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## Identification of Endogenous Bacteria in Micropropagated *Helleborus ×nigercors*

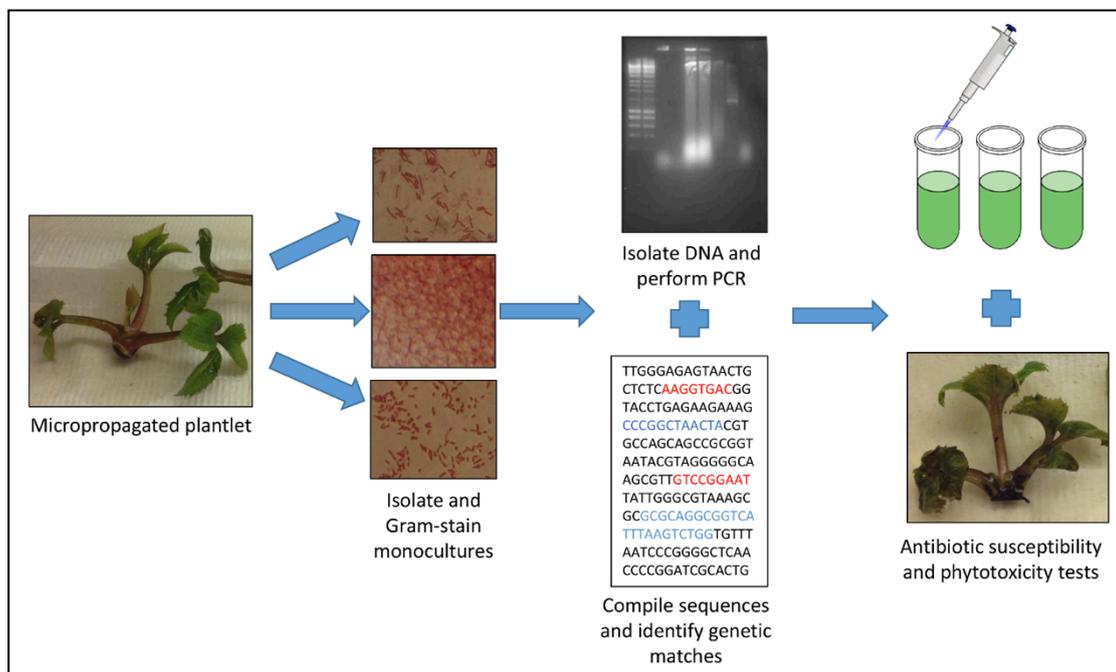
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### Graphical Abstract:



**Abstract:** During micropropagation of *Helleborus ×nigercors*, plantlets were observed to be bacterially contaminated. To determine the identity of contaminants, bacteria resistant to surface sterilization were isolated and Gram stained. Polymerase Chain Reaction (PCR) and 16S rRNA sequencing were used to identify bacterial isolates H7G and H7S as belonging to the *Paenibacillus* and *Luteibacteri* genera, respectively. Strain H7R had highest sequence similarity to the *Pseudomonas*, *Stenotrophomonas*, and *Lysobacter* genera. Strains H7R and H7S were unable to grow in the absence of plant tissue and other bacterial species. *Paenibacillus* sp. H7G was screened using combinations of antibiotics including streptomycin sulfate, gentamicin sulfate, and cefotaxime, and was only eradicated by concentrations of gentamicin sulfate above phytotoxic levels. This is the first documented exploration of bacterial endophytes associated with *Helleborus* species.

**Keywords:** Endophytes; *Helleborus ×nigercors*; Micropropagation; Antibiotics

### Introduction

An endophyte is an organism living within plant tissue. The term is associated with microbial organisms that are symbionts, often mutualists, which provide plants with a benefit of some kind, such as protection against pathogens or aid in rooting (Wilson 1995). Endophytic organisms are of special interest to the scientific community due to the close biological complexes they develop with their hosts. Because of these close interactions, they may possess a range of biological activities that contribute to the plant's success in nature (Strobel 2003).

Endophytes found in micropropagated plantlets have in some cases been shown to lower plant quality due to the lack of competition that the 'biological vacuum' within sterile, carbohydrate-rich vessels promotes (Cassells 1997). However, in other cases, bacterial endophytes have contributed to plant growth by promoting hormone production and aiding in rooting (Dias et al. 2009; Lata et al. 2006).

During the multiplication stage, micropropagated plantlets of *Helleborus ×nigercors* were observed to be visibly contaminated with bacteria as indicated by the presence of a gray halo around the base of plantlets. Following sterilization with sodium hypochlorite, three bacteria remained prevalent

in culture. These three bacteria were likely harbored within the plant tissue, allowing them to survive surface sterilization. To better understand the biological complex found in hellebore cultures, putative endophytic bacteria were isolated and characterized. Although fungal endophytes have been isolated from other members of the *Helleborus* genus (Spadaro et al. 2014), this is the first study in which bacterial endophytes have been identified.

## Materials and Methods

**Plant Material and Reinitiation Procedure.** Plant material for micropropagation was obtained from Pine Knot Farms in Clarksville, Virginia, where the original cross was completed. Parent plants were grown in containers with a combination of native soil characterized by silt loam on top of fine, kaolinitic red clay, PermaTill® (Arden, NC), and Metro Mix® 852 heavyweight bark mix (BFG Supply Co.; Burton, OH). Shoot tips were initially sterilized at the Institute for Sustainable and Renewable Resources® (ISRR; Danville, Virginia), following which they were transferred to Clemson University for propagation.

Initiated plantlets were multiplied for several cycles before contamination was evident by clouding of the media. Thirty-two contaminated plantlets were disinfested using four possible treatment combinations: high or low bleach concentrations (0.83% and 0.41% sodium hypochlorite, respectively), and short and long time intervals (1 and 4 min). After soaking in the bleach solution, plantlets were rinsed twice with sterile deionized water and placed into Smithers-Oasis flexible Tissue Culture Vessels (Smithers-Oasis, Kent, OH). Vessels contained 25 mL of liquid WPM salts (Lloyd and McCown 1980) at pH 5.7, 3% sucrose, and 9  $\mu\text{M}$  TDZ. Plants were placed under 28  $\mu\text{mol m}^{-2} \text{s}^{-1}$  delivered from monochromatic LED lights (33.3% blue, 66.7% red) at 10-13 °C on a rocker shaker (EW-51301-00, Cole-Parmer® Portable Rocker Shaker, Vernon Hills, IL) set to 3 rpm.

**Isolation and Characterization of Endogenous Bacteria.** Slices of callus from reinitiated plantlets were streaked onto Petri plates containing tryptic soy agar (TSA) (470193-226, VWR Scientific Products, Suwanee, GA) and incubated for 24 h at 30 °C to detect contamination. To establish purity of culture, individual colonies were subcultured, incubated for 24 h, and stored in a refrigerator at 10 °C in darkness. Individual colonies from subcultured plates were restreaked to maintain vigor. Individual colonies were selected at random from the agar plates for genetic analysis. Gram reactions and colony and cell morphological characteristics were repeated in triplicate.

Polymerase chain reaction (PCR) was conducted on culture isolates using a transfer loop to inoculate each culture into a sterile Eppendorf tube (20901-547, VWR Scientific Products, Suwanee, GA) containing 50  $\mu\text{L}$  of Promega nuclease free water (PAP1195, VWR Scientific Products, West Chester, PA). Tubes containing the culture and water mixtures were placed into boiling water for 10 min. After boiling, 12.5  $\mu\text{L}$  of the mixture was transferred to a sterile PCR tube to be used as a DNA template. Two primers were added to the

tube: 1  $\mu\text{L}$  of the forward oligonucleotide primer (16S rRNA For, 5'AGAGTTTGATCCTGGCTCAG 3', ReadyMade™ Primers, Integrated DNA Technologies, Coralville, IA), and 1  $\mu\text{L}$  of the reverse oligonucleotide primer (16S rRNA Rev, 5'ACGGCTACCTTGTTACGACTT 3', ReadyMade™ Primers, Integrated DNA Technologies, Coralville, IA), as well as 10.5  $\mu\text{L}$  of Promega GoTaq® Green Master Mix (PAM7122, VWR Scientific Products, West Chester PA).

Each PCR reaction tube containing the DNA template, primers, and GoTaq® Green Master Mix was placed in a thermocycler (iCycler iO, BioRad Laboratories, Inc., Richmond, CA). The thermal cycle program consisted of 1 cycle of 95 °C for 2 min, followed by 30 repeating cycles of 94 °C for 30 s, 50.6 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. The cycling program ended by holding the tubes at 4 °C until removal from the thermocycler (Hayes et al. 2012; Promega Corporation 2012). Prior to sequencing, the concentration and 260:280 ratio of the PCR-amplified products was measured using a NanoDrop 2000 spectrophotometer (ND-2000, Thermo Fischer Scientific, Pittsburg, PA).

The PCR-amplified products were observed by gel electrophoresis in 1.5% agarose gels. Ten  $\mu\text{L}$  of each PCR product and the HyLadder™ molecular mass marker (Denville Scientific Inc., CB4225-2, Metuchen, NJ) were examined using agarose gel electrophoresis with subsequent ethidium bromide staining (97064-970, VWR Scientific Products, Suwanee, GA). The amplified DNA fragments were visualized by UV illumination.

Purification of PCR products was completed with Promega Wizard® SV Gel and PCR Clean-Up System (PAA9281, VWR Scientific Products, Suwanee, GA). The 16S rRNA sequencing was completed through the Clemson University Genomic Institute® (CUGI). BioEdit (Version 7.2.5) was used to align sequences and to compile consensus sequences from forward and reverse primers. Sequences were examined using the National Center for Biotechnology Information (NCBI) BLAST database (Altschul et al. 1997). The top BLAST nucleotide/16S rRNA database results with maximum identity greater than 97% were reported.

**Choice of Antibiotics and Susceptibility Tests.** Minimal bactericidal concentrations of single antibiotics and antibiotic combinations were determined using a tube dilution method. Original bacterial isolates were transferred into broth cultures containing 3% sucrose and ½ strength MS medium (Murashige and Skoog 1962). Following clouding of medium, Gram stains were done to confirm the presence of original strains. Twelve single antibiotic treatments were tested in triplicate: streptomycin sulfate (S; 1000, 500, 250, or 125  $\mu\text{g mL}^{-1}$ ), cefotaxime (C; 500, 250, 125, or 62.5  $\mu\text{g mL}^{-1}$ ), and gentamicin sulfate (G; 50, 25, 12.5 or 6.25  $\mu\text{g mL}^{-1}$ ). In addition, twelve combination treatments were prepared: streptomycin sulfate + cefotaxime (250 + 125, 250 + 62.5, 125 + 125, and 125 + 62.5  $\mu\text{g mL}^{-1}$ ), streptomycin sulfate + gentamicin sulfate (250 + 12.5, 250 + 6.25, 125 + 12.5, and 125 + 6.25  $\mu\text{g mL}^{-1}$ ), and cefotaxime + gentamicin sulfate (125 + 12.5, 125 + 6.25, 62.5 + 12.5, and 62.5 + 6.25

$\mu\text{g mL}^{-1}$ ). Antibiotics were diluted in 5 mL of deionized water containing 3% sucrose and  $\frac{1}{2}$  strength MS liquid medium (Murashige and Skoog 1962). Following preparation of media, 1 mL of broth cultures containing bacterial isolates were added to each treatment and inoculated in flat-bottomed vials (O.D. x H.: 29x94 mm; Fisher glass shell vials, Thermo Fisher Scientific Inc., Pittsburg, PA) for 7 days at 25 °C.

After 7 days, 1 mL of the inoculated antibiotic cultures was transferred into flat-bottomed vials containing the same media formulation without antibiotics. These tubes were inoculated at 25 or 35 °C for an additional 7 days. Following inoculation, growth was assessed. Tubes showing no growth were designated as bactericidal. The lowest concentration in each treatment showing no growth was termed the minimal bactericidal concentration (MBC).

**Phytotoxicity tests and antibiotic treatment of plantlets.** *H. ×nigercors* plantlets were treated with single antibiotics ( $\mu\text{g mL}^{-1}$ ): 12.5 (G) and 50 (G), and combinations of two antibiotics: 125 (S) + 12.5 (G), 250 (S) and 25 (G), 62.5 (C) + 12.5 (G), and 125 (C) + 25 (G). Plantlets of *H. ×nigercors* known to be contaminated with three endogenous bacteria were totally submerged in  $\frac{1}{2}$  strength liquid MS medium with and without the aforementioned antibiotic treatments in Magenta™ GA7 boxes (Magenta® Corp., Chicago, IL) and placed at 10-12 °C under  $28 \mu\text{mol m}^{-2} \text{s}^{-1}$  monochromatic LED lights (33.3% blue, 66.7% red) for 12 days on a rocker (EW-51301-00, Cole-Parmer® Portable Rocker Shaker, Vernon Hills, IL) set to 3 rpm. Each of the four treatments contained three boxes, with three plantlets per box.

Following antibiotic treatment, bases of plants were streaked onto TSA medium and plantlets were transferred to a multiplication medium. Phytotoxicity was ranked on a subjective numerical scale from 0-4, with a score of 0 indicating no phytotoxicity symptoms and a score of 4 indicating severe phytotoxicity or death. Plantlets showing phytotoxicity symptoms were judged on the presence of chlorosis, tissue browning, softening of tissue, and morphological changes. Plantlets were subcultured in four-week cycles, and plant condition and bacterial contaminants recorded during transfer.

## Results and Discussion

**Genetic Identification and Morphological Characteristics of Bacterial Isolates.** After plantlet reinitiation and subsequent streaking of *Helleborus ×nigercors* callus on TSA plates, bacterial colonies were visible after three days. All bacterial isolates were Gram-negative and individual cells were rod-shaped. Using 16S rRNA sequence analysis, Gram stain results, and colony morphology, isolates were identified to the genus level (Table 1).

Strain H7G (GenBank accession no. KU721939) was identified as a member of the *Paenibacillus* genus and was characterized by colonies that were grey, round, and opaque. Members of this genus have been found as endophytes in

plant species including pine, coffee, poplar (Ulrich et al. 2008), and barley (Rasimus et al. 2012). Many endophytic *Paenibacilli* produce compounds that aid in plant growth, such as auxins, cytokinins, and antibiotics (Ulrich et al. 2008; McSpadden Gardener 2004). Most *Paenibacilli* grow in cool temperatures (Rasimus et al. 2012), and some have been isolated from Alaskan tundra (Nelson et al. 2009). The cold-loving tendencies of this genus are consistent with their growth and persistence within tissues of the frost-tolerant and winter-flowering *Helleborus ×nigercors*.

Strain H7S (GenBank accession no. KU721940), with small, light yellow colonies, was identified as a member of the *Luteibacter* genus. This genus was discovered in 2005 from the rhizosphere of spring barley (Johansen et al. 2005). All members of this genus are yellow-pigmented, aerobic, Gram-negative rods (Kämpfer et al. 2009), which is consistent with the morphological characteristics of strain H7R. *Luteibacter rhizovicinus*, the first species discovered from the genus, has been isolated from micropropagated *Malus domestica* “Golden Delicious” plantlets (Guglielmetti et al. 2013), where it lowered shoot regeneration abilities (Piagnani et al. 2007), and from micropropagated barley plantlets, where rooting was stimulated (Guglielmetti et al. 2013).

Strain H7R (GenBank accession no. KU729138) had high sequence similarity (> 97%) with three genera ubiquitous in soils: *Stenotrophomonas*, *Pseudomonas*, and *Lysobacter* (Table 1). Because these genera also share many genotypic and phenotypic similarities, 16S rRNA is often inadequate to differentiate between members of these genera (Svensson-Stadler et al. 2012; Hayward et al. 2010). The genus *Stenotrophomonas* is comprised of several species of bacteria that show a range of activities including plant-growth promotion (Zhu et al. 2012), antibiotic production (Hayward et al. 2010), and pathogenicity (Ryan et al. 2009; Nyč and Matějková 2010). Endophytic strains of *Stenotrophomonas maltophilia* have been isolated from cucumber, oilseed rape, potato, strawberry, alfalfa, maize, sunflower, rice, wheat, willow, and poplar (Ryan et al. 2009), but some have developed antibiotic resistance to chloramphenicol and quinolone antibiotics (Nyč and Matějková 2010; Alonso and Martinez 1997). Members of the *Pseudomonas* genus include endophytic species such as those isolated from tulip poplar trees (*Liriodendron* spp.) and willows (*Salix gooddingii*), where they may produce rooting hormones through multiple pathways (Taghavi et al. 2009). *Lysobacter* species have been isolated from the rhizosphere of rice (Aslam et al. 2009) and ginseng (Srinivasan et al. 2010). *Lysobacter* spp. are often studied as biological control agents due to their predatory activity against Gram-negative and Gram-positive bacteria, blue-green algae, yeasts, fungi, and nematodes (Hayward et al. 2010; Sullivan et al. 2003).

**Choice of Antibiotics and Susceptibility Tests.** Streptomycin sulfate and gentamicin sulfate are aminoglycoside antibiotics that show effectiveness in controlling Gram-negative bacteria. Cefotaxime, a beta-lactam antibiotic, was chosen in addition to these two aminoglycosides due to the effectiveness of beta-lactams against resistant strains of

Table 1. Endophytic bacteria isolates identified using Gram reaction and cell morphology and 16S rRNA sequencing analysis.

Isolate	Gram Reaction and Cell Morphology	Colony color (on tryptic soy agar)	16S Bacteria Identification (>97% top identity matches) <sup>a</sup>	16S rRNA/ nucleotide database % match <sup>b</sup>
Isolate 1 (H7G)	Negative rod	Grey	<i>Paenibacillus xylanexedens</i> <i>Paenibacillus tundrae</i> <i>Paenibacillus amylolyticus</i> <i>Paenibacillus taichungensis</i>	98%, 99% 99%, 99 98%, 98% 98%, N/A
Isolate 2 (H7S)	Negative rod	Yellow	<i>Luteibacter rhizovicinus</i> <i>Luteibacter anthropi</i> <i>Luteibacter yeojuensis</i>	98%, 98% 98%, 98% 97%, N/A
Isolate 3 (H7R)	Negative rod	Beige	<i>Stenotrophomonas maltophilia</i> <i>Stenotrophomonas pavanii</i> <i>Stenotrophomonas chelatinphaga</i> <i>Stenotrophomonas humi</i> <i>Stenotrophomonas terrae</i> <i>Stenotrophomonas nitritireducens</i> <i>Stenotrophomonas panacihumi</i> <i>Stenotrophomonas ginsengisoli</i> <i>Stenotrophomonas rhizophila</i> <i>Stenotrophomonas daejeonensis</i> <i>Stenotrophomonas acidiminiphilia</i> <i>Stenotrophomonas koreensis</i> <i>Pseudomonas geniculata</i> <i>Pseudomonas hibiscicola</i> <i>Pseudomonas pictorum</i> <i>Lysobacter enzymogens</i> <i>Lysobacter soli</i> <i>Lysobacter rushenii</i> <i>Lysobacter oryzae</i> <i>Lysobacter yangpyeongensis</i>	100%,100% 100%, N/A 100%, N/A 100%, N/A 99%, N/A 99%, N/A 99%, N/A 99%, N/A 99%, N/A 99%, N/A 99%, N/A 98%, N/A 100%, 100% 99%, 100% 99%, 100% 99%, N/A 98%, N/A 98%, N/A 98%, N/A 98%, N/A

<sup>a</sup> Bacterial candidates not fitting the Gram reaction and morphology observed were not included in the top identity matches

<sup>b</sup> Percentages from 16S rRNA database listed first, followed by percent matches from the nucleotide database, if applicable

*Stenotrophomonas maltophilia*, a candidate genus for strain H7R (Alonso and Martinez 1997). Before antibiotic treatment, however, broth cultures containing bacteria were Gram stained to confirm monocultures. *Luteibacter* H7S and strain H7R were not included in the remainder of the experiments due to lack of growth in control tubes after three weeks. Possibly, these strains showed initial growth in culture due to storage of a vital nutrient obtained directly from the plant tissue. Following isolation and initial proliferation, the amount stored may have become inadequate for growth.

Initial experiments indicated that *Paenibacillus* sp. H7G was not affected by streptomycin sulfate, cefotaxime, or combinations of the two. Gentamicin sulfate was most effective, with an MBC of 12.5 µg mL<sup>-1</sup>. MBCs were used to determine plant treatments. Since only treatments containing at least 12.5 µg mL<sup>-1</sup> of gentamicin sulfate were bactericidal,

only these treatments were evaluated for phytotoxicity and effective elimination of bacteria from plant material.

Shoot tips of *H. ×nigercors* showed some symptoms of phytotoxicity (browning of outer leaves and minor chlorosis) immediately after treatment with single treatments and combinational treatments containing 12.5 µg mL<sup>-1</sup> gentamicin sulfate. Interestingly, control plants often had minor symptoms, indicating that submersion in media, even without antibiotics, causes some stress to plantlets. Plantlets treated with 25 µg mL<sup>-1</sup> gentamicin sulfate and 125 µg mL<sup>-1</sup> cefotaxime or 25 µg mL<sup>-1</sup> gentamicin sulfate and 250 µg mL<sup>-1</sup> streptomycin sulfate showed more severe phytotoxicity, with shoots showing tissue browning and tissue softening. Plantlets in the 50 µg mL<sup>-1</sup> gentamicin treatment were affected most severely, and 22% of the plantlets did not survive the treatment. After a month of growth in non-antibiotic containing media, plantlets remained low quality. All plantlets from the 50 µg mL<sup>-1</sup> treatment and 33% of

plantlets from the 25  $\mu\text{g mL}^{-1}$  gentamicin sulfate and 250  $\mu\text{g mL}^{-1}$  streptomycin sulfate died, showing a failure to re-acclimate after antibiotic treatment. Surviving plantlets were very low quality, showing tissue blackening, shoot tip necrosis, and tissue softening (Table 2).

**Table 2.** Phytotoxicity rankings and survival percentages of *H. ×nigercors* plantlets treated with two single treatments of gentamicin sulfate (G), two combination treatments of gentamicin sulfate (G) and cefotaxime (C), and two combination treatments of gentamicin sulfate (G) and streptomycin sulfate (S) for 12 days.

Antibiotic Treatment ( $\mu\text{g mL}^{-1}$ )	Average Phytotoxicity Ranking <sup>a</sup>	Survival Percentage <sup>b</sup>
Control	1.00	100%
G(12.5)	1.67	100%
G(50)	3.67	0%
G(12.5) + C(62.5)	1.67	100%
G(25) + C(125)	2.00	100%
G(12.5) + S(125)	1.67	100%
G(25) + S(250)	3.00	67%

<sup>a</sup> Phytotoxicity scores were subjectively determined from 0-4, with a score of 0 indicating no phytotoxicity symptoms and a score of 4 indicating extremely severe symptoms, including blackening of plant tissue and death.

<sup>b</sup> Results taken after the second cycle as a percentage of the original number of plantlets placed in treatment. Original treatments used 3 Magenta GA7 boxes with 3 plantlets/box.

**Antibiotic Effectiveness on Plant Tissue.** Initial experiments indicated that the 12-day antibiotic treatments of all single and combinational treatments containing 12.5  $\mu\text{g mL}^{-1}$  gentamicin were ineffective for eliminating bacteria from culture, with all treatments showing the growth of at least one species. MBCs are used as starting points for determining effective treatments, and treatment concentrations required to penetrate plant tissues and maintain bactericidal effects are often two to four times higher than the MBCs (Leifert *et al.* 1991). When concentrations of gentamicin sulfate were quadrupled (50  $\mu\text{g mL}^{-1}$ ), up to 44% of plantlets showed no bacterial growth after the first 4-week cycle of treatment. After the second cycle, bacterial growth resumed completely, indicating that initial success was due to a slowing of bacterial growth rather than colony death (Table 3).

**Table 3.** Ratio of bacteria-free hellebore shoots to contaminated shoots in plantlets treated with two single treatments of gentamicin sulfate (G), two combination treatments of gentamicin sulfate (G) and cefotaxime (C), and two combination treatments of gentamicin sulfate (G) and streptomycin sulfate (S) for 12 days.

Treatment ( $\mu\text{g mL}^{-1}$ )	Cycle 1	Cycle 2
	Bacteria-free plants/treated plants	
G(12.5)	0/9	--
G(50)	0/9	--
G(12.5) + C(62.5)	0/9	--
G(25) + C(125)	4/9	0/9
G(12.5) + S(125)	0/9	--
G(25) + S(250)	3/9	0/9

## Conclusions

The establishment of aseptic culture is one of the major challenges associated with micropropagation of *H. ×nigercors*. Antibiotic treatments were unsuccessful at eliminating these endogenous bacteria due to the severe phytotoxicity associated with high antibiotic concentrations. Combinational treatments showed initial success, but proved to have bacteriostatic properties rather than bactericidal properties when penetrating plant tissues.

This is the first study in which endophytic bacteria have been characterized from members of the *Helleborus* genus. While the evidence points to internal habitation by these strains, further studies are necessary to determine if these bacteria have beneficial, neutral, or negative effects on the growth of *H. ×nigercors*. Since antibiotic treatment was unsuccessful, plants containing endogenous bacteria could not be compared to aseptic plants. As such, additional studies are necessary to define protocols to eliminate internal contaminants in order to characterize these organisms and assess their impact on performance of micropropagated plantlets of *H. ×nigercors*.

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