Oak Bark (*Quercus* sp. cortex) Protects Plants Through the Inhibition of Quorum Sensing Mediated Virulence of *Pectobacterium carotovorum*

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Abstract

Bacterial intercellular communication mediated by small diffusible molecules, known as quorum sensing (QS), is a common mechanism for regulating bacterial colonisation strategies and survival. Influence on QS by plant-derived molecules is proposed as a strategy for combating phytopathogens by modulating their virulence. This work builds upon other studies that have revealed plant-derived QS inhibitors extracted from oak bark (*Quercus* sp.). It was found that co-incubation of *Pectobacterium carotovorum* VKM-B-1247 with oak bark extract (OBE) reduced the production of acyl-HSL. This was accompanied by a dose-dependent decrease in the bacterial cellulolytic and protease activity. The effect of OBE treatment at the transcriptomic level is the suppression of the main QS-related genes *expR* / *expI*. Potato tubers pre-treated with OBE showed resistance to a manifestation of soft-rot symptoms. Analysis of the component composition of the OBE identified several biologically active molecules, such as n-hexadecanoic acid, 2,6-di-tert-butyl-4-methylphenol, butylated hydroxytoluene (BHT), gamma-sitosterol, lupeol, and others. Molecular docking of the binding energy of the identified molecules with homology models of LuxR-LuxI type proteins showed high potential binding of a few plant-derived molecules to these proteins.

1. Introduction

Plant diseases of microbial aetiology are a serious threat to human biosafety around the globe. Until recently, it was believed that bacteria, as a cause of spoilage of agricultural products, cause relatively less economic damage comparing to fungi and viruses (Sundin *et. al.* 2016) However, the situation changes every year, and the importance of bacteriosis and the attention of agronomists to has increased. There has been an increase in plant infections caused by *Pseudomonas syringae*, *Xanthomonas translucens*, *Pectobacterium* sp., *Xanthomonas* sp., *Clavibacter michiganensis*, and *Ralstonia solanacearum* (Leadbeater *et. al.* 2014).

Good agricultural practices, cultivation and crop rotation are important tools to minimize the impact of pathogenic bacteria on crops. However, these measures do not lead to complete success, and bacterial diseases of crops are found to be difficult to control. At present, the situation is such that there are almost no effective tools means to protect plants from bacterioses. If the list of fungicides contains hundreds of preparations, then a number of antibacterial drugs is limited to copper compounds in combination with fungicides, less often, these can be antibiotics aminoglycosides or tetracyclines used to treat fruit trees in some countries (Leadbeater *et. al.* 2014). In addition, the usage of chemical pesticides and antibiotics often leads to the development of field resistance (Travis *et. al.* 2012). Thus, it is necessary to search for new bactericidal substances with a new mechanism of antimicrobial action to overcome resistant forms which already exist.

The phenomenon of density-dependent intercellular communication of bacteria by chemical molecules known as quorum sensing (QS), was discovered at the end of the 20th century (Fuqua *et. al.* 1994). This finding made it possible to look differently at the functioning of microbial communities, including how the pathogenic potential of a bacterial population is realised. Moreover, it became clear that dissociation

of intercellular communication could appear a promising approach to fighting infections, as long as cells remain alive, which means that the selective pressure on the population decreases, and the selection of resistant forms is unlikely (Ji *et. al.* 2014). The suppression of the QS system functionality of some organisms by others is widespread in nature and called quorum quenching (Grandclément, *et. al.* 2016).

In addition to bactericidal substances, for self-defence plants synthesise many different low molecular weight molecules that suppress the functioning of the QS system of phytopathogens (Koh *et. al.* 2013, Deryabin *et al* 2019, Janak *et. al.* 2021). The inhibition of QS is realised by various mechanisms, including enzymatic degradation, adsorption on various macromolecules, and interference with the production or perception of autoinducers (AIs) (Grandclément *et. al.* 2016). Quorum quenchers of plant origin implement their own action through interference with the perception and biosynthesis of QS AIs (Koh *et. al.* 2013, Deryabin *et. al.* 2019, Janak *et. al.* 2021). Since numerous plant-derived molecules have structural similarities to natural AIs, this allows them to competes for AI binding sites. Thus, several chemical classes of molecules related to terpenes, phenylpropanoids, flavonoids, tannins, and ellagitannins bind and disturb the functionality of LuxR/I-type receptor proteins (Deryabin *et. al.* 2019).

Pectobacterium carotovorum is a Gram-negative bacterium that is widespread throughout the world. The ability to produce a number of plant cell wall degrading enzymes (PCWDEs) including pectinases, cellulases, and proteases (Põllumaa *et. al.* 2012) determines the presence of this species in the list of top-10 phytopathogenic microorganisms (Mansfield *et. al.* 2012). *P. carotovorum* responsible for causing soft-rot in numerous types of plants, including economically important potato crop (*Solanum tuberosum*).

The production of PCWDEs is triggered by a quorum-dependent mechanism when conditions for cell growth are favourable and there is a high requirement for nutrients and habitat expansion (Põllumaa *et. al.* 2012). The functioning of the QS system of *P. carotovorum* based on biosynthesis of acylated homoserine lactones (C6-oxo-HSL or C8-oxo-HSL) and the perception of them by ExpR receptor proteins (Põllumaa *et. al.* 2012). Several studies have demonstrated the effectiveness of plant-derived substances as a quorum quencher to prevent plant diseases caused by *Pectobacterium* sp. strains (Janak *et. al.* 2020, Pun *et. al.* 2021).

Previously, suppression effects of extracts derived from tissues of *Quercus virginiana* Mill (Fagaceae) (Adonizio *et. al.* 2021) and *Quercus robur* (Tolmacheva *et. al.* 2014) were shown against the QS system of *Chromobacterium violaceum.* However, the details of this phenomenon are still unknown.

In this work, we describe the chain of events from the moment of contact of the phytopathogen with plant molecules (*Quercus* sp.), and the possibility of using the revealed QS-inhibitory phenomenon in plant protection. We studied the effects of OBE on the expression of the main QS-related genes and the manifestation of QS-dependent virulence *in vitro* and *in vivo*. Using gas chromatography coupled with to mass spectrometry and molecular docking analysis, we described the molecular composition of OBE and identified the most likely inhibitors.

2. Material And Methods

2.1 Bacterial strains

Pectobacterium carotovorum (Jones) Waldee VKM B-1247 was obtained from the All-Russian Collection of Microorganisms (Pushchino, Russia). This strain produces 3-oxohexanoyl-L-homoserine lactone (3-oxo-C6- HSL) as it was revealed by HPLC-MS method (Figure S5).

Chromobacterium violaceum CV 026 is *a* mini-Tn5 mutant of wild type ATCC 31532, deficient in QS dependent violacein production was used as a biosensor for detecting exogenous acyl- HSL. *C. violaceium* CV026 produces purple pigment in response to short chain acyl- HSL.

Bioluminescent biosensors strain of *Escherichia coli* MG 1655 pVFR1-lux is a bioluminescence-based QS-biosensor (Manukhov, et. al. 2011). This strain carries the plasmid pVFR1, which possesses the *luxRI':luxCDABE (*meaning *luxI* mutated) bioluminescent reporter gene. This system can detect acyl- HSL with acyl chains ranging from six to twelve carbons in length (C6 to C12 acyl-HSLs).

2.2 Extract preparation

Pharmacological preparation of oak bark (registrational number N001007/01) produced by pharmacological company “Farmatsvet” (Russia) has been used in this work. The preparation formulated as a chopped oak bark of *Quercus sp.* plants which were collected in Krasnodar region of Russia in August 2020.

Extract from oak bark was prepared by extraction with an organic solvent. A weighted portion of 10 g of oak bark was poured into 100 ml of 70% ethanol (v/v), and stirred for an hour at 25 C. The resulting suspension was centrifuged at 9000 rpm for 15 min, and then supernatant was filtered through a filtration system (0.22 μm pore diameter). The resulting solution was frozen in liquid nitrogen and lyophilized.

2.3 Gas-MS-chromatography of Oak bark extract

Chromato-mass spectrometric study was carried out on a Trace GC Ultra chromatograph with a DSQ II mass-selective detector in the electron ionization mode (70 eV). Thermo TR-5 MS quartz capillary column 15 m long, 0.25 mm inner diameter, with a stationary phase film thickness of 0.25 μm was used. Split-free input mode was used. Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. Evaporator temperature 250°C, junction chamber temperature 250 ° C, ion source temperature 250°C. The temperature of the column thermostat was changed according to the program: from 80 (delay 5 min) to 290°C at a rate of 15 ° C/min. The total analysis time is 29 minutes. The volume of the injected sample is 10.0 μl. Chromatograms were recorded in TIC mode. Mass scanning range 40–500 amu.

2.4 Growth curves

Pectobacterium carotovorum VKM-B-1247 was grown overnight at 28°C in 4 mL liquid medium under continuous shaking at 150 rpm. Prepared bacteria for 10^6 CFU/ml subsequently have been inoculated to 96-well microtiter plates (Eppendorf, USA) containing growth media and various concentrations of oak bark extract in series of two-fold dilutions. The dynamics of bacterial growth were assessed by reading and plotting the absorbance data at 620 nm obtained by the spectrophotometer Multiscan GO (Thermo Scientific, USA).

2.5 Qualitative determination of acyl-HSL formed by *P. carotovorum* under the influence of oak bark extract

The overnight grown *P. carotovorum* culture was seeded on soft agarose LB-medium (0.75% v/v) supplemented with Oak bark extract (0.5 and 1.0 mg/mL). *P. carotovorum* was checked for acyl-HSL production by striking against biosensor strain *C. violaceium* CV026 on soft agarose medium. The plates were incubated at 28°C for 24 h and acyl-AHL-dependent pigment production in CV026 was observed. The extent of purple pigment production in CV026 was indicative of diffusible acyl-AHL produced by *P. carotovorum*.

2.6 Quantification of acyl-HSL formed by *P. carotovorum* under the influence of oak bark extract

P. carotovorum was cultured in a volume of 10 mL in LB medium (tryptone − 10, yeast extract − 10, glucose − 0.5%, NaCl − 2 g/L) at 28 C, 95 rpm. Upon reaching the stage of the exponential growth, 100 µl of oak bark extract dilutions were added to the cells up to the final appropriate concentrations. The cells were subsequently cultured.

At hourly intervals, 1.5 ml of each cell suspension was collected. Bacteria were precipitated by centrifugation at 10 000 rpm / 15 min, 6°C. The supernatant was transferred into a separate tube for the quantitative analysis of autoinducers using the bioluminescence assay. Bacterial biomass was frozen in liquid nitrogen and stored at -80°C for subsequent isolation of nucleic acids.

2.7 Bioluminescence assay

Quantitative detection acyl-HSL using biosensor E.coli MG1655 pVFR1-lux

Supernatants of *P. carotovorum* cultures were mixed with biosensor *E. coli* MG 1655 pVFR1-lux in 96-well microtiter plates to a final volume of 100 µL. Bioluminescence was recorded as relative light units (RLU) using Multiscan FL reader (Thermo, USA) every 5 min at 25°C. The resulting values of bioluminescence were estimated in induction factor (R), which was processed according to the following Eq. 1:

$$R = It_{120\ min} - It_{0min} / Ic_{120\ min} - Ic_{0min} \quad (1)$$

Where *It* is intensity of bioluminescence of a treated sample; *Ic* is intensity of bioluminescence of the control sample (in the absence of inductor).

2.8 RNA sample collection and gene expression analysis using reverse transcription and quantitative Real-Time PCR (RT-qPCR).

RNA extraction and cDNA preparation

Total RNA was isolated using the Quick-RNA Microprep kit (Zymo Research) according to the manufacturer's instruction. RNA was quantified using Qubit RNA HS Assay Kit (Thermo Fisher Scientific). Total RNA was further treated with DNase I (New England Biolabs) followed by the RNA Clean & Concentrator-5 kit (Zymo Research). The absence of contaminating DNA was verified by PCR. Then, using the reverse transcription reaction, complementary DNA was obtained using the iScript reverse transcription supermix for RT-qPCR reagent (Bio-Rad, USA).

Quantification of mRNA by quantitative real-time PCR (qRT-PCR)

Quantitative PCR was carried out with the obtained cDNA using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA). Each reaction mixes with a volume of 20 µl was prepared with 300 nM each primer (final concentration) and 20 ng of cDNA. Light Cycler96 Real-Time PCR detection system (Roche, Switzerland) was used for the measurements using a protocol with the following thermal cycling conditions: DNA denaturation at 95 ℃ for 2 min, followed by 40 cycles of denaturation at 95 ℃ for 10 s and annealing/extension at 60◦C for 15 s. After the last amplification cycle, a melting curve analysis was carried out by heating from 65 to 95 ℃ in increments of 0.5 ℃/s. Negative controls (without template or reverse transcriptase enzyme) were included in each run. Gene-specific primers (described in Table S1) for the genes used in RT-qPCR experiments were designed using online service Integrated DNA technologies (https://www.idtdna.com/Primerquest/Home/Index). Fold changes in the expression levels of the investigated genes were normalized in relation to the levels of the housekeeping genes *rho* and *rec*A mRNA.

2.9 Effect of oak bark extract on production of plant cell wall degrading enzymes (PCWDEs)

Semi-quantitative assays for protease and cellulase activities were performed using a plate assay. Protease activity was assessed by cultivation of bacteria on LB- medium (BD, USA) with 3% gelatin and 1% agar (w/w). Bacteria let grow for 24 hours, then Petri dishes were filled with ammonium sulfate solution to contrasting halo areas around colonies (Pitt, et. al. 1970).

Cellulase activity was assessed as described by Gupta *et al* (Pratima et. al. 2012). Bacteria seeded on medium contained 0.2% carboxymethyl cellulose and 25 mM sodium phosphate, 0.75% agarose, Congo-Red 0.02%, and gelatin 0.2% (pH 7.0). *P. carotovorum* was inoculated to the medium, and the plates were incubated at 28C for 48 h. Cellulose-degrading potential was qualitatively estimated by calculating hydrolysis capacity, that is the ratio of diameter of halo zone and colony.

2.10 Effect of oak extract on virulence of *P. carotovorum* by potato maceration assay

Assay based on methodic described in Singh et al. with our modifications (Singh, A.A., et. al. 2021). Potato tubers were purchased from the market and washed under tap water. The potato slices (~ 0.5 cm) were surface sterilized by treating with 3% hydrogen peroxide for 30 minutes followed by washing with sterile distilled water. Each slice was placed in a sterile Petri plate containing wet filter paper. Prepared water suspension of the oak bark extract (1 mg/mL) or sterile water (control group) were scattered to the surface of potato slices and dried in the air. Bacterial suspension at 10^6 CFU/mL was dropped at the center of each slice. The Petry dishes were incubated at 28 C for 76 hours.

2.11 In silico analysis

Homology modeling of CviI/CviR of Chromobacterium violaceum

Homology model of acyl-homoserine-lactone synthase CviI of *C. violaceum* is based on its UniProt sequence (Q83XU6) and crystal structure of the homologous protein TofI from *Burkholderia glumae* (PDB ID: 3P2H), which has endogenous ligand 5'-deoxy-5'-methylthioadenosine resolved in the catalytic site (Chung et. al. 2011) and shares 26% of identity with the target. These values are above the 20% threshold considered to be appropriate for homology modeling (Joshi et. al. 2016).

The 3D homology model of CviI was built using Prime in Schrödinger Suite (Schrödinger, Inc., USA), sequences were aligned with Prime STA. Protein Reliability Report tool was utilized to assess binding pocket stability revealing no deviations from acceptable values.

CviR is a transcriptional regulator of *Chromobacterium violaceum* which structure was downloaded from the RCSB website (PDB ID: 3QP5) in the form of crystal structure bound to antagonist chlorolactone (Chen, et. al. 2011). The protein structures were prepared using the Protein Preparation Wizard implemented in Maestro.

Docking

The ligand structures were downloaded from the PubChem website. LigPrep tool was utilized to prepare ligands using the OPLS3 force field generating low energy ionized and tautomeric states at pH 7.0 ± 2.0, while chirality was determined from ligands 3D structure. Receptor grid box was centered using co-crystallized ligands and extended to 12Å. Bound ligands were extracted and then was re-docked along with a set of optimized ligands of interest using Glide XP. Docking scores are demonstrated in the results section.

2.12 Statistical analysis

The experiments were performed using two independent series with 3 to 10 technical replicates each. The obtained results were statistically manipulated with Origin 2018 (OriginLab Corporation, Northampton, Massachusetts, USA) software.

The Shapiro-Wilk test was used to assess the normal distribution of values. In the presence of a normal distribution, the Student's t-test has been used, indicating the mean and standard deviation (Mean ± SD). Differences were considered significant at p-value ≤ 0.05.

3. Results

3.1 Antibacterial effect of oak bark extract, and Its influence on biosynthesis of acyl-homoserine lactone

First, the oak bark extract (OBE) was evaluated for its antimicrobial properties. We were unable to detect the inhibition of *P. carotovorum* cells growth by OBE taken at concentrations of 0.25–1.0 mg/mL (Figure S1).

In the second step, we assessed the OBE quorum quenching activity. The growth of *P. carotovorum* is accompanied by the diffusion of C6-oxo-HSL AIs, which are perceived by the sensory strain *Chromobacterium violaceum* CV026 (Fig. 1). It has been shown that pectobateria produces less AIs when growing on a medium containing OBE compared to a medium without any supplements (Fig. 1).

Thus, a qualitative suppression of the production of *P. carotovorum* AIs by OBE was established, and the range of effective concentrations was determined. The quantitative effect of the addition of OBE on AI production was assessed using the bioluminescent strain of *E. coli* pVFR1-lux. The induction of bioluminescence is proportional to the inducer concentration in the medium. It was found that OBE inhibited the biosynthesis of C6-oxo-HSL in dose-dependent manner. The inhibition effect was most pronounced when adding OBE at a concentration of 1 mg/mL, this concentration delayed AIs biosynthesis by 86% (Fig. 2). At the same time, no suppression of the viability of bacterial cells was observed (Figure S2).

3.2. Expression of quorum sensing-regulated genes in the presence of oak bark extract

The effect of the extract at the molecular level was assessed using quantitative PCR, targeting the main regulatory genes of the QS system. It was found that the expression of *expI* and *expR* genes was affected under the influence of OBE. The most significant dysregulation was shown for the *expI* gene (Fig. 3a). Co-incubation of *P. carotovorum* with 0.5 mg/mL of OBE for 2 hours did not lead to a decrease in *expI* expression compared to the control, but 1.0 mg/mL was able to reduce gene expression. During the third hour of treatment, *expI* expression was significantly decreased at all tested concentrations, compared with the intact sample. In contrast, the transcription level of the *expR* gene in the intact samples had no changes over time, but under the treatment (0.5 and 1.0 mg/mL) were decreased (Fig. 3b).

3.3. Effect of oak bark extract on the production of cell wall degrading enzymes

The used strain of *P. carotovorum* VKM-B-1247 was tested for its ability to produce various enzymes involved in the degradation of plant tissues. The inhibition of protease and cellulase activity was found at all concentrations of OBE (Fig. 4). The virulence factor that was the most affected by the treatment was the protease activity. Thus, the addition of 0.5 mg/mL of OBE reduced the protease activity of pectobacterium by 50%, while 1.0 mg/mL of OBE reduced the protease activity of pectobacterium by more than 90%. At the same time, the cellulase activity was reduced by 60% in response to 1.0 mg/mL OBE in comparison with the intact control.

3.4. Effect of oak bark extract on *P. carotovorum* virulence

It is expected that a decrease in the biosynthesis of C6-oxo-HSL leads to a reduction in PCWDE activity and, in turn, to a decrease in the manifestations of the disease caused by *P. carotovorum.* To assess this, we infected the potato tuber with a suspension of *P. carotovorum.*

It turned out that the pre-treatment of potato tubers with OBE slowed down the development of symptoms of tissue maceration, compared with the control group. So, on the first day on the untreated potato slices signs of maceration appeared (Fig. 5a). After 72 hours, the mass of diseased tissue was about 20% of the mass of the whole potato (Fig. 5g). In turn, the blackening of the tissues of potatoes treated with OBE was not observed even by the third day of incubation (Figs. 5d–f), and the percentage of affected tissue remained less than 10% (Fig. 5g).

3.5 Gas chromatography mass spectrometry (GC-MS) analysis of oak bark extract

Gas chromatographic analysis of the oak bark extract allowed us to obtain mass spectra for 11 different substances with varying degrees of homology according to the NIST reference database (Table 1, Figure S3).

Among the identified components, the main part presented by lipophilic polyphenolic substance 2,2'-methylenebis(6-tert-butyl-4-methylphenol) (relative content 21.3%), n-hexadecanoic acid (8.6%), plant phytosteroid gamma-sitosterol (4.3%), terpenoid friedelan-3-one (3.4%), phenolic antioxidant 2,6-di-tert-butyl-4-methylphenol (3.3%), (5), pentacyclic triterpenoid lupeol (3.2%), 1-(5-(hydroxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)propan-2-ol (1.76%) and (9β)-9,19-Cyclolanostan-3β-ol acetate (1.2%). Other identified components presented in insignificant amounts (< 1%).

3.6. Molecular docking analysis

The mechanism determining the suppression of QS-dependent bacterial virulence must be sought in the intermolecular interaction of small molecules with the main components of the QS system. There are two crucial components in QS system: acyl-HSL producing enzymes and LuxR-type signal receptors (Chen, G.,

et. al. 2011). We used molecular docking to assess the binding capacity of the identified molecules (Table 2) to the active sites of these proteins.

Molecular docking of various ligands with LuxR-LuxI proteins requires their resolved crystal structures. However, only the structure of LuxR-type protein CviR is presented in Protein Data Bank (PDB ID: 3QP5). Considering the ability of oak bark extract to inhibit QS-dependent violacein biosynthesis in *Chromobacterium violaceum* ATCC 31532 (Figure S4), we performed *in silico* analysis using available structure CviR and generated homology model CviI.

CviI acyl-homoserine-lactone synthase homology model was built on the crystal structure of the homologous protein TofI (PDB ID: 3P2H) analogous to the previously described approach [26]. Using homology modelling, we calculated the active site composition of *C. violaceum* CviI acyl-homoserine-lactone synthase to provide a model for testing acyl-HSL producing enzyme inhibitors. Gamma-sitosterol demonstrated a slightly lower docking score (−6.888 kcal/mol) compared to the reaction by-product 5'-deoxy-5'-methylthioadenosine (−7.553 kcal/mol), which was resolved in the TofI crystal structure and redocked to the CviI homology model (Fig. 6a,b). Other candidates for potential acyl-HSL producing enzyme inhibitors (9β)-9,19-Cyclolanostan-3β-ol acetate (-6.364 kcal/mol), 1-(5-(hydroxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)propan-2-ol (-6.296 kcal/mol), 2,2'-methylenebis(4-methyl-6-tert-butylphenol) (-5.835 kcal/mol) (Table 2).

The *C. violaceum* CviR transcriptional regulator was analysed to model LuxR-type signal receptors. The best binding poses for every compound are shown in Table 2. According to our predictive model for the CviR transcriptional regulator, 2,2'-methylenebis(4-methyl-6-tert-butylphenol) (Fig. 6d) and 1-(5-(hydroxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)propan-2-ol have slightly higher binding energy (-7.876 and − 7.739 kcal/mol, respectively) than the known inhibitor chlorolactone (-7.222 kcal/mol) (Fig. 6c) and thus can be analysed as potential QS signal blockers. 2,2'-Methylenebis(4-methyl-6-tert-butylphenol) and 1-(5-(hydroxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)propan-2-ol bind several amino acids that play role in the chlorolactone interaction with the protein (Trp84, Tyr88, Asp97, Ser155) (Fig. 6d), therefore, a similar mechanism can be proposed. Docking analysis revealed that several compounds in OBE can possibly affect the functioning of both acyl-HSL producing enzymes and LuxRtype signal receptors.

4. Discussion

This work is a continuation of a series of studies devoted to discover and structure-functional analysis of plant-derived substances with quorum quenching activity. The bark and leaves of some oaks (*Querqus* spp.) are recognised pharmacological agents used in various countries for the treatment and prevention of infectious processes caused by viruses, bacteria, fungi, and other pathogens (Assessment report EMA/HMPC/3206/2009).

Regarding bacteria, the oak bark extract (OBE) is well known as a substance with bactericidal and bacteriostatic properties, while its quorum quenching activity was discovered recently (Adonizio et. al. 2006). Subsequently works in this direction have revealed details on the molecular composition of OBE

which determine its quorum quenching properties (Deryabin et. al. 2015). However, the molecular details on how quorum quenching is realised is still unknown, and how it could be useful to fight microbial virulence is an ongoing research topic.

We estimated what extract of oak bark is potentially be considered as natural active substance to prevent plant disease caused by *P. carotovorum.* Interestingly, we found that the direct antimicrobial activity of OBE against *C. violaceum* and *P. carotovorum* is weak. In contrast, the anti-quorum activity of the OBE was pronounced against *C. violaceum*, as well as *P. carotovorum.* This is not surprising, given that the QS system of *P. carotovorum*, like *C. violaceum*, is arranged as the LuxI-LuxR regulatory system.

At the next stage, we carried out a quantitative assessment of the ability of oak bark extract to suppress the functioning of quorum sensing of pectobacteria. It turned out that co-incubation of the test strain with the studied drug inhibited the biosynthesis of QS-autoinducers. The implementation of suppression begins from the first hour and reaches a maximum by the third hour of exposure.

The mechanism of blocking quorum sensing was investigated at the transcriptomic and phenotypic levels. It turned out that oak bark extract reduces the expression of *expR* and *expI*, the products of which are proteins of reception and synthesis of acylhomoserine lactones, respectively.

Thus, oak bark extract affects the expression of QS-related genes, that led to reducing biosynthesis of AIs and subsequently affects enzymatic activity of cellulases and proteases. In the ecology of pectobacteria, the processes of colonization of plant tissues are under the control of the QS system. After fixing on the surface of plants, pectobacteria produce various enzymes that destroy plant tissues. Therefore, the production of cellulases, pectinases and glycosyl hydrolases allows plant pathogenic bacteria to destroy the cell wall firstly, which provides the bacterial population with carbohydrates and allows it to penetrate inside plant tissues (Gorshkov et. al. 2017). In turn, proteases of phytopathogens destroy lectins, deactivate PR-proteins, mitogen-activated protein kinase (MAPK) and activate plant immunity and extensins that strengthen the structural integrity of the plant cell wall (Dow et. al. 1998; Savidor et al., 2012; Ek-Ramos et al., 2019; Gur-Arie et al., 2020).

Analysing the molecular composition of the oak bark extract, we used the method of gas chromatography with mass spectrometric detection. This method has proven itself well in the study of the chemical composition of various plant extracts (Husain et. al. 2017) including oak bark extract (Deryabin et. al. 2015). We found that none of the substances we identified had previously been found in the composition of the oak bark extract. The oak bark extract obtained in the work of Deryabin et. al. (2015) contained at least five different QS inhibitors, including vanillin, and coumarin derivatives. These molecules were not found in our samples. This result can be explained by two main reasons: first of all, an origin of biological material and composition of bark which admittedly belongs to different *Querqus* spp. (for instance, *Q. robur* and *Q. pubescens*); secondly, in this work we made soft trial preparation excepting high pressure and temperature during autoclaving that may lead to partial chemical degradation of components located inside the bark.

Nevertheless, a number of identified molecules can potentially act as inhibitors of quorum sensing. It was previously reported that n-hexadecanoic acid (palmitic acid) demonstrated the ability to inhibit bacterial biofilm development and reduce quorum sensing mediated gene expression in *V. harvey*i (Santhakumari et. al. 2017). The antioxidants 2,6-di-tert-butyl-4-methylphenol (butylated hydroxytoluene) (Jiang et. al. 2013) and 2,2'-Methylenebis (6-tert-butyl-4-methylphenol) (Jang et. al. 2017) are known for their antiviral and anticarcinogenic properties. Lupeol is a pharmacologically active pentacyclic triterpenoid (Margareth et. al. 2009). It has anti-cancer and anti-inflammatory activity and slightly antimicrobial activities. It is important to note that lupeol has been shown to inhibit the violacein production of *C. violaceum* (Bodede et. al. 2018). The triterpenoid friedelan-3-one has been reported to exhibit antioxidant and anti-inflammatory activities (Christudas et. al. 2013) derived from the leaf of *Pterocarpus santalinoides* showed antibacterial activity (Ichiko et. al. 2016). Then docked in CviR, the higher scores were calculated for 2,2'-methylenebis(4-methyl-6-tert-butylphenol) and 1-(5-(hydroxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)propan-2-ol. It is better cores than for the known antagonist chlorolactone. Interaction modeling with CviI demonstrated the best docking scores for gamma-Sitosterol and 2,2'-Methylenebis(4-methyl-6-tert-butylphenol) compared to other tested compounds.

Thus, we determined that oak bark hydro-ethanol extract is a plant material that quite effectively inhibits quorum sensing mediated virulence and soft-rot symptoms caused by *P. carotovorum*, ant these effects can be achieved because of a number of secondary metabolites possess a combined or complementary action, and thereby, makes it possible to be a biopesticide. Molecular modelling has shown that a number of molecules can be incorporated into the active centre of both the CviR receptor protein and the CviI protein, which possibly determines their quorum quenching properties. Thus, a combination of such molecules well be used as in a plant protection agent against *Pectobacterium* spp.

Declarations

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Author contributions: ASV conceived and designed the study. DVP, AVI, RS, AVV, EAR performed the experiments. ASV, DVP, RS, AVV, EAR analyzed and interpreted the data. ASV, EAR drafted and wrote the manuscript. ASV supervised the study. All authors approved the final version of the manuscript.

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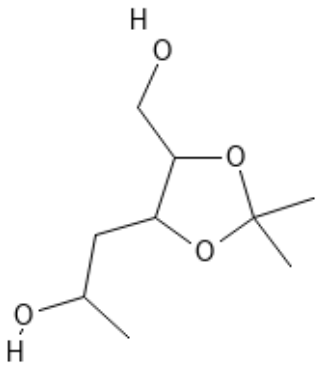
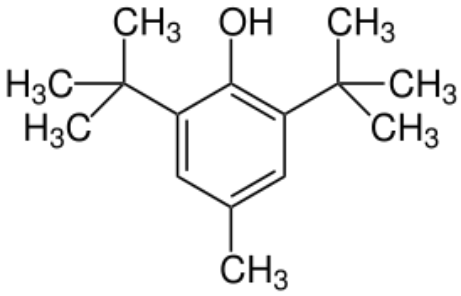
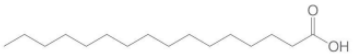
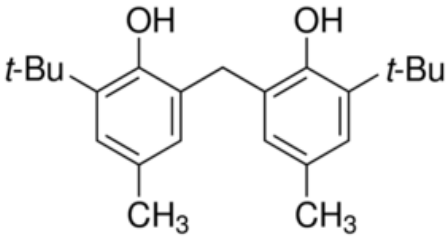
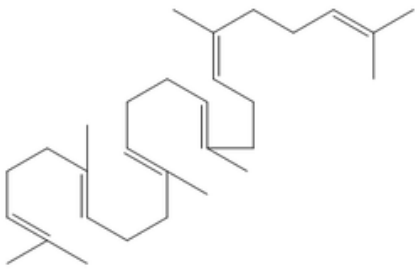
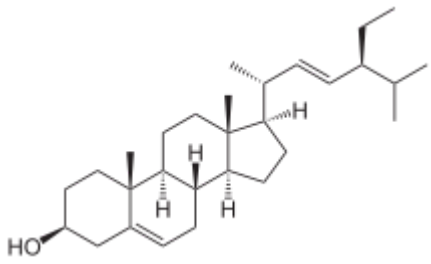
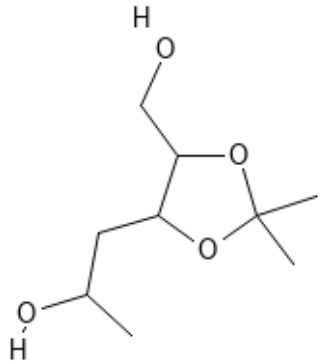
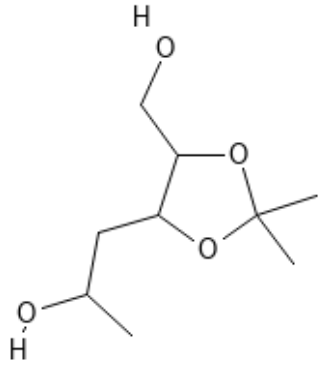
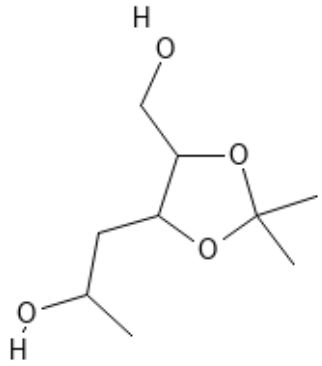
Tables

Table 1. The phytochemicals identified in the *Quercus cortex* extract by gas chromatography-mass spectrometry

No	Component	Retention time, min	Relative content, %	Structural formula
1	1-(5-(hydroxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)propan-2-ol	5.22	1.76	
2	2,6-di-tert-butyl-4-methylphenol	9.40	3.25	
3	n-Hexadecanoic acid	13.04	8.6	
4	2,2'-Methylenebis(6-tert-butyl-4-methylphenol)	15.60	21.3	
5	Squalene	17.60	1.61	
6	Stigmasterol acetate	18.80	0.98	

				
7	Gamma-sitosterol	20.11	4.34	
8	Lupeol	20.06	3.17	
9	(9β)-9,19-Cyclolanostan-3β-ol acetate	20.70	1.2	
10	Friedelan-3-one	27.7	3.43	

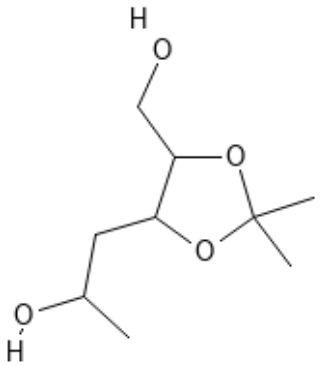


Table 2. Docking results of complexes from CviI and CviR proteins.

CviR / Ligand	Docking score (kcal mol^{-1})
2,2'-Methylenebis(4-methyl-6-tert-butylphenol)	-7.876
1-(5-(hydroxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)propan-2-ol	-7.739
(9β)-9,19-Cyclolanostan-3β-ol acetate (-6.364 kcal/mol)	-7.540
Butylated hydroxytoluene	-7.397
Chlorolactone (resolved in the crystal structure, re-docked)	-7.222
gamma-Sitosterol	-6.555
[5-(Hydroxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl]methanol	-5.675
Hexadecanoic acid	-5.485
Squalene	-4.751
Friedelan-3-one	-3.221
CviI / Ligand	
5'-Deoxy-5'-methylthioadenosine (resolved in the crystal structure, re-docked)	-7.553
gamma-Sitosterol	-6.888
(9β)-9,19-Cyclolanostan-3β-ol acetate (-6.364 kcal/mol)	-6.364
1-(5-(hydroxymethyl)-2,2-dimethyl-1,3-dioxolan-4-yl)propan-2-ol	-6.296
2,2'-Methylenebis(4-methyl-6-tert-butylphenol)	-5.835
Lupeol	-4.497
Butylated hydroxytoluene	-4.438
Squalene	-2.538
Stigmasterol acetate	-2.468
Friedelan-3-one	-2.438

Target: *Chromobacterium violaceum* CviR transcriptional regulator (PDB ID: 3QP5); and *Chromobacterium violaceum* CviI acyl-homoserine-lactone synthase homology model built on TofI from *Burkholderia glumae* structure (PDB ID: 3P2H).

Figures

Figure 1

C6-oxo-HSL autoinducers synthesis by *P. carotovorum* VKM-B-1247 and its perception by *C.violaceum* CV026, when growing on medium with the addition of oak bark extract for 0 (a), 0.25 (b), 0.5 (c), and 1.0 mg/mL(d).

Figure 2

The bioluminescent response of the *E. coli* pVFR1-lux sensor is proportional to the amount of C6-oxo-HSL contained in the *P. carotovorum* VKM B-1247 culture medium. Each bar represents three replicates, from two independent experiments. Treatments not connected by the same letter in each panel are significantly different from each other ($p < 0.05$; means ± SD).

Figure 3

Effects of oak bark extract on the transcript levels of quorum sensing (QS) system genes in *P. carotovorum* VKM-B-1247. The transcript levels of the QS system genes *expI, expR* were determined by quantitative real time-polymerase chain reaction (qRT-PCR) of cDNA samples prepared from RNA extracts of bacterial cultures grown in LB-Broth with or without (control) the oak bark extract. Treatments not connected by the same letter in each panel are significantly different from each other ($p < 0.05$; means ± SD).

Figure 4

Oak bark extract reduces exoenzyme activities of *P. carotovorum* VKM-B-1247. Enzymatic activity was estimated on the size of substrate degradation haloes during 24 h cell growth on agar medium without the addition (intact) and with the addition of oak bark extract at concentrations of 0.5 - 1.0 mg/mL (a). Results are expressed as the percentage of activity relative to controls without the compounds. Treatments not connected by the same letter in each panel are significantly different from each other ($p < 0.05$; means ± SD) (b).

Figure 5

Inhibition of *P. carotovorum* virulence on potatoes. Potato discs coated with oak bark extract (1 mg /mL) before bacteria were inoculated (10^6 CFU / ml). Representative photos of development of soft-rot symptoms on untreated (a-c) and pretreated with OBE (d-f). Photos were made at 24 (a, d), 48 (b, e), and 72 hours (c, f). Plant-protection effect was determined as the percentage of macerated tissue 72 h after the inoculation of potato tubers with *P.carotovorum* (g). Treatments not connected by the same letter in each panel are significantly different from each other ($p < 0.05$; means ± SD).

Figure 6

Docked complex of CviI from *Chromobacterium violaceum* homology model built on TofI from *Burkholderia glumae* (PDB ID: 3P2H). Complex CviI with endogenous ligand 5'-Deoxy-5'-methylthioadenosine (a) and gamma-sitosterol (b).

Docked complex of the CviR transcriptional regulator from *Chromobacterium violaceum* (PDB ID: 3QP5) with native antagonist chlorolactone (c), and 2,2-Methylenebis(4-methyl-6-tert-butylphenol) (d). Only key amino acids of proteins are shown for clarity. Ligands are shown as grey sticks, hydrogen bonds as yellow lines, and pi-pi stacking interactions as blue lines.

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