Korean J. Chem. Eng., **27**(4), 1252-1257 (2010)
DOI: 10.1007/s11814-010-0196-5

Inhibiting biofilm formation of *Enterobacter* sp. prevented premature withering in cut flowers

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(*Received 21 October 2009 • accepted 8 December 2009*)

Abstract−Early withering and premature flower fall are a growing menace to the cut flower industry, the reasons of which were considered to be varied from loss of water uptake, bacterial proliferation, decay in tap water etc. In the present study, we identified the bacterial biofilm formation by *Enterobacter* sp. and subsequent water uptake blockage as the root cause of early withering in cut flowers using Chrysanthemum, Yellow Daisy and Maroon Rose as model plants. The biofilm-forming *Enterobacter* sp was identified by 16 S rDNA sequencing data. Studies on biofilm were conducted by using field emission scanning electron microscope electron back scattering diffraction (FE-SEM-EBSD), Atomic force microscopy (AFM) and fluorescence microscopy. *In vitro* and *in vivo* studies were conducted with different antimicrobials to prevent biofilm formation in both conditions. Most antimicrobials were toxic to plants, but we found citric acid 1,000 μg/ml and calcium hypochlorite 50 μg/ml to be most effective in preventing biofilm formation and extending the vase life of cut flowers. We studied the synergistic action of different combinations *in vivo* and suggest citric acid 1,000 μg/mL, Ca hypochlorite 50 μg/mL and glucose 1,000 μg/mL as the best combination to be used for prolonging vase life of cut flowers from 10 days (non-treated) to 30 days (treated).

Key words: Water Uptake, Cut Flower, Withering, *Enterobacter* sp., Biofilm

INTRODUCTION

The vase life in cut flowers is often limited to 10-15 days and the major reason is vascular occlusion development due to various factors, such as bacteria, air emboli and physiological responses of stems to cutting [1-5]. Bacterial proliferation is largely responsible for vascular occlusion, which shortens the vase life of cut flowers. Our present study was intended to find a suitable antimicrobial agent to inhibit bacterial proliferation so as to increase the vase life in cut flowers. We also identified the biofilm-forming bacteria on the stem bottom which was causing the vascular blockage and subsequent early withering in cut flowers. The aggregates of microbial cells that exist in close association with the surfaces are referred to as biofilms and have tremendous impact on the local environment [6]. Biofilm formation is akin to the developmental pathway [6]. Initial attachment of bacteria to a surface is often mediated by filaments that extend from the bacteria. These proteinaceous appendages can serve as anchors, transiently fixing the bacteria on location. Once attached to the surface, biofilm-associated bacteria initiate the synthesis of an extra cellular matrix, which generally signifies their commitment to a sedentary existence [7]. Thus during biofilm formation, the *milieu* at or near the surface of bacteria undergoes dramatic alterations.

In the present study, the biofilm community was isolated and identified as *Enterobacter* sp. Vascular blockage was studied by SEM, AFM and fluorescence microscopy. Antimicrobial susceptibility was tested first *in vitro* and the effective agents were tested *in vivo* to study its toxicity to plant, effect on water uptake and potency to disrupt biofilm and enhance water uptake. SEM pictures of the present study clearly indicated that the surface attachment of bacteria is not by pili but by a protoplasmic extension from its cell. Biofilm formation by *Enterobacter* sp. is not previously reported and its mode of attachment and embedding in extra polymer substance (EPS) matrix is very peculiar.

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MATERIALS AND METHODS

1. Isolation and Identification of Biofilm Community

The cut flowers were put in tap water for 10-15 days. After that the bottom portion of the stem was observed for presence of biofilm indicated by jelly-like transparent matrix and gently pressed on to the Mueller Hinton (MH) or nutrient agar (NA) surface and incubated overnight. The plate showed thick growth of film and organisms were separated on fresh nutrient agar plates by quadrant streak method. The single isolates were identified by 16S rDNA based PCR [8].

2. Antibacterial Screening of Antimicrobials Against the Biofilm Community *In Vitro* and *In Vivo*

Different antimicrobials (natural and synthetic) were tested for their activity against biofilm-forming *Enterobacter* sp. *in vitro* by agar diffusion technique incorporating 20,000 μg/ml of compound on to sterile disc and placing them in MH agar and zone of inhibition measured after overnight incubation. Those found to be active *in vitro* were tested *in vivo* adding 0.1% of antimicrobial to 400 ml tap water and putting the cut flowers for 20 days. After 20 days the presence of biofilm was checked by replica plating of stem bottom to MH and NA plates followed by overnight incubation.

3. MIC Determination and Measuring Water Uptake Rate

Those antimicrobial compounds which effectively prevented biofilm both *in vitro* and *in vivo* were considered for determining MIC provided that the compounds *in vivo* did not show any kind of toxicity to plants. The above criterion was included in studies since a compound toxic to plants cannot be exploited commercially. One ml of *Enterobacter* suspension grown in peptone broth (1×10^6 CFU/ml equivalent to 1 : 100 dilution of a suspension of turbidity equal to McFarland standard 0.5) was added to tubes containing 1 ml of peptone broth and serial dilutions of antimicrobial agent ranging from a concentration of 20 mg/ml reaching up to a final dilution of 1 μg/ml were tested. A serial dilution of antimicrobial agent was prepared in sterile double distilled water. After overnight incubation, visual turbidity was noted and the highest dilution showing no turbidity was taken as the minimum inhibitory concentration of antimicrobial against the biofilm forming community [9].

In *in vivo* experiments, water uptake rate was determined by measuring the tap water remaining after fixed days. Citric acid was included in the *in vivo* experiments since it is considered to be a low pH provider enhancing water uptake [10].

4. Observation of Antimicrobial Effect on Plant Leaves and Stem

In *in vivo* experiments, after the cut flowers were put in tap water with 0.1% of antibacterial agents, they were observed daily for toxicity to plants. This was done by visual examination everyday and recording the results**.** Water uptake rate was determined after 15 days by measuring the volume of water remaining and taking the average as per day water uptake. CFU counting was done by serial dilution spread plate method at five days interval and recorded.

5. Studies of Calcium Hypochlorite Alone and in Combination on Extending the Vase Life of Cut Flowers

In vivo effect of calcium hypochlorite alone and in combination with citric acid and glucose on extending the vase life of cut flowers was studied. Control with tap water (sterilized and nonsterilized) was used for this experiment.

6. FE-SEM-EBSD, AFM and Fluorescence Microscopy

After the 15th day, stem cuttings from the lower portion of cut flowers of 5 mm length were taken keeping aseptic conditions and freeze dried overnight for SEM. Instrument specification: S-4300 SE Hitachi FE-SEM-EBSD (field emission scanning electron microscope electron back scattering diffraction). Ion sputter specification: E-1030 Ion sputter–Hitachi. Freeze dried stem cuttings (5 mm length) were attached to stainless steel metal stub with NEM tape (Nisshin Emco Ltd). The sample was coated with Pt-Palladium using Hitachi 1030 ion sputter and observed with S-4300 SE FE-SEM-EBSD. Three samples were scanned under different resolutions including a control which was from cut flowers put in autoclaved tap water.

A new technique was devised since AFM observation can be done only with flat samples with thickness <0.5 mm. The bottom portion of cut flower stem was gently pressed onto the middle portion of a cover slip after the cover slip was fixed to a glass slide with cello tape. After drying in air the cover slip was removed and specimen portion was cut with glasribe F 44150 Bel Art Diamond glass cutter pen. The specimen with cover slip was mounted onto the steel tub with Axia 025 gold gum. Images of different resolution from 1 μm to 20 μm were observed.

For fluorescence microscopy, biofilm was impregnated onto a glass slide by gently pressing the bottom portion of cut flower onto the glass surface of the slide and air dried. A Nile red stock solution was prepared in acetone under subdued light conditions in dark room and diluted with 75% aqueous glycerol to a final concentration of 2.5 μg/mL and stored in a colored bottle after degassing in vacuum to remove bubbles [11]. The specimen was stained with one drop of Nile red/glycerol staining solution in a dark room and examined in fluorescence microscope (Olympus BX51) using mirror unit U-MWB2 with excitation filter 460-490 and emission filter 520 IF with 500 dichromatic mirror. Software used was DP Controller.

RESULTS AND DISCUSSIONS

1. Sequencing of PCR Products: The 16S r DNA Sequence Analysis Data

1-1. Forward Sequencing Data

NNNNNNGGACGTACGGATACGGGCGTAAGCGCACGC
AGGCGGTCTGTCAGTCGGATGTGAAATCCCCGGGCTCA
ACCTGGGAACTGCATCCGAAACTGGCAGGCTTGAGTCT
CGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAAT
GCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGG
CCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCG
TGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTG
GGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT
CGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCC
ACAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAAC
TGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTT
GTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTATCCTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAA
GGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGAT
GACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACA
CACGTGCTACAATGGCGCATACAAAGAGAAGCAATCTC
GCGAGAGCTAGCGGACCTCATAAAGTGCGTCGTAGTCC
GGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAAT
CGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGT
TCCCGGGCCTTGTACACACCGC

1-2. Backward Sequencing Data

NNGGGTTNNNNNNNGGACTTTTCACTGACGTCGTCT
TCGTCCGGGGGCCGCCTTCGCCACCGGTATTCCTCCAG
ATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCC
CCCTCTACGAGACTCAAGCCTGCCAGTTTCGGATGCAG
TTCCCAGGTTGAGCCCGGGGATTTCACATCCGACTTGA
CAGACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGAT
TAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCA
CGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATC
GACGTGGTTATTAACCACATCGCCTTCCTCCCCGCTGAA
AGTACTTTACAACCCGAAGGCCTTCTTCATACACGCGG
CATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCC
CCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCA
GTTCCAGTGTGGCTGGTCATCCTCTCAGACCAGCTAGG
GATCGTCGCCTAGGTGAGCCGTTACCCCACCTACTAGCT
AATCCCATCTGGGCACATCTGATGGCAAGAGGCCCGAA
GGTCCCCCTCTTTGGTCTTGCGACGTTATGCGGTATTAGC
TACCGTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTTC

CCAGACATTACTCACCCGTCCGCCACTCGTCAGCGAAG
CAGCAAGCTGCTTCCTGTTACCGTTCGACTTGCATGTGT
TAGGCCTGCCGCCAGCGTTCAATCTGAGCAGNNNNAA
AANCTTAAAAANNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNN

Both forward and backward sequences were pasted onto the NCBI website and analyzed with BLAST software (website: http://www.ncbi.nlm.nih.gov/blast). 99% Similarity was obtained with different species of *Enterobacter* sp. and therefore the biofilm forming species was designated as *Enterobacter* sp. Extension of vase life of cut flowers by inhibiting bacterial proliferation was the primary aim of this study, but the gathering of primary data culminated in the identification of biofilm formation and subsequent water uptake blockage caused by it resulting in premature withering of cut flowers. The bacterial species showed 99% homology with different strains of *Enterobacter* sp. in 16S rDNA sequence analysis using BLAST of NCBI. The phylogenic tree diagram also indicated its closest relation to different species of *Enterobacter (*data not shown).

2. Antibacterial Screening of Antimicrobials Against the Biofilm Community and MIC Determination

The activity of compounds against biofilm-forming *Enterobacter* sp. *in vitro* was recorded as given in Table 1. Antimicrobials with zone of inhibition 25 mm were considered sensitive and were used in *in vivo* experiments. *In vivo* effect of antimicrobials on plants and their effectiveness in preventing biofilm formation is recorded as Table 2. Only calcium hypochlorite was found to be effective against *Enterobacter* sp. both *in vitro* and *in vivo* without causing toxicity to the plant. Its MIC was determined as 100 μg/ml by macro broth serial dilution method. Neomycin sulphate and citric acid which were active *in vitro* were not active *in vivo* in breaking down the biofilm. Water uptake rate was measured and recorded as Fig. 1. Tap water contains 2×10^5 CFU/ml, which increased to 3×10^7 CFU/ml on 5th day and 5×10^9 CFU/ml on 10th day and 7×10^{12} CFU/ml on 15th day.

Table 1. Antimicrobial activity of different antimicrobials *in vitro* against *Enterobacter*

Name of antimicrobial (20,000 μg/ml)	Zone of inhibition
Ampicillin	R
Grape seed extract	R
CTC-HCl	12 mm
Colistin	14 mm
Scutella root extract	R
Apigenine	R
Grape fruit extract	R
Ciprofloxacin	S (45 mm)
Citric acid	16 mm
Neomycin sulphate	S (3 mm)
*T-Neem oil 10 μl+2.5 μl+997.5 μl DW	R
**Negative C- 2.5 μl+997.5 μl DW	R
Tea seed extract	R
$KMnO_4$	R
Tobacco extract	R
Garlic aqueous extract	R
Thymol	R
Sodium chlorite	26 mm (S)
Chloramine T trihydrate	R
Sodium hypochlorite	70 mm (S)
Glutaraldehyde	15 mm
Phenol	30 mm (S)
Calcium hypochlorite	62 mm (S)

R: Resistant (No zone of inhibition) S: Sensitive (≥25 mm zone inhibition)

*Test **Negative control DW: Distilled Water

Table 2. *In vivo* effect of antimicrobials and their effect on plant and effectiveness in preventing biofilm formation

Antimicrobial name	Toxicity to plant	Biofilm resistance
Ciprofloxacin	−	−
Neomycin sulphate	−	+
Citric acid	−	+
Sodium chlorite	+	−
Sodium hypochlorite	+	−
Phenol	+	−
Calcium hypochlorite	−	−

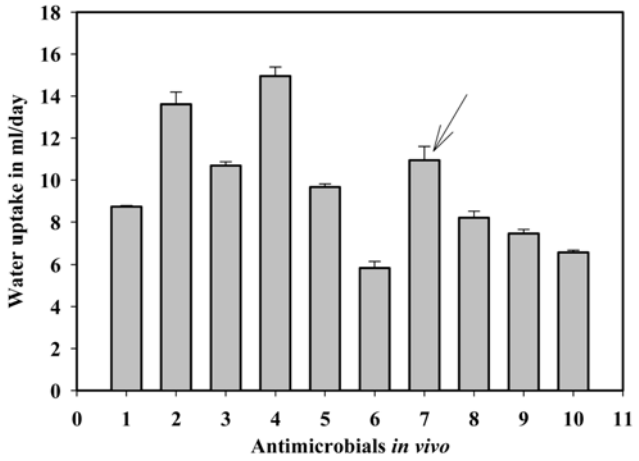


Fig. 1. The water uptake rate of different antimicrobials *in vivo*. 1. Glucose; 2. Citric acid; 3. Tap water; 4. Autoclaved tap water; 5. Neomycin sulphate; 6. Ciprofloxacin; 7. Calcium hypochlorite; 8. Sodium chlorite; 9. Phenol; 10. Sodium hypochlorite. Arrow indicates comparatively higher water uptake rate of cut flowers in calcium hypochlorite solution.

Table 3. *In vivo* effect of calcium hypochlorite alone and in combinations on extending the vase life of cut flowers (the data indicated is for chrysanthemum). Control with tap water (sterilized and nonsterilized) also shown

Medium used	Vase life in days
Tap water	5
Autoclaved tap water	10
Ca hypochlorite 50 μg/mL in tap water	28
Citric acid 1,000 μg/mL+Ca hypochlorite 50 μg/mL+Glucose 1,000 μg/mL in tapwater	39

3. Studies of Calcium Hypochlorite Alone and in Combination on Extending the Vase Life of Cut Flowers

Studies of calcium hypochlorite alone and in combination on extending the vase life of cut flowers *in vivo* clearly showed its effectiveness in breaking biofilm and increasing the water uptake rate. Results are shown in Table 3.The best combination was found to be citric acid 1,000 μg/mL, Ca hypochlorite 50 μg/mL and glucose 1,000 μg/mL which can be used for prolonging vase life of cut flowers beyond 30 days. The other tested antimicrobials were found to be not significant for this purpose because of toxicity to plants, biofilm resistance and low water uptake rates.

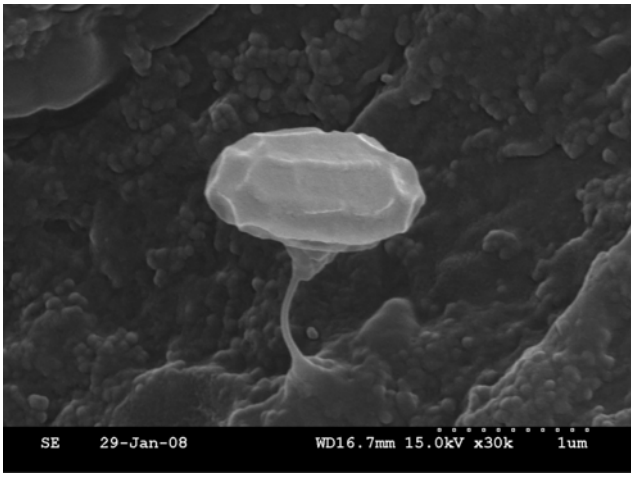
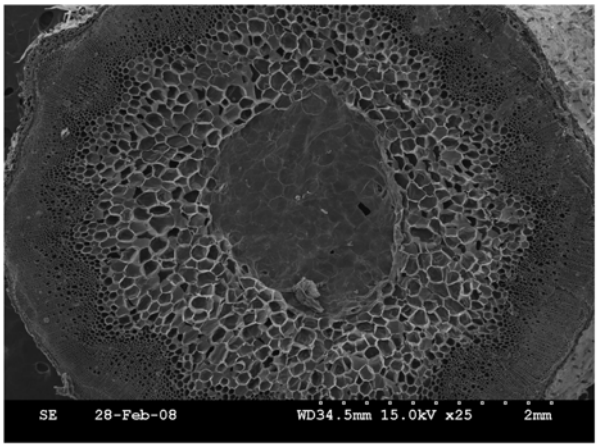
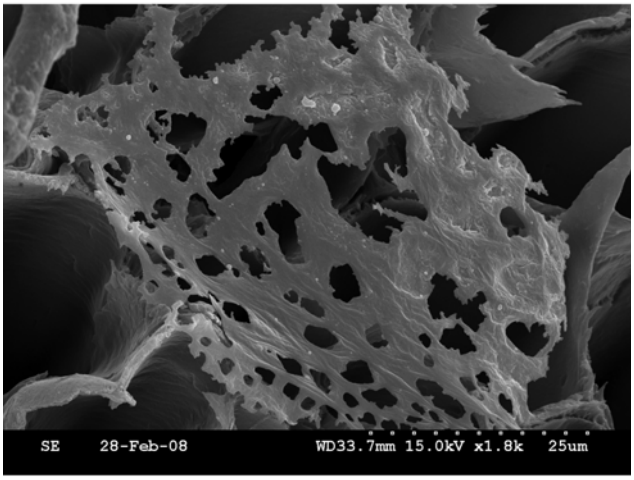


Fig. 2. Attachment and anchoring of *Enterobacter* to surface by protoplasmic extension.



(a)

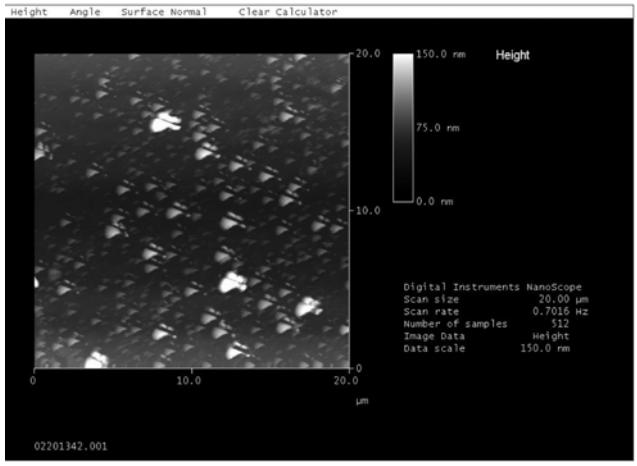


(b)

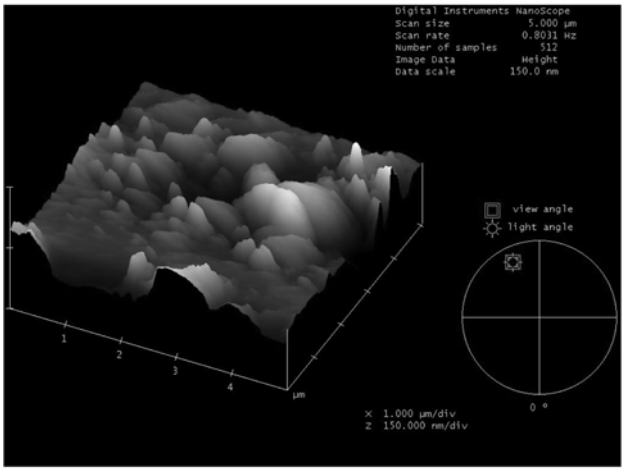
Fig. 3. Surface of cut flowers in (a) autoclaved tap water and (b) non-autoclaved tap water. Note the biofilm formed in (b).

4. SEM, AFM and Fluorescence Microscopy

Results are given as Figs. 2, 3(a), 3(b), 4(a), 4(b) and 5. Vascular blockage was evidenced in the SEM pictures [Fig. 3(b)], which might probably have a detrimental effect on water uptake leading to withering of leaves and flowers. As per AFM, sample thickness data is 150 nm-200 nm [Fig. 4(a) and 4(b)]. The fluorescence microscopy revealed red fluorescence, indicating the layer of hydrophobic layer of phospholipids, other amphipathic lipids and strongly hydro-



(a)



(b)

Fig. 4. (a) AFM. The arrangement of bacteria as a mosaic pattern embedded in hydrophobic lipid matrix. Brown colored regions are the lipid matrix indicating the surface height 0 nm, and whitish regions indicate projecting bacterial cells up to 150 nm thickness forming biofilm. (b) AFM. 3D view of biofilm surface: sample height 150 nm, intestinal villi like projections to the outer surface clearly visible. Matrix is uniform but not flat in the 3D view.

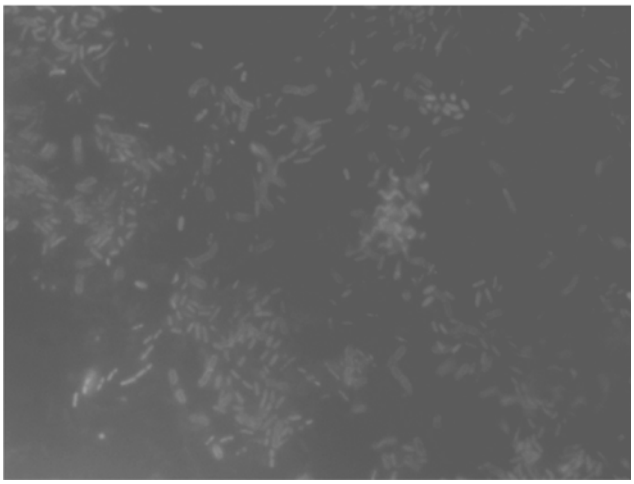


Fig. 5. Lipid hydrophobic layer showing red fluorescence after Nile Red Staining Neutral lipids-yellow gold fluroscence. Hydrophobic lipids-red fluorescence (100 X objective view).

phobic proteins [12]. Yellow gold fluorescence indicated the surface lipids of bacteria which are neutral (Fig. 5).

The bacteria was anchored to dead plant surface by protoplasmic extension as evidenced by the SEM studies and there is secretion of exopolysaccarides (EPS) to create a protective matrix surrounding the cells. The biofilm is reported to offer resistance to antimicrobials in *in vivo* conditions [13]. Fluorescence microscopy by using Nile red indicated the presence of a hydrophobic lipid barrier which gave red fluorescence indicating the presence of strongly hydrophobic lipids and proteins [12]. The bacterial membrane lipids which are neutral lipids were visible as yellow gold fluorescent structures which were embedded in the hydrophobic lipid matrix with strong red fluorescence, indicating the strong hydrophobic nature of biofilm. AFM indicated the presence of bacterial cells being embedded in the lipid matrix, and the source of these lipids is quite unknown from the present studies.

Biofilm formation of *Enterobacter* sp. has not been previously reported. Even though many antibacterial agents were used for *in vitro* and *in vivo* studies against the biofilm-forming community, only calcium hypochlorite was found to be suitable for industrial use at low concentrations, i.e., ≤1,000 μg/ml. Increased concentrations of this antimicrobial are found to have negative impact on plant vigor and freshness as per our repeated observations and studies.

Citric acid, even though not effective in breaking down the biofilm *in vivo*, it was found to decrease the pH of tap water from 7.6 to 2.8, and that might be the reason for its enhancement of water uptake. The advantage of using citric acid is that it did not have any detrimental effect on the plant even at higher concentrations. Water uptake was maximum in the case of cut flowers put in autoclaved tap water, indicating bacterial growth and biofilm formation as the major cause for lessened water uptake of cut flowers which are put in tap water in florist shops. The antibiotic resistance offered in biofilm may be due to the hydrophobic barrier created by the presence of lipids which slow down the antimicrobial penetration. Identification of suitable antibacterial agent to be used in industry faces limitations due to the toxicity of most agents to the plant resulting in plant foliage loss and withering. The effectiveness of calcium hypochlorite may be due to the fact that it is based on lime and provides active chlorine in water, which prevents bacterial growth.

Chemotaxis has an important role in biofilm formation in several microorganisms and it guides bacteria to swim toward nutrients (hydrophobic pollutants) that are adsorbed to a surface, which is followed by surface attachment using the bacterial flagellum [14,15]. Flagella are required for attachment to abiotic surfaces and facilitate the initiation of biofilm formation [14,16]. In addition, chemotaxis and/or motility might be required for bacteria within a developing biofilm to move along the surface to grow and spread [16]. But *Enterobacter* sp which formed the biofilm community in our present studies was attaching to surface not by pili or flagella but a kind of extension from its own protoplast (Fig. 2: SEM).

Neomycin sulphate and citric acid, which were active *in vitro,* were not active *in vivo* in breaking down the biofilm. Even though they were active *invitro* against *Enterobacter* sp, *in vivo* they gave negative results.This confirms the role of biofilm formation in offering resistance to a pathogen in infections involving biofilm formation. The persistence of *Pseudomonas aeruginosa* during cystic fibrosis infections has been linked, in part, to its ability to form biofilms [17]. Biofilms play a significant role in infectious diseases [18]. Bacteria within biofilms are usually embedded within a matrix, which can consist of protein, polysaccharide, and nucleic acid. The matrix plays a crucial role in the biofilm resistance phenotype. Bacteria in a biofilm are less susceptible to antimicrobial agents and are protected from the host immune response, giving rise to chronic infections that are notoriously difficult to eradicate [19,20]. A complete understanding of the organization and composition of the Enterobacter sp. biofilm matrix may assist in the development of novel therapeutics aimed at disrupting biofilms, which will translate into improved water uptake prolonging the vase life of cut flowers in industry.

CONCLUSION

Premature withering of cut-flower is an economic loss in the flower industry. In this study, we have identified the microbe, *Enterobacter* sp., which forms biofilm in the base of cut flowers. Prevention of biofilm by various combinations of ingredients, including antimicrobials and citric acid, significantly improved the water uptake of cut-flower, increasing the vase-life of cut flowers from 10 to 30 days. This result provides practical and beneficial information to the cut-flower industry.

ACKNOWLEDGEMENTS

This research was supported by Inha University for which the authors are grateful.

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