mixture with LAB suspension with initial pH 5.0-5.5 caused 1-2 fold decrease in viable pathogen cell quantity. If initial pH of LAB suspensions was 4.1-4.5, one-hour of co-incubation was sufficient for 4-folds decrease in amount of viable cells (Table 3).

The experiments carried out with *L. ivanovii* ATCC 19119 as indicator strain showed that the strains *E. faecium* C8 and *E. durans* 3y inhibited its growth, when incubated at 30°C or 37°C, suggesting the production of antimicrobial metabolites other than organic acids, such as bacteriocins. However, these metabolites were inactive against *A. tumefaciens*.

Tests on *Kalanchoe daigremontiana* Mill

Strain *L. plantarum* ONU 12 with the best result in crown gall inhibition on carrot explants was used for further investigations.

When the mixture of bacterial cultures "C58/ONU 12" was injected in kalanchoe leaf tissues, no tumors were formed in any of repeats (Figure 2).

Treatment of the scars with this mixture resulted in tumor formation just in one case that was evaluated as 1.1% from the total amount of infected plants.

If scars were first infected with *A. tumefaciens* C58 and after 30 min inoculated with LAB culture, the percentage of galled plants decreased in 3.5 times (20.0 ± 4.2% comparing with 73.3 ± 4.6% in positive control) and mean weight of tumors was 4.4 times less (0.0309 ± 0.0181 g comparing with 0.1325 ± 0.0582 g in the positive controls).

Crown gall was not detected on the scars treated with LAB cultures 30 min before inoculation with the pathogen.

Variant	Amount of viable cells (CFU/mL)		
	Before incubation	After incubation	
		pH 5.0-5.5	pH 4.1-4.5
C58 + ONU 311	$(6.4 \pm 0.8) \times 10^9$	$(4.2 \pm 0.7) \times 10^7$	$(1.5 \pm 0.4) \times 10^5$
C58 + ONU 312		$(2.2 \pm 0.5) \times 10^7$	$(4.8 \pm 1.2) \times 10^5$
C58 + ONU 12		$(1.6 \pm 0.3) \times 10^7$	$(5.2 \pm 1.1) \times 10^5$
C58 + ONU 87		$(2.4 \pm 0.8) \times 10^7$	$(2.1 \pm 0.2) \times 10^5$

Table 3: Effect of LAB on the survival of A. tumefaciens C58 cells after one hour of co-incubation.

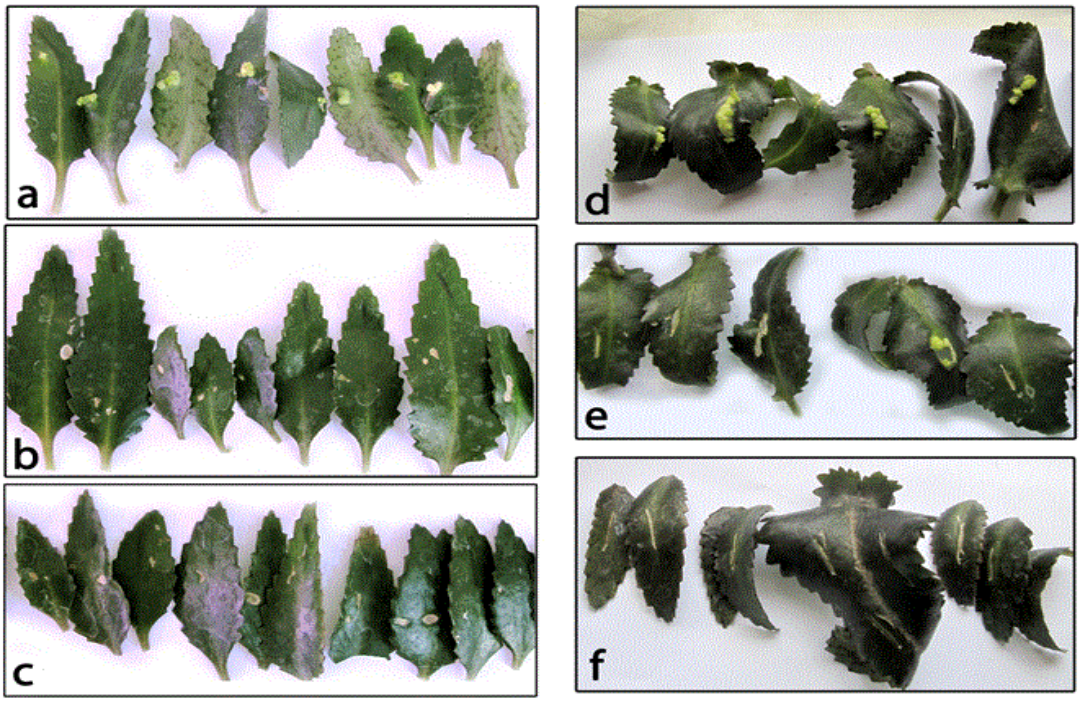

Figure 2: Kalanchoe leaves infected by injections (left) and by scars (right): a, d - inoculated only with *A. tumefaciens* C58; b, e - co-inoculated with *A. tumefaciens* C58 and *L. plantarum* ONU 12; c, f - inoculated with *L. plantarum* ONU 12.

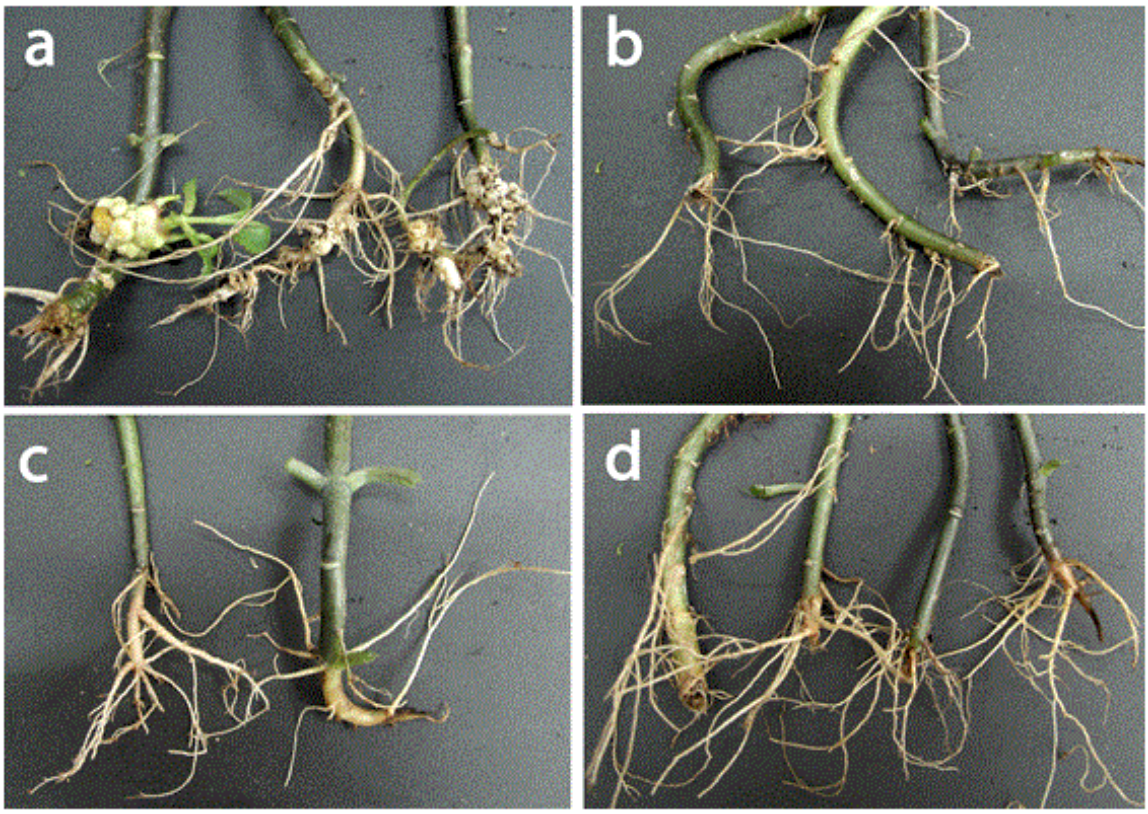

Figure 3: Kalanchoe plants infected via wounded crowns and roots: a - inoculated only with *A. tumefaciens* C58; b - co-inoculated with *A. tumefaciens* C58 and *L. plantarum* ONU 12; c – soaked in water; d - inoculated with *L. plantarum* ONU 12.

After the treatments with a mixture "*A. tumefaciens* C58/*L. plantarum* ONU 12" and in some cases with LAB suspension, necroses were observed but they were restricted to wounded sites.

Treatment with LAB simultaneously with the inoculation with agrobacteria via root system allowed to protect plants in 100% of cases (Figure 3).

The obtained results indicated the high efficacy of using *L. plantarum* ONU 12 to inhibit crown gall on kalanchoe.

Tests on grapevine cuttings

Inoculation with *A. tumefaciens* C58 resulted in the induction of necroses on basal ends of 100% of the treated cuttings in positive controls. 24% of inoculated cuttings died (Figure 4) and the surviving grapevines showed 9.4% smaller amount of buds that grew (Figure 5).

Inoculation with *L. plantarum* ONU 12 had a positive effect on the amount of buds that grew, but the effect was small (11.2%). But the highest stimulating effect on buds that grew was revealed in case of the treatments with a mixture "*A. tumefaciens* C58/*L. plantarum* ONU 12". Soaking the cuttings in the mixture "*A. tumefaciens* C58/*L. plantarum* ONU 12" during 1 h resulted in 45.0% increase in amount of buds that grew. One-hour treatments with LAB culture followed by subsequent inoculation with *A. tumefaciens* C58 during 15 min lead to 35.0% increase. The mixture of nutritional media MRS and LB in the same ratio as in the bacterial cultures did not demonstrate any stimulation of the treated cuttings.

When inoculation with *A. tumefaciens* C58 was carried out simultaneously with the treatment with *L. plantarum* ONU 12 culture, the negative effect of phytopathogen was not observed. All the evaluated characteristics of plants did not differ from that in the negative control (water). Thus, the number of surviving cuttings reached 85.3 ± 3.5% in negative control, 64.8 ± 5.6% in positive controls infected with pathogenic agrobacteria – and 89.7 ± 5.0% in cuttings co-inoculated with *A. tumefaciens* C58 and *L. plantarum* ONU 12. Mean lengths

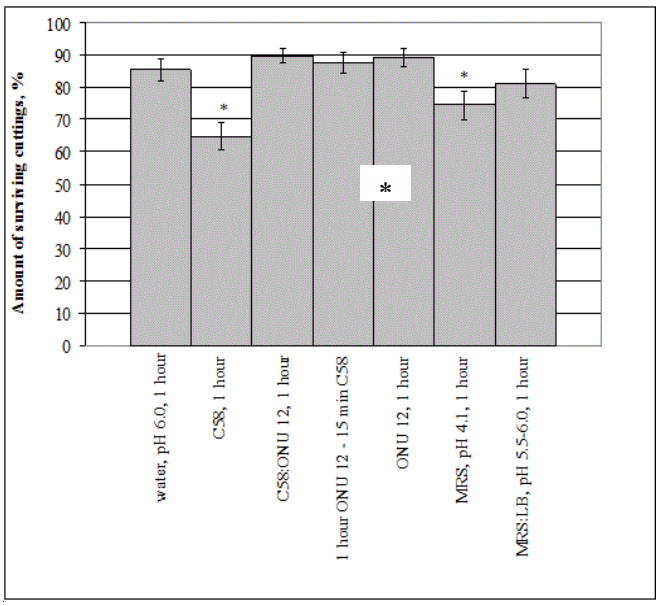

Figure 4: Survival of grape cuttings after the treatments with *A. tumefaciens* C58, controls and *L. plantarum* ONU 12 (expressed as a mean ± SE): * - significant differences between means after soaking in SDW and soaking in agrobacteria and MRS medium with pH 4.1 ($p<0.05$, *t* test).

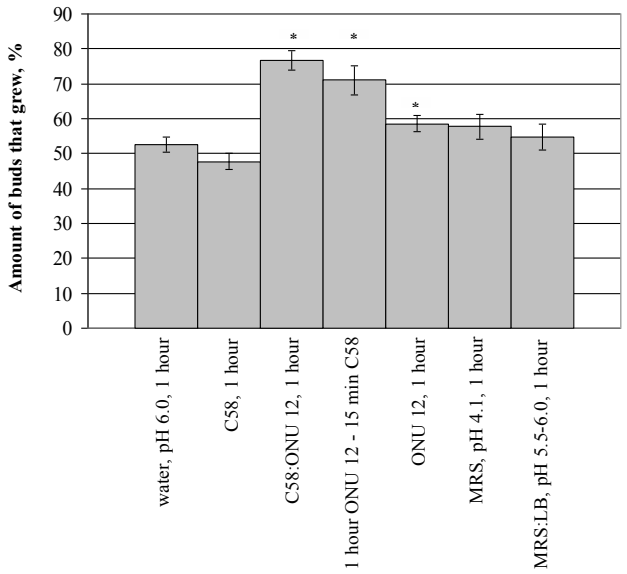

Figure 5: Effect of the treatments of grape cuttings with *A. tumefaciens* C58 and *L. plantarum* ONU 12 on amount of buds that grew (expressed as a mean ± SE): * - significant differences between means after soaking in SDW and soaking in lactobacilli alone and in combinations of bacteria ($p<0.05$, *t* test).

of green shoots formed in grape cuttings treated with *A. tumefaciens* C58 and *L. plantarum* ONU 12 in different combinations didn't differ significantly from the control (data not shown).

PCR with DNA of bacteria isolated from the treated cuttings showed the presence of pathogenic agrobacteria in tissues of 87.2% of samples inoculated in positive controls (Table 4). After a treatment with a mixture "*A. tumefaciens* C58/*L. plantarum* ONU 12" the amount of infected cuttings decreased to 17.0%. One-hour preliminary treatment with *L. plantarum* ONU 12 culture followed by a 15 min inoculation with *A. tumefaciens* C58 helped to decrease the amount of infected plants to 27.5% [52].

Discussion

Investigations of LAB use in plant protection against bacterial and fungal infections, for plant growth stimulation and for soil treatment have been largely described [1-3,5-9,53]. However, the possibility of crown gall biocontrol with LAB has not been evaluated yet. For the preliminary tests, we used the carrot disc model, what allowed to obtain rapid results and to carry out the simultaneous estimation of possibility of LAB strains to cause fermentation of plant tissues. It is mostly because of their fermentative activity and consecutive acidification of fermented media that LAB are used for food preservation [54]. For plant protection it was necessary to find strains with low fermentative activity not causing significant damage of wounded tissues.

Whereas *in vitro* tests have showed that the main inhibitory effect was based on low pH, different strains of LAB with the same pH of overnight cultures still varied in levels of their antagonistic activities *in vivo* on carrot explants showing the presence of other factors involved in suppression of phytopathogens. Still on this stage of the experiment it is unclear what is the mechanism of such inhibition. The suppression of phytopathogens can occur on the next stage as well when bacteria of both species are applied on carrot disk surface and LAB compete for the nutrients, attachment sites and excrete metabolites such as organic acids (lactic acid) and oxygen peroxide, which affect negatively

Variant	Amount of tested cuttings	
	Total	Infected (%)
A. tumefaciens **C58**	94	87.2
A. tumefaciens C58:*L. plantarum* ONU 12	88	17.0
1 hour *L. plantarum* ONU 12 then 15 min *A. tumefaciens* C58	80	27.5

Table 4: Presence of pathogenic agrobacteria in grapevine tissues after the experimental inoculation.

the survival of agrobacteria on plant surfaces. Different levels of antagonistic activity among strains of *L. plantarum* species described remain in agreement with the results of Visser et al. [1], Trias et al. [2], El-Mabrok et al. [5], who described the strain-specific effect of LAB against phytopathogens.

Strain *L. plantarum* ONU 12 with the highest antagonistic activity on carrot explants was also effective in suppressing crown gall on *Kalanchoe daigremontiana* and agrobacterial infection on grapevine cuttings. Depending on the method of inoculation, its suspension protected 100% of wounded tissues (via injections of the mixture "*A. tumefaciens* C58/*L. plantarum* ONU 12" or via soaking damaged roots and crowns in such a mixture), or decreased the number of galled plants by 98.5% (treatments of leaf scars with the mixture "*A. tumefaciens* C58/*L. plantarum* ONU 12") and by 72.7% in case of inoculation with agrobacteria followed by the treatment with LAB after 30 min interval.

Inoculation of grapevine with *A. tumefaciens* C58 culture reduced the number of viable cuttings, amount of buds that grew and mean length of green shoots from the cuttings that survived. *A. tumefaciens* in some cases can be a crown gall agent on grapevine, the same as *A. vitis* does [15,16]. Certain pathogenic strains originally isolated from grapevine as *A. tumefaciens* FACH was aggressive on kalanchoe but did not cause tumor formation on grapevine [51]. As for *A. tumefaciens* C58, its high tumorigenic activity on grapevines was reported by Holden et al. [54]. In our experiments, necroses instead of tumors were formed on all inoculated in the positive control grape cuttings. It was revealed by Bazzi et al. [27] that high concentrations of pathogenic *A. vitis* (approximately 10^8 CFU/mL) caused vast necroses of woody parenchyma instead of gall formation [55]. Plants with necrotic tissues exhibited poor growth and high mortality [28]. In the case of *A. vitis*, the necrogenic response of grape tissues can be explained by activity of polygalacturonase degrading cell walls [56] and other necrosis factors inducing inoculum-dependent damages on shoot explants and grape leaves [12]. *A. tumefaciens* C58 genome does not carry *pehA* gene responsible for polygalacturonase synthesis [51] and the mechanism of necrogenesis in case of *A. tumefaciens* is different from that induced by *A. vitis* [12]. Thus, strain *A. tumefaciens* A281 being super virulent on many plant species induced a necrogenic response (84%) rather than gall formation on several grapevine cultivars and such necroses seemed to be cultivar specific and related to genes of Ti-plasmid. Exogenous auxin increased the level of necrogenesis and this implied that plant hormones influenced the process of necrogenesis limiting the tumor formation. Deng et al. [57] proposed the hypothesis that *A. tumefaciens*-induced necrotic response is a consequence of increased levels of auxin or precursors of auxin affecting grapevine cells. The greatest degree of necrogenesis was observed in case of inoculation of basal ends [58]. Treatments of basal ends of cuttings in our experiments resulted also in vast necroses and subsequent high mortality of grapevines.

Co-inoculation with agrobacteria and LAB removed completely the negative effect of phytopathogen on grapevines as it was shown by comparison of numbers of survived cuttings, amounts of buds and mean lengths of green shoots. If grapevine cuttings were inoculated simultaneously with *A. tumefaciens* C58 and *L. plantarum* ONU 12, amount of infected cuttings decreased by 81% whereas preliminary inoculation with *L. plantarum* ONU 12 reduced this number by 68.4% showing the effect of pre-treatments with LAB suspensions.

As for the number of buds that grew, higher stimulating effect of lactobacilli in a mixture with a pathogen was rather unexpected. Locke et al. [58] showed similar effect on buds distal to tumors caused by the attenuated culture of agrobacteria due to cytokinins produced by tumors (reviewed in Binns, [59]). In our study, tumors were not formed but the stimulation activity was observed.

Stimulating activity of the overnight culture of *L. plantarum* ONU 12 on number of buds that grew was not associated with the components of MRS medium but only with the substances synthesized by LAB. Indeed, soaking in MRS, pH 4.1 as a negative control simulating the medium for lactobacilli with the lowest possible pH of the bacterial culture after appropriate time of incubation influenced negatively the grapes. The number of survived cuttings and mean length of green shoots decreased. Grapevine is not an acid tolerant plant, although vineyards with acid subsoils with pH lower than 5.0 are known (reviewed in Kirchhoff et al., [60]). Significant decrease in reduction of root and shoot biomass was observed in soils with pH below 4.5 [61,62]. Hence, it may be proposed that the positive effect of metabolites of *L. plantarum* ONU 12 compensated in executed experiments the unfavorable influence of low pH of overnight LAB culture.

Our previous investigations showed the stimulating effect of *L. plantarum* ONU 12 on development of tomato seedlings. In that case we used LAB cells washed from MRS with metabolites synthesized overnight and resuspended in SDW with final pH 5.0-5.2 [63]. Thus, the treatment with washed cells in case of tomato seeds and with overnight culture in MRS in case of grapevine cuttings had stimulating effect on test plants in both experiments.

Positive effects of treatments of the plants with lactobacilli under field [6] and laboratory conditions [63] has been described. Improving of root and shoot lenghts [53,63], including plants on plots infested with *Fusarium oxysporum* [6] indicated the possibility of treatments with lactobacilli not only as antimicrobial agents but also as plant stimulating microorganisms.

Taking into account the marked antagonistic activity *in vivo*, *L. plantarum* ONU 12 can be proposed as a microorganism with the high potential for protection of plants against *A. tumefaciens*. However, experiments with more of other plant varieties and species in green house and under field conditions would be still needed for deeper evaluation of all potential of practical use of studied LAB.

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