MORPHOLOGICAL AND PHYSIOLOGICAL RESPONSE OF *Pantoea vagans* BACTERIUM TO FOUR ANTIMICROBIAL COMPOUNDS

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ABSTRACT

Pantoea vagans is an opportunistic pathogen for humans. This organism is frequently isolated from various horticultural commodities. Active compounds from some essential oils have been used to control food-borne pathogens. The objectives of this investigation were to determine the effect of four active constituents of essential oils on the morphological changes and membrane affectations of this microorganism. Scanning Electron Microscopy was used to observe morphological changes, while Fluorogenic techniques (LIVE/DEAD BacLight Kit, CFDA-SE, $DiBAC_4$) were applied to evaluate membrane changes. The active compounds thymol, eugenol, carvacol and cinnamaldehyde altered and broke the bacterial membrane causing deformations and increase in permeability, a decrease in cytoplasmic pH (121.8 RFU, Relative Fluorescence Units) and depolarization (1841.025 URF). These active constituents could be an alternative for the control of *P. vagans* once they are tested in *in situ* studies.

Keywords: natural compounds, membrane integrity, essential oils, derived compounds.

RESPUESTA MORFOLÓGICA Y FISIOLÓGICA DE LA BACTERIA *Pantoea vagans* A CUATRO COMPUESTOS ANTIMICROBIANOS

RESUMEN

Pantoea vagans es un patógeno oportunista en humanos. Este organismo es frecuentemente aislado en varios productos hortícolas. Los compuestos activos de algunos aceites esenciales han sido utilizados en el control de microorganismos de los alimentos. Los objetivos de esta investigación fueron determinar el efecto de cuatro constituyentes activos derivados de aceites esenciales en los cambios morfológicos y daños en la membrana de este microorganismo. La microscopía electrónica de barrido se utilizó para observar los cambios morfológicos mientras que técnicas fluorogénicas (LIVE/DEAD BacLight Kit, CFDA-SE, $DiBAC_4$) se aplicaron para evaluar los cambios en la membrana. Los compuestos activos como el timol, eugenol, carvacrol y cinamaldehido, alteraron y rompieron la membrana de la bacteria causando deformaciones e incremento en la permeabilidad, un decremento en le pH citoplásmico (121.8 URF) y despolarización (1841.025 URF). Estos compuestos activos pudieran ser una alternativa para el control de *P. vagans* una vez que se hayan probado en estudios *in situ*.

Palabras clave: compuestos naturales, integridad de membrana, aceites esenciales, compuestos derivados.

INTRODUCTION

Pantoea vagans (syn. *Pantoea agglomerans*, *Erwinia herbicola*) is a bacterium classified within the family Enterobactereaceae and it is often isolated from a wide range of other environmental habitats including edible horticultural commodities [32]. Reports indicate that *P. vagans* is able to infect hospitalized patients receiving antibiotic treatment, or people suffering diabetics, cancer, and patients with burns or wounds. It may also colonize respiratory and urinary tracts, making it an opportunistic bacterium [8, 12, 23]. Thus, methods to control this

bacterium in foods are necessary [14, 25]. Currently, the resistance of microorganisms to synthetic antimicrobials is increasing. In this regard, numerous antimicrobial systems from plants have been developed [24]. Currently, the essential oils of numerous plants are widely used in pharmaceuticals, cosmetics and food products because they effectively inhibit the growth of a wide range of microorganisms, they are considered safer alternatives to synthetic compounds and they could have fewer adverse effects than synthetic antimicrobial compounds [6, 19]. The antimicrobial activity of thyme, clove and oregano essential oils is due to the presence of the phenolic compounds thymol, eugenol and carvacrol, respectively [34]. Although the properties of essential oils and their components are known, their antimicrobial mechanisms of action have not been studied in great detail [5]. For example, some oils have a better effect on Gram-positive than on Gram-negative bacteria. Therefore, studies made in this area are useful to provide a better understanding of the mechanism of action and to develop appropriate measurements to control these microorganisms. The bacterial cell membrane is one of the common sites of action of these compounds, thus affecting other physiological processes as a result. The aim of this research was to evaluate the antimicrobial effect of four essential oils derived compounds on *P. vagans* morphology and determine their activity on membrane physiology.

MATERIALS AND METHODS

Inoculum

The test microorganism for each antimicrobial evaluation was isolated from fresh dahlia flowers. The organism was morphologically (selective and differential media), biochemically (Kit Enteric/ Nonfermenter ID System/ BD BBLCRYSTAL E/NF) and molecularly (ZR Fungal/Bacterial DNA MidiPrep™; GeneJet PCR Purification Kit) identified as *Pantoea vagans*.

Preparation of antimicrobials

The antimicrobial agents evaluated in this study were four derived compounds from essential oils: eugenol, carvacrol, thymol and cinnamaldehyde (Sigma-Aldrich Inc.). For these compounds a mixture of ethanol-water in a ratio 1:1 was used. Each antimicrobial solution was prepared at the time of the test.

Scanning electron microscopy

Preparation of inoculum

Fifteen microliters of *Pantoea vagans* was inoculated in tubes with 5 mL of Brain Heart Infusion broth (BHI) and incubated for 18 - 24h at 37°C. A subculture in flasks with lactose broth (L) was made and incubated for 18 - 24h at 37°C. Then the solutions of each derived compounds at 0.25 % concentration were added to the cultures. Each antimicrobial was allowed to stand for 30 min.

Sample preparation for microscopic observation

The *P. vagans* cultures treated with the four antimicrobials were fixed in 2% glutaraldehyde and then centrifuged at 10000 rpm for 20 min to obtain a pellet, after which 2 washes were performed, the supernatant was discarded and each pellet washed with phosphate buffer at pH 7.2-7.4. The samples were allowed to stand for 24h. A fixation was made with osmium tetraoxide for 2h and then the samples were washed three times with phosphate buffer solution. Then 30% ethanol was added to each culture and allowed to stand for 30 min. The sample was centrifuged and the supernatant discarded. Then the sample was subjected to subsequent concentrations of ethanol (40, 50, 60%, 70, 80, 90 and 100%). The samples were stored in microporous capsules in 100% ethanol. Then samples were critical-point dried (CPD) and mounted on carbon film. A JOEL 7600F high-resolution field emission scanning electron microscope with a voltage of 1kV without conductive coating was used. The images were obtained with secondary electrons.

Assay membrane integrity

Pantoea vagans cultures treated with the four antimicrobial compounds at 1, 2 and 4% concentrations. Bacterial cell membrane integrity was determined using the LIVE/DEAD BacLight Kit and an epifluorescence microscope (Axioskop 40; Carl Zeiss). The negative control consisted of cultures treated with solvent [30]. The software (ImageJ version 1.41) was used to determine statistical differences among treatments.

Determination of cytoplasmic pH (pH_{in})

For loading of cells with fluorescent probe (cFDA-SE), soybean agar tripticasein broth (5mL) was inoculated with activated overnight cultures of the strain *P. vagans* and incubated at 37 ºC for 3 h. Cells were harvested by centrifugation (1500 g for 10 min) and washed twice with 50mM HEPES buffer with 5 mM EDTA, pH 8. The cell pellet was resuspended in 10 mL of this buffer added with 1.0 µM fluorescent probe, carboxyfluorescein diacetate succinimidyl ester (cFSE). Cells were incubated for 10 min at 37 ºC, washed once in 50mM potassium phosphate buffer with 10 mM $MgCl_2$, pH 7.0, and resuspended in 10 ml buffer. To eliminate nonconjugated cFDA-SE, glucose 10 mM was added and cells were incubated for an additional 30 min at 37 ºC. Cells were washed twice, resuspended in 50 mM phosphate buffer pH 7.0 and placed on ice until needed [30].

pH_{in} assay

The pH_{in} of *P. vagans* was analyzed according to the method described by Breewer *et al.* [5] with minor modifications. A 96-well polystyrene microplate (Corning N.Y.) was loaded with 100 µL of fluorescently labeled (1×10^7 CFU/ml) cell suspension previously treated with the antimicrobial compounds (carvacrol, thymol, eugenol, cinamaldehyde) at concentrations of 1%, 2% and 4% were added. Fluorescence intensities were measured in a spectrofluorometr Victor X2 multilabel reader (Perkin Elmer) immediately and every minute for 10 min using and excitation wavelength of 490 nm and emission wavelength of 520 nm. During the assay, the system was maintained at room temperature (25°C). A fluorescence reading of the cell-free supernatant (background fluorescence) was measured after the 10 min assay. In this case, treated bacterial suspensions were centrifuged (1500 g 10 min) and fluorescence of the cell-free supernatant was measured and deducted from the treated suspensions.

Fluorometric assay for membrane potential

The method described by Pag *et al.* [27] was followed with minor modifications to measure changes in membrane polarity caused by the addition of carvacrol, thymol, eugenol and cinamaldehyde. *Pantoea vagans* cells were grown in 3 ml of 0.5 × Mueller Hinton (Difco) broth at 37°C to an OD610 of 0.5 (approximately 1×10^7 CFU/ml). Then, 1 µM membrane-potential-sensitive fluorescent probe bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC4_{(3)}$; Molecular Probes, Eugene, Oregon] was added for 5 min, followed by the addition of the antimicrobial compounds at concentrations of 1%, 2% and 4%. After 5 min, a 96-well polystyrene microplate (Corning N. Y.) was loaded with 100 µL of treated cell suspensions and fluorescence was measured at the excitation and emission wavelengths of 492 and 515 nm, respectively using the spectrofluorometer referred to above. Background fluorescence resulting from the extracts added to the medium was determined and the results corrected.

Statistical analysis

All experiments were performed in duplicate. A completely randomized design was used for the arrangement of the experiments. Data are presented as the mean plus/minus the standard deviation of three measurements. Mean values were compared using Tukey's test at $p< 0.05$ to consider significant differences.

Data were processed using JMP statistical software, version 5.0.1.

RESULTS AND DISCUSSION

Response of *P. vagans* isolated from Dahlia flowers to derived compounds from essential oils

Effect of antimicrobial compounds on morphology

The effect of antimicrobial compounds on *P. vagans* is shown in Fig. 1. The effect of antimicrobial compounds on P. vagans was similar among them. Compared with the control, changes are observed in the morphology of the treated bacterium. The culture exposed to the antimicrobials evaluated presented loss of turgor and the bacilli were deformed, with holes and in some cases cleaved. In studies with cinnamaldehyde applied to *E. coli* and *Salmonella* [36], similar behavior was observed in both organisms since applying the antimicrobial resulted in morphological alterations on these bacteria. Di Pascua *et al.* [10] report that derived of essential oils such as cinnamaldehyde and thymol cause structural alterations in the outer envelope of Gram-negative bacteria, while Burt and Reinders [7] observed that applying cinnamaldehyde to *E. coli* O157: H7 cells caused them to collapse and resulted in the release of the cell contents due to the damage to the outer membrane.

Effect of antimicrobial compounds on membrane integrity

To assess the effect of antimicrobial compounds on *P. vagans*, we used the LIVE/DEAD BacLight kit, which to give us an indication of the fraction of cells that are viable and nonviable (green bacteria and red bacteria, respectively) [22], the images are shown in Fig. 2. As is typical for this kit, the viable bacterial population demonstrated strong green fluorescence and weak red fluorescence, while a completely permeabilized population showed weak green fluorescence and strong red fluorescence.

There were not statistically significant differences among treatments; however, with respect to the control significant differences were found $p < 0.05$ (Fig. 3). All treatments exceeded the control (solvent) treatment. Red stained bacteria (dead cells) were higher than in the control. Percentage dead cells of the treated bacterium were in a range of 52 to 87 % approximately, indicating that the membrane of these cells had been damaged, and therefore became permeable to propidium iodide.

The mode of action by which microorganisms are inhibited by essential oils and their derived compounds could involve different mechanisms. Regarding bacterial susceptibility towards antibacterial agents, membrane permeability is part of the early bacterial defense [3]. Helander *et al.* [15] analyzed the effect of carvacrol on *E. coli* and *S. thyphimurium* and reported that carvacrol and thymol decrease the intracellular ATP content while extracellular ATP increases, indicating the disruptive action of these compounds on the cytoplasmic membrane. It has also been hypothesized that inhibition mechanisms may involve phenolic compounds, because they sensitize the phospholipid bilayer of the cytoplasmic membrane [18, 20]. Thus, for example, carvacrol increases the passive permeability of the microbial cytoplasmic membrane, by its ability to dissolve into the lipid bilayer, aligning itself between fatty acids and resulting in distortion of the physical structure of the cytoplasmic membrane [21].

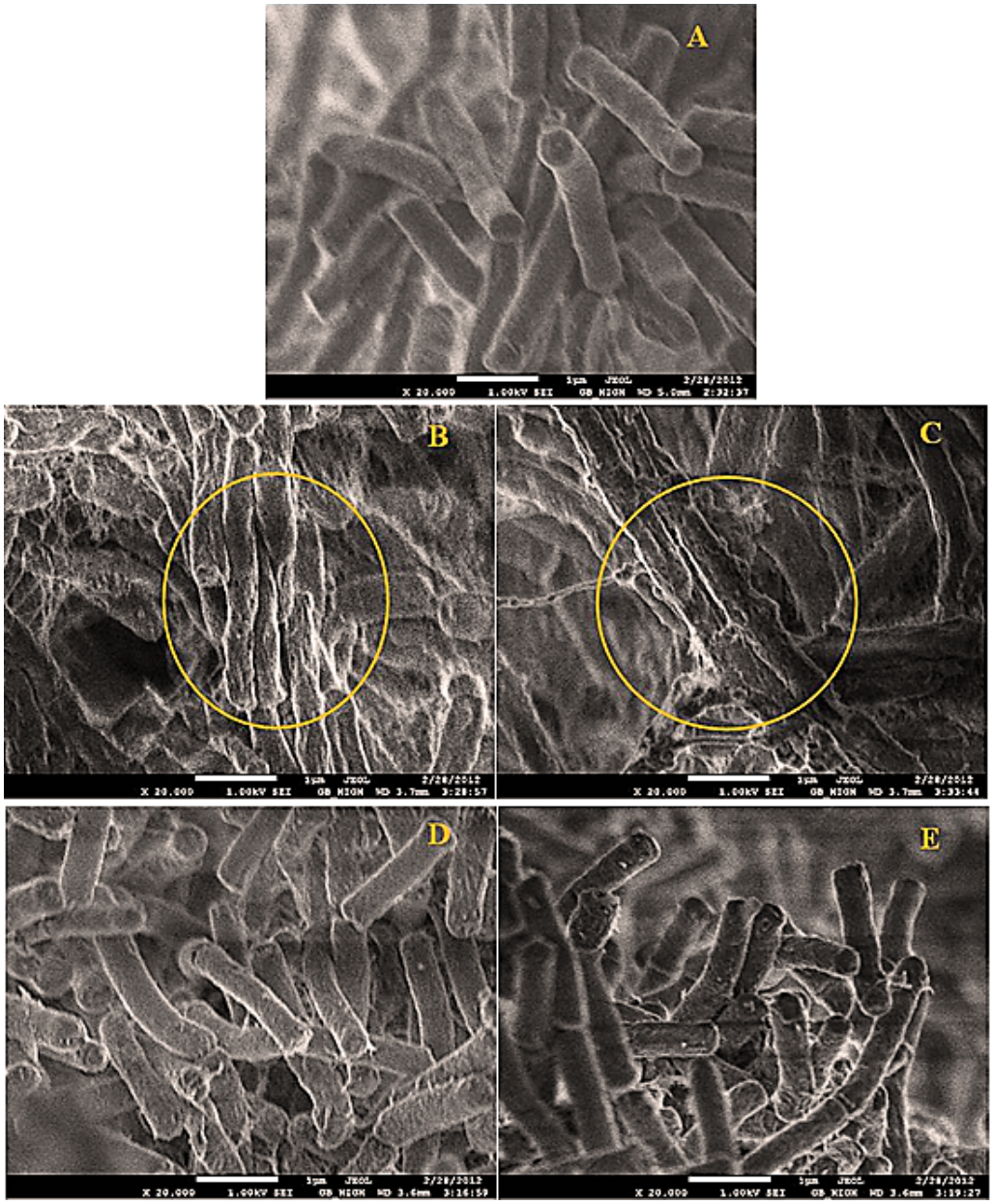

Fig. 1. SEM images (20,000X) of the **e**ffect of compounds tested on the morphology of *Pantoea vagans*. Bacteria treated with 50% ethanol (blank) (A), eugenol (B), thymol (C), carvacrol (D) and cinnamaldehyde (E). Bar corresponds to 1 µm.

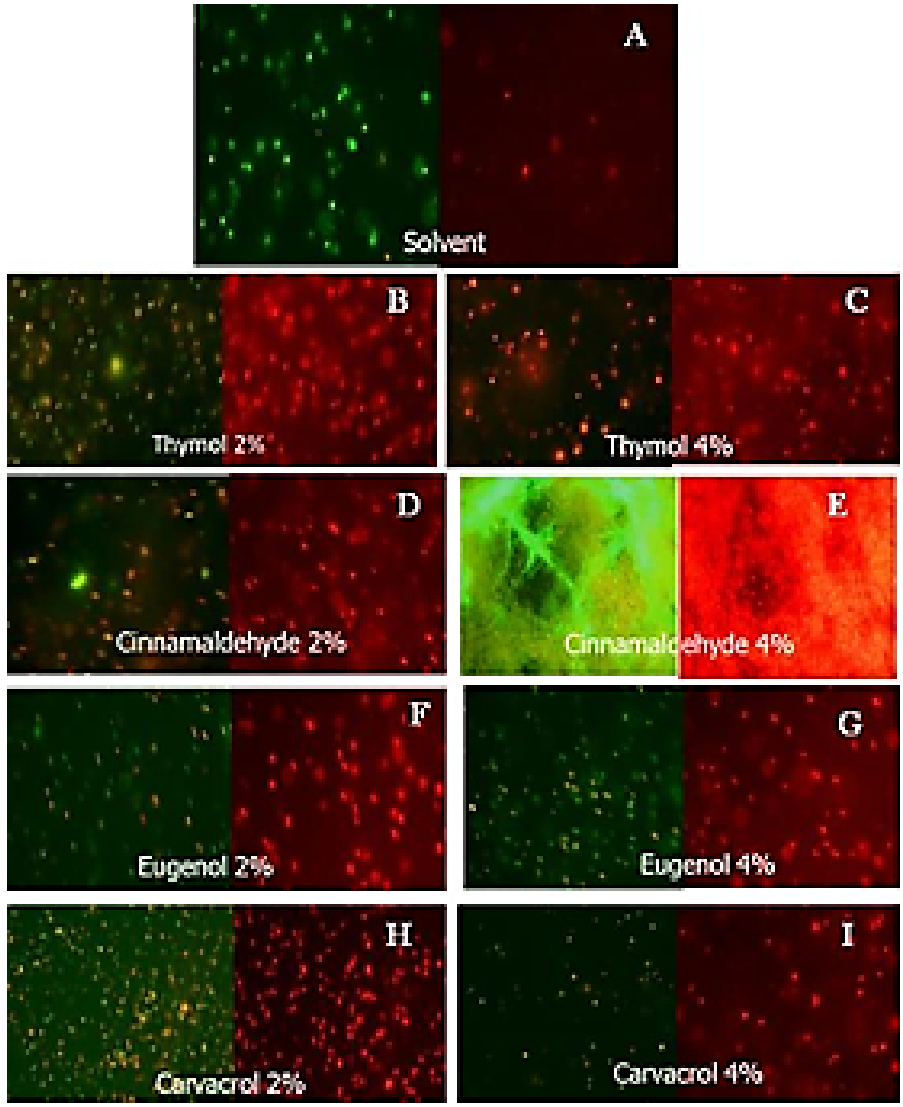

Fig. 2. Epifluorescence micrographics (100X) illustrating the effect of exposure to 2% and 4% thymol, cinnamaldehyde, eugenol and carvacrol on the integrity of the membrane of cells of *Pantoea vagans* in Mueller Hinton Broth (pH 7) by the LIVE/DEAD BacLight Kit.

Another compound derived from essential spice oils and tested in this work was eugenol (main component of clove oil), which has been tested against some Gram-negative bacteria such as *E. coli*, *Enterobacter aerogenes*, *Pseudomona aeruginosa*, *Salmonella typhimurium*, and *Proteus vulgaris*. It causes an increase in membrane permeability and interacts with proteins [16]. Eugenol's action on membranes occurs mainly by a non-specific permeabilization [17]. The hydroxyl group of eugenol is thought to bind to and affect the properties of proteins, accordingly contributing to eugenol's inhibitory effect at sub-lethal concentrations. Consistent with this, it has been shown that the eugenol inhibits the activity of the enzymes: ATPase, histidine decarboxylase, amylase, and protease [13, 33, 35,]. Inhibition of the ATPase may be important for cell killing at high eugenol concentrations (as tested in *P. vagans*) because energy generation needed for cell recovery is impaired [13]. On the other hand in many studies have demonstrated that cinnamaldehyde a phenylpropene aldehyde (analyzed also against *P. vagans*) interacts with the cell membrane, but it is not yet clear how it perturbs membranes. Bouhdid *et al*. [4] observed that *Cinnamomum verum* essential oil (73% cinnamaldehyde) caused membrane depolarization, loss of membrane integrity, reduced respiratory activity, and coagulation of cytoplasmic material of *Pseudomona aeruginosa.*

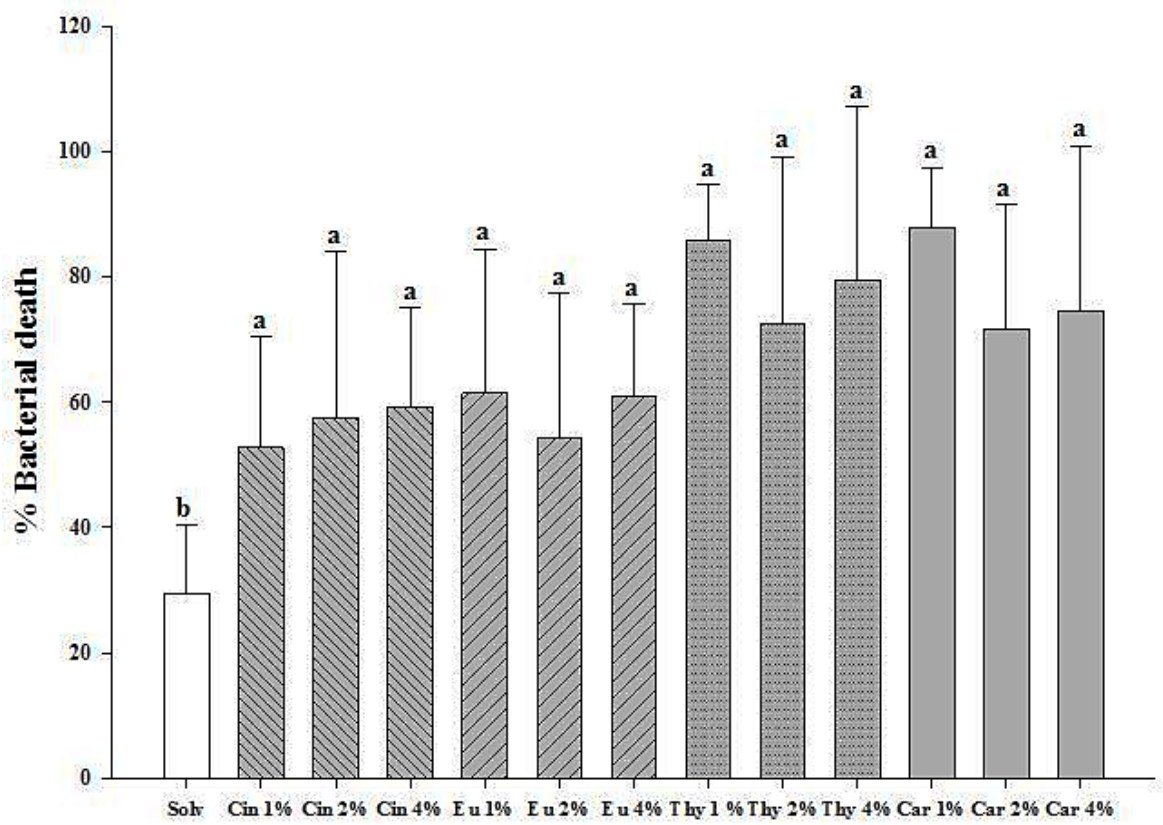

Fig. 3. Effect of exposure to 2% and 4% thymol, cinnamaldehyde, eugenol, and carvacrol on the integrity of the membrane of cells of *Pantoea vagans* in Mueller Hinton broth (pH 7) by the LIVE/DEAD BacLight system. The results are expressed as percent of dead bacteria. The results are the mean ± standard derivation of twenty-five measurements. Comparison of all means performed using the Tukey test with a value of $p < 0.05$. Different letters indicate samples are significantly different.

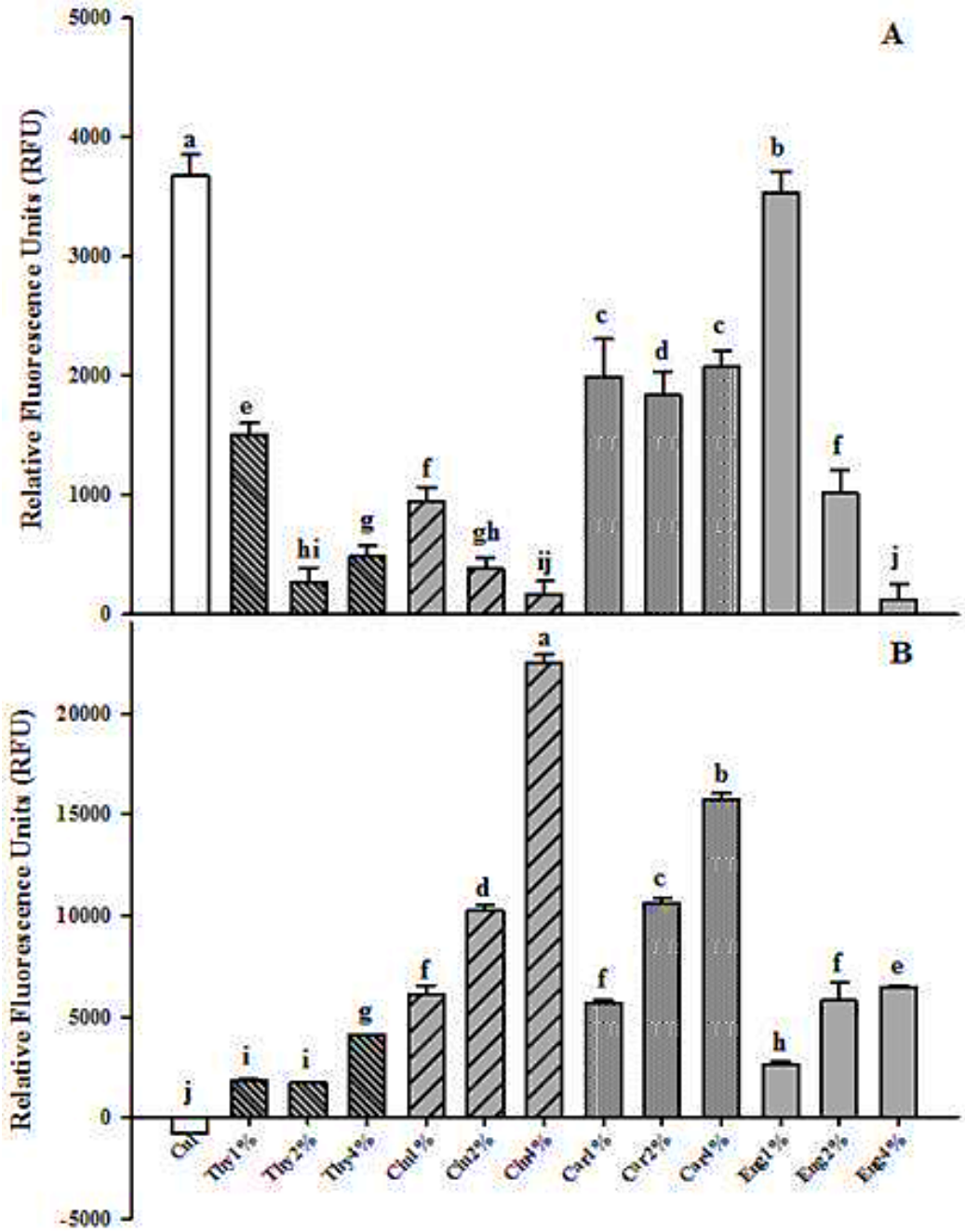

Fig. 4. Effect of antimicrobial compounds at different concentrations on intracellular pH_{in} (A) and membrane potential (B) of *Pantoea vagans*. The results are expressed as Relative Fluorescence Units (RFU). The results are the mean ± standard deviation of three measurements. Comparison of all means performed using the Tukey test with a value of $p < 0.05$. Different letters indicate samples are significantly different.

In others studies the cinnamaldehyde altered the membrane lipid profile with large increases in saturated fatty acids, yielding a more rigid membrane probably compensating for a fluidifying effect of cinnamaldehyde, and cell structure of *E. coli*, *S. enterica*, *P. fluorescens*, and *Bacillus thermosphacta*, while only *Staphylococcus aureus* demonstrated disintegration of the cell envelope [9, 10].

Effect of antimicrobial compounds on cytoplasmic pH (pHin)

The results are shown in Fig. 4A. It can be seen that unlike the control (culture treated only with the alcohol water solvent 1:1), overall, as the concentration of each antimicrobial compound increased (1% - 4%) there was a decrease in fluorescence in the treated cultures.

Fluorescence microscopy revealed that the treated cells with the antimicrobial compounds were poorly labelled with cFSE, due probably to an increased efflux of cFSE resulting from the cell membrane damage caused by the antimicrobial treatment. Sánchez *et al.* [30] evaluated the effect of extracts of edible and medicinal plants on *Vibrio cholerae* using CFDA-SE dye to monitor changes in internal pH (pHin) in bacteria treated with the antimicrobials and noted that the increase in fluorescence is directly related to changes in pH within the bacteria. Thus, in almost all cases examined in our study, pH decreases by increasing the concentration of the antimicrobial compound.

Depending on the microorganism and its growth condition, changes in internal or external pH can lead to subsequent changes in various primary physiological parameters such as the concentration of ions (K +, Na +), differences between internal and external pH, $\Delta\Psi$ (membrane potential) and ΔP (proton-motive force) [26]. Using the cFSE fluorescent test was useful in assessing changes in the pHin of the bacteria. cFSE has been previously used to determine cell division in lymphocytes and bacteria but can potentially be applied as a pH test, because the fluorescence emitted is dependent on pH. The bacteria can easily take the cFSE during incubation with its diacetate ester, cFDASE. Once incorporated, it is thought that its succinimidyl group forms conjugates with aliphatic amines in cells presenting fluorescence [8]. The pH decrease that occurs can be explained by the disruption of the cell membrane which controls among other processes DNA transcription, protein synthesis and enzymatic activity [29].

Effect of antimicrobial compounds on membrane potential

It can be seen in Fig. 4B, that increasing the concentration of the antimicrobial compound on the culture (*P. vagans*) resulted in an increase in fluorescence (relative fluorescence units, RFU), which results in the depolarization of the bacterial membrane. The DiBAC4(3) is a lipophilic and anionic bisoxonol. The uptake of this membrane potential-sensitive dye is restricted to depolarized cells or cells with broken cytoplasmic membranes. The fluorescent dye accumulates within cells by binding to intracellular proteins and membranes [2]. It is therefore used to monitor changes in membrane potential by measuring the fluorescence of cells that have been stained because of their interaction with the dye. The fluorescence emitted by the dye is increased when it crosses the cell membrane as a result of the depolarization, due to the increase in membrane permeability leading to ion release.

Membrane potential plays an essential role in bacterial physiology. As a component of proton-motive force, it is closely linked to ATP production [11]. It is also involved in various processes such as bacterial autolysis, glucose transport and chemotaxis, and it survives at low pH. In metabolically-active bacteria, membrane potentials are generated by differences in the concentration of ions on

opposite sides of the bacterium [31]. Regarding the results of the effect of the antimicrobial compounds against *P. vagans*, these again affected the membrane by increasing its permeability to some particles, including ions which produced as earlier mentioned changes in membrane potential. The same behavior has been previously observed in bacteria such as *Salmonella* spp., *Brucella* spp., *Pseudomona* spp., *E. coli*, *S. aureus*, and *Bacillus subtillis*, among others [1].

CONCLUSIONS

The use of essential oils derived compounds as antimicrobial compounds have been evaluated in previous studies, but the mechanisms by which these compounds act has not been fully elucidated. In our study, **t**he antimicrobial activity of derived compounds against growth of *P. vagans* was demonstrated. Thymol, eugenol, carvacol and cinnamaldehyde altered and disrupted the bacterial morphology and membrane causing deformation and increase in permeability. A decrease in cytoplasmic pH and depolarization was also observed.

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