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Micropropagation of Hybrid Hellebores and their Endogenous Bacteria

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MICROPROPAGATION OF HYBRID HELLEBORES AND THEIR ENDOGENOUS BACTERIA

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ABSTRACT

Hellebores (*Helleborus* spp.) are winter-flowering ornamental plants that are difficult to propagate in tissue culture. To improve the performance of hellebores during micropropagation, preliminary tests were done to standardize temperature and light quality. The effects of growth hormone type and concentration on plant multiplication were also assessed. Performance remained low, and plantlets often displayed visible contamination.

During Stage II micropropagation, plantlets of *Helleborus* ×*nigercors* were observed to be bacterially contaminated. To assess the effects of contaminants on performance *in vitro*, 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 at the genus level, belonging to *Paenibacillus* and *Luteibacter* genera, respectively. Strain H7R had high sequence similarity to the *Pseudomonas, Stenotrophomonas,* and *Lysobacter* genera. *Paenibacillus* sp. H7G was screened using multiple antibiotic treatments, including streptomycin sulfate, gentamicin sulfate, and/or cefotaxime, to determine bactericidal doses. Treatments were bactericidal if they contained at least 12.5 μg/mL gentamicin sulfate. Minimal bactericidal concentrations (MBCs) were unsuccessful at penetrating plant tissue, with none of treated plants being bacteria-free. When MBCs were quadrupled, severe phytotoxicity of plant tissues was noted, and viable bacteria remained.

To improve performance of hellebores (*Helleborus* spp.) in micropropagation, the influence of sucrose [1-3%], phosphate (PO_4^{3-}) [1.25-6.25mM], nitrate (NO_3^-) [39.8-69.8 mM], ammonium (NH_4^+) [5-35 mM], plant density, dilutions of micronutrients (from Woody Plant Medium; WPM), and thiadiazuron (TDZ) [1-9 µM] on multiplication ratios, plant quality, and callus development were evaluated in liquid medium. Each factor was tested across a range of concentrations, and individual treatments were chosen using D-optimal criteria. Two hellebore genotypes (*Helleborus* ×*nigercors* and *Helleborus* ×*ballardiae* 'Raulston Remembered') were propagated in each treatment. Analysis of variance (ANOVA) indicated that sucrose, NO_3^-, and the sucrose × PO_4^{3-} interaction had the largest positive effects on plant multiplication. Although *Helleborus* ×*ballardiae* 'Raulston Remembered' performed better overall, responses to nutrient factors were consistent across genotypes. Nitrate levels were within the range tested. Multiplication was highest at maximum concentrations of phosphate and sucrose, indicating that ideal concentrations may be greater than the range tested. A second, 3x3 factorial study extending the range of sucrose and phosphorus levels was conducted, and found that the factors were not significant in gelled medium. The D-optimal response surface approach was successful for defining factors of interest and optimal concentrations to improve micropropagation of these hellebore genotypes.

DEDICATION

I would like to dedicate this thesis to my mother, Alison Strever, for fueling my passion for plants. Her words of encouragement have kept me centered, even in times where my research seemed fruitless. I thank her for her guidance and for always accepting me and supporting my goals in life. Our mutual love for plants and their undiscovered mysteries have brought us even closer, and for that I am forever grateful.

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PREFACE

Tissue Culture: An Overview

Plant tissue culture is an important industry dedicated to the rapid generation of genetically identical, disease-free progeny. Despite the high production costs, plants produced in such a way are valuable to growers because of their uniformity and freedom from pests. Disease-indexed plantlets are valuable to the tissue culture laboratory itself, due to reduced laboratory losses, improved rooting rate and quality of shoots, and reduced losses during acclimatization (Holdgate and Zandvoort 1997). This allows for commercial laboratories to supply quality plant products to consumers that have superior and reliable growth rates, easier crop management, and better yields (Holdgate and Zandvoort 1997).

In this paper, contamination refers to an unintentional introduction of microorganisms into a tissue culture system, rendering it non-sterile. Many micropropagated plantlets may be contaminated without visible evidence. This can compromise the benefits gained through tissue culture, costing laboratories unnecessary time and money. Surface sterilization during initiation is often inefficient, especially if microorganisms are protected within the plant tissue (Leifert and Woodward 1997). Consequently, explants can appear clean during Stage I, and may pass into clonal propagation with bacterial contaminants within or on the plant tissue (Cassells 1997). Plants, even if initially clean, should be continually tested to verify cleanliness. Nutrient broths can be used to detect microorganisms. If organisms are present, DNA sequencing or serological tests can

be used to determine specific pathogens and eradication procedures (Holdgate and Zandvoort 1997).

Hellebores in the Plant Trade

The genus *Helleborus*, a member of the *Ranunculaceae* family, is comprised of 17-20 species of herbaceous perennial flowering plants. Most hellebores are native to European mountainous regions, particularly in the Balkans, often found in open woodlands and on rocky slopes with calcareous, humus-rich soils (Burrell and Tyler 2006).

Helleborus niger, the Christmas Rose, has been an important ornamental plant in European and American cultures since the late 18th century (Burrell and Tyler 2006). Before they were recognized for their ornamental traits, hellebores were used for medicine, food, and clothing (Burrell and Tyler 2006; Maior and Dobrota 2013). In traditional medicine, these plants were used to treat edema, arthritis, and ulcers, as well as for anti-inflammatory purposes. Recently, active compounds have been characterized and shown to have antioxidant, anti-inflammatory, and antimicrobial effects (Maior and Dobrota 2013). Although they have a rich medicinal history and great medical potential, hellebores today are most popular in the ornamental trade both as blooming perennials and cut flowers, providing delicate drooping flowers and rich-textured foliage in the winter landscapes (Burrell and Tyler 2006; Seyring 2002).

Helleborus niger is popular in the ornamental trade due to its extensive range and durability. It is hardy in Zones 3 to 7 and can tolerate light to full shade, as well

as acidic and alkaline soils. It is widely variable in terms of leaf size and shape and flower color, making it popular for cultivar development (Burrell and Tyler 2006). *Helleborus niger* hybridizes easily with other species, and is a parent plant of 75% of the hybrids included in this study. Often, interspecific crosses are more accessible in the market (Beruto et al. 2013). A summary of the species included in this research is found in Table A1.

Helleborus plants reproduce both sexually by seed and vegetatively by rhizomes in nature. Hellebores are most commonly propagated by seed, due to prolific seed production and slow division rates in nature and commercial production. Interspecific hybrids such as the plants included in this study, however, often produce little or no seed. Additionally, the hybrid nature of these plants will result in seed that does not breed true (Burrell and Tyler 2006; Beruto et al. 2013). Due to the slow nature of in vivo propagation methods, the best-quality hellebore plants, including rare interspecific hybrids, are often prohibitively expensive. Because commercialization of these cultivars is dependent on their propagation, high division rates may be possible using a suitable tissue culture method.

Problems in Micropropagation

Although tissue culture methods have been successfully utilized for many ornamental plants, effective micropropagation of *Helleborus* spp. resulting in high multiplication ratios has not yet been attained. Beruto et al. (2013), were successful in achieving multiplication ratios between 1.4-2.1 for genotypes of *Helleborus* ×*nigercors,* but noted several genus-specific problems associated with the plant.

The first of these difficulties is that of establishment. Formation of axenic cultures is difficult and limited by contamination of source materials with microorganisms, resulting in <40% of healthy shoots resulting from sterilization procedures (Beruto et al. 2013; Seyring 2002). Even after establishment, development is slow, often requiring up to 11 months before propagation can begin (Seyring 2002).

Genotype, even within a particular species, also influences propagation success. Genotypes of *Helleborus niger* and *Helleborus* ×*nigercors* observed in previous studies showed differential responses to tissue culture in the ability to divide and develop roots (Seyring 2002; Beruto et al. 2013). Multiplication ratios decreased after 12-15 months in some studies, after which Stage I initiation of new material was required (Beruto et al. 2013). Micropropagation procedures for *Helleborus* spp. require refinement in terms of multiplication, rooting, and acclimatization protocols before successful commercial exploitation of the genus can be achieved.

The objectives of this work were to maximize *in vitro* shoot multiplication of four interspecific *Helleborus* hybrids. Conventional techniques were taken to optimize temperature and light quality and to assess the effect of growth hormone type and concentration on performance. Following conventional experiments, nonconventional approaches were utilized to improve performance of these hybrids, including (1) contamination identification and antibiotic treatment, and (2) assessment of main and interactive effects of mineral nutrients, sucrose, thiadiazuron (TDZ), and plant density on performance of hellebores *in vitro.*

Preliminary Experiments

Four hellebore hybrids, *Helleborus* ×*ballardiae* 'Raulston Remembered', *Helleborus* ×*nigercors, Helleborus* ×*ashwoodensis* 'Briar Rose', and *Helleborus* ×*hybridus,* were obtained as initiated Stage II plantlets (Kane 2011) from Dan River Plants in Danville, Virginia. Plantlets showed poor performance in tissue culture, supporting previous claims of the low performance of the genus during micropropagation (Beruto et al. 2013; Seyring 2002). Multiplication ratios and plant quality were consistently low, with plantlets often displaying physiological abnormalities such as engorged petioles, excessive callus development, and shoot tip necrosis. Additionally, plantlets of these four clones were contaminated with bacteria during Stage II multiplication. Bacterial colonization was visualized by a grey halo around the bases of plantlets and occasionally by a bacterial film on the surface of the media.

Early attempts at improving micropropagation procedures for these plants included environmental changes to temperature and light quality. Environmental conditions were standardized based on the results of the temperature (Table A2) and light quality experiments (Tables A3 and A4). The optimal temperature range for micropropagation was 10-12°C. Light quality did not influence propagation success. To further evaluate micropropagation procedures, a media trial was conducted to determine effects of growth hormone type and concentration on multiplication, plant quality, and callus development and to compare performance between pre-formulated Murashige and Skoog (MS; Murashige and Skoog 1962) and

Woody Plant Medium (WPM) salts (Lloyd and McCown 1980). Hormones tested included TDZ, kinetin, and benzyladenine (BA) either alone or in combination with indole-3-acetic acid (IAA) or N6-[2-isopentyl]adenine (2ip). There were no significant differences due to salt type, or hormone type on multiplication ratios. Results of this media trial can be seen in Tables A5-A7.

Understanding the influence of nutrient concentrations and plant density can help to formulate media that prevent nutrient deficiencies that occur during micropropagation (Adelberg et al. 2013). Spent medium analysis is a useful tool to identify specific elements that may be involved in limiting plant quality or growth within a particular micropropagation system (Leifert et al. 1995; Adelberg 2010). Following the first cycle of the media trial, spent liquid media from 11 liquid cultures were analyzed for remaining nutrient residuals. Only cultures showing high plant performance and thus high nutrient utilization were chosen and sent to Clemson University Agricultural Services for analysis.

Residual nitrate concentrations were determined using a flow injection/cadmium nitrate reduction system in a FIALab Nitrate Analyzer (Bellevue, WA). Standard solutions ranging from 1 to 4 ppm NO_3 were prepared in deionized water, and an ammonium chloride buffer (1.6 M, pH 8.5) and a colorimetric sulfanilamide solution (LabChem Inc., No. LC132802; Zelienople, PA) were used as reagents. FIALab for Windows 5.0 was used to complete the analysis, and set to autosample standards, blanks, and samples. Frequency of Quality Control/Driftcor sampling was set to 20, and the position was set to 3. Ammonium levels remaining

were determined via steam distillation using a Kjeltec ™ 2300 Distillation unit. All other remaining nutrient concentrations were determined via Inductively Coupled Plasma (ICP) analysis using a Spectro© ARCOS radial plasma analyzer (Kleve, Germany). Wavelengths were set as follows: B (249.7 nm), Ca (370.6 nm), Cl (134.7 nm), Cu (324.8 nm), Fe (259.9 nm), K (766.5 nm), Mg (382.9 nm), Mn (257.6 nm), Na (589.6 nm), P (177.5 and 214.9 nm), S (182.0 nm), and Zn (213.9 nm). Nutrient levels remaining in spent media are shown in Table A8. Plant nutrient-use was expressed as a percentage of the total original specified concentration of MS (4.43 g/L) or WPM (2.41 g/L) salts added to the original media. Deficiencies or imbalances identified through this preliminary experiment were selected as potential elements limiting plant growth and were included as individual factors in the D-optimization design, discussed in Chapter 2.

Although plant quality and callus growth were affected by factors tested in the media trial, the results were not viewed as horticulturally relevant due to negligible differences in multiplication ratios. This preface documents a number of experiments conducted using conventional techniques, such as manipulation of plant growth regulators and environment, for improving micropropagation procedures for perennial plants using standard experimental designs used to account for random error through replication. Since these techniques did not provide notable improvements in multiplication ratio, less conventional experiments were conducted, including 1) antibiotic treatment of endogenous

bacteria and, 2) multivariate designs rebalancing media components. The two chapters of this thesis describe the two aforementioned experiments.

CHAPTER 1: IDENTIFICATION AND ANTIBIOTIC TREATMENT OF ENDOGENOUS BACTERIA IN MICROPROPAGATED *HELLEBORUS* ×*NIGERCORS*

ABSTRACT

During Stage II micropropagation, plantlets of *Helleborus* ×*nigercors* were observed to be bacterially contaminated. To assess the effects of contaminants on performance *in vitro*, 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 at the genus level, belonging to *Paenibacillus* and *Luteibacter* genera, respectively. Strain H7R had high sequence similarity to the *Pseudomonas, Stenotrophomonas,* and *Lysobacter* genera. *Paenibacillus* sp. H7G was screened using multiple antibiotic treatments, including streptomycin sulfate, gentamicin sulfate, and/or cefotaxime, to determine bactericidal doses. Treatments were bactericidal if they contained at least 12.5 μg/mL gentamicin sulfate. Minimal bactericidal concentrations (MBCs) were unsuccessful at penetrating plant tissue, with none of treated plants being bacteria-free. When MBCs were quadrupled, severe phytotoxicity of plant tissues was noted, and viable bacteria remained.

Keywords: Contamination management, endophytes, *Helleborus* ×*nigercors,* Antibiotics, Micropropagation

INTRODUCTION

Tissue culture is an important tool for rapid multiplication of genetically identical clones. Micropropagation protocols can be applied to breeding, propagation, disease elimination, and genetic resource conservation (Lal et al. 2014; Cassells 1997; Holdgate and Zandvoort 1997). Disease-indexing was the first practical application of tissue culture, and was first practiced with orchids. Clean culture practices result in plants that are more productive in terms of multiplication and rooting, higher quality, and easier to acclimatize (Holdgate and Zandvoort 1997; Leifert and Woodwart 1997).

Contamination is not always visible during Stage I establishment of plantlets and may only become evident after multiple subcultures (Reed et al. 1997). Often, contamination during the clonal micropropagation stage can be visualized by a gray halo around the base of the plant and, in extreme cases, by a visible slime layer on the surface of the medium or plant (Benjama and Charkaoui 1997). Microbial contamination can contribute to reduced multiplication ratios, low plant quality, limited ability to acclimatize, or cause mortality (Tanprasert and Reed 1997; Leifert and Woodward 1997).

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). However, many endogenous bacteria may have both mutualistic and pathogenic associations with the host plant at different points in

their lifetime (Wilson 1995). Bacteria resistant to surface sterilization, such as endophytic bacteria found within plant tissue, lead to ongoing difficulties in tissue culture, and represent the highest source of loss for tissue culture laboratories in terms of labor, media components, space, and plants (Holdgate and Zandvoort 1997; Reed et al. 1997; Benjama and Charkaoui 1997; Leifert and Woodward 1997; Lal et al. 2014). Even if these bacteria may be beneficial in a field setting, they are often problematic in tissue culture, due to the fact that sterile micro-cuttings in carbohydrate-rich medium, represent a 'biological vacuum' in which a lack of competition allows for uninhibited colonization by bacterial or fungal contaminants (Cassells 1997).

In cases where plantlets show contamination in later stages, antibiotic treatments may be required. Antibiotic treatments, like tissue culture protocols in general, vary greatly depending on both the plant and the bacterial contaminant involved (Tanprasert and Reed 1997). In order for antibiotic treatment to be successful, bacterial strains should be isolated, genetically identified, and subjected to susceptibility tests. In addition to determining the susceptibility of the bacteria, it is important to determine if bactericidal concentrations are toxic to plant tissue (Falkiner 1997). Often, antibiotics that show bactericidal effects on isolated colonies are not effective in practice, due to phytotoxic effects or inability to penetrate plant tissues effectively (Tanprasert and Reed 1997). Often, combinations of antibiotics can be used to avoid phytotoxic effects and increase the likelihood of tissue penetration (Falkiner 1997).

In this study, plantlets of *Helleborus* ×*nigercors* were observed to be visibly contaminated with bacteria during Stage II multiplication (Figure 1.1). Most explants survived with the contamination in vitro, but multiplication ratios were small, and plant quality was low. To better understand the biological complex found in hellebore cultures, putative endophytic bacteria were isolated, characterized, and subjected to a variety of antibiotic treatments in order to compare the growth and division of aseptic and bacterially inhabited plant material.

MATERIALS AND METHODS

Plant Material

Plant material for micropropagation was obtained from Pine Knot Farms in Clarksville, Virginia. The original cross to produce this clone of *H.* ×*nigercors* was completed at Pine Knot Farms Nursery. Original 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 (SpecCast Collectibles; Dyersville, IA).

Initiation Procedure

Shoot tips were produced from Pine Knot Farms' potted source plants and initiated at the Institute for Sustainable and Renewable Resources© (ISRR; Danville, Virginia). At the ISSR, source plants were rinsed to remove visible debris, and outer leaves and roots were removed. Plants were placed under running tap water with two drops of dishwashing liquid per liter for one hour, and rinsed three times with sterile deionized water. Under a laminar flow hood, plants were soaked in 80%

ethanol for 30 seconds and rinsed for three, five-minute intervals in sterile deionized water. Plants were then placed in a 1.65% bleach solution (20% dilution of Clorox ™, 8.25% sodium hypochlorite solution) and stirred for 20 minutes. Axillary buds from sterilized explants were removed and plated. The remaining basal portions containing the meristems were cut into additional sections and plated for entry into culture.

Reinitiation Procedure

Visibly contaminated Stage II plantlets were surface sterilized for reinitiation. Thirty-two 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 minutes). After soaking in the bleach solution, plantlets were rinsed twice with sterile deionized water and placed into SmithersOasis Flexible Tissue Culture Vessels (SmitherOasis, Kent, OH). Vessels contained 25 mL of liquid WPM salts (Lloyd and McCown 1980) at pH 5.7, 3% sucrose, and 9 µM TDZ. Plants were placed on a rocker (EW-51301-00, Cole-Parmer® Portable Rocker Shaker, Vernon Hills, IL) at 3 revolutions per minute in a low-temperature growth chamber (10-13 °C) and maintained under 28 µmol delivered from monochromatic LED lights (33.3% blue, 66.7% red).

Screening for Bacterial Contaminants

Slices of callus from reinitated plantlets were streaked onto Petri plates containing tryptic soy agar (TSA) and incubated for 24 h at 30 °C to detect

contamination. To establish purity of culture, individual colonies were subcultured, incubated for 24 hours, and stored in a refrigerator at 10 °C in darkness.

Identification and Characterization of Endophytes:

Individual colonies from subcultured plates were restreaked to maintain vigor and ensure accurate Gram stain results. Individual colonies were selected at random from the agar plates for genetic analysis. Gram reactions and colony and cell morphological characteristics were repeated to ensure use of pure bacterial cultures and recorded.

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 µL 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 µL 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 µL of the forward oligonucleotide primer (16S rRNA For, 5'AGAGTTTGATCCTGGCTCAG 3', ReadyMade™ Primers, Integrated DNA Technologies, Coralville, IA), and 1 µL of the reverse oligonucleotide primer (16S rRNA Rev, 5'ACGGCTACCTTGTTACGACTT 3', ReadyMade™ Primers, Integrated DNA Technologies, Coralville, IA), as well as 10.5 µL 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, Pittsburgh, PA).

The PCR-amplified products were observed by gel electrophoresis in 1.5% agarose gels. Ten µL of each PCR product and the HyLadder™ molecular mass marker (Denville Scientific Inc., CB4225-2, Metuchen, NJ) was 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.

Screening of Antibiotics

Bactericidal activity: 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 media, Gram stains were done to confirm the presence of original strains. Two cultures were confirmed and moved into antibiotic treatment. Twelve single antibiotic treatments were used: streptomycin sulfate (S; 1000, 500, 250, or 125 µg/mL), cefotaxime (C; 500, 250, 125, or 62.5 µg/mL), and gentamicin sulfate (G; 50, 25, 12.5 or 6.25 µg/mL). In addition, twelve combination treatments were prepared: streptomycin sulfate + cefotaxime (250+125, 250 +62.5, 125+125, and 125+62.5 µg/mL), streptomycin sulfate + gentamicin sulfate (250 + 12.5, 250 +6.25, 125 + 12.5, and 125 + 6.25 µg/mL), and cefotaxime + gentamicin sulfate (125 + 12.5, 125+6.25, 62.5+12.5, and 62.5+6.25 µg/mL). Antibiotics were diluted in 5 mL of deionized water containing 3% sucrose and ½ 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 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).

Phytotoxicty: H. ×*nigercors* plantlets were treated with single antibiotics (µg/mL): 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).

Antibiotic treatment of plants: Plantlets of *H.* ×*nigercors* known to be contaminated with three endogenous bacteria were totally submerged in ½ strength liquid MS medium with and without the antibiotic treatments listed above in Magenta GA7 boxes (Magenta Corp., Chicago, IL) and placed on a rocker (EW-51301-00, Cole-Parmer® Portable Rocker Shaker, Vernon Hills, IL) set to three revolutions per minute in a low-temperature growth chamber (10-12 °C) under 28 µmol monochromatic LED lights (33.3% blue wavelength, 66.7% red wavelength) for 12 days (Figure 1.2). 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. Plantlets were subcultured in four-week cycles, and plant condition and bacterial contaminants recorded during transfer.

RESULTS AND DISCUSSION

Identification of Bacterial Isolates

After plantlet reinitiation and subsequent streaking of *Helleborus* ×*nigercors* callus on TSA plates, bacterial colonies were visible after three days (Figure 1.3). All

bacterial isolates were Gram negative and individual cells were rod-shaped (Figure 1.4). The colonies ranged in color from grey to beige to light yellow. Using 16S rRNA sequence analysis, Gram stain results, and colony morphology, isolates were identified at the genus level. Strain H7G was identified as belonging to the *Paenibacillus* genus. Members of this genus have been found as endophytes in numerous woody plant species, including pine, coffee, and poplar (Ulrich et al. 2008), as well as in 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 at chilled 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 hellebores grown in low-temperature micropropagation chambers.

Isolate H7S was identified as belonging to the *Luteibacter* genus. This genus was discovered in 2005 from the rhizosphere of spring barley (Johansen et al. 2005), and all members of the genus are yellow-pigmented, aerobic, Gram-negative rods (Kämpfer et al. 2009). *Luteibacter rhizovicinus,* the first species discovered from the genus, has been isolated from micropropagated apple plantlets of *Malus domestica* "Golden Delicious" (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).

The third isolate, strain H7R, had high DNA sequence similarity (>97%) with members of three genera: *Stenotrophomonas, Pseudomonas,* and *Lysobacter*. These genera are all ubiquitous in soils. They also share many genotypic and phenotypic similarities, and 16S rRNA is often inadequate to differentiate between members of these genera (Svensson-Stadler et al. 2012; Hayward et al. 2009).

The genus *Stenotrophomonas* is comprised of several species of bacteria that show a diverse range of activities including plant-growth promotion (Zhu et al. 2012), antibiotic production (Hayward et al. 2009), 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, sunflower, maize, 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). *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. 2009; Sullivan et al. 2003). Results are summarized in Table 1.1. Due to the similarity in antibiotic susceptibility of these genera, further characterization was not necessary to fulfill the goals of this experiment. The third candidate genus for strain H7R, *Pseudomonas,* is also comprised of endophytic species such as those isolated from tulip poplar trees

(*Liriodendron* spp.) and willows (*Salix gooddingii*) where they may produce IAA through multiple pathways (Taghavi et al. 2008). These abilities of *P. putida* make it well-adapted to regulating phytohormonal balance by degrading compounds that can inhibit plant growth (Taghavi et al. 2008) and produce those that help promote root development.

Screening of Antibiotics

Choice of antibiotics: Streptomycin sulfate and gentamicin sulfate are aminoglycoside antibiotics commonly used in tissue culture due to their low phytotoxicity and effectiveness in controlling Gram-negative bacteria. Cefotaxime, a beta-lactam antibiotic, was chosen in addition to these two aminoglycosides due to effectiveness of beta-lactam antibiotics against resistant strains of *Stenotrophomonas maltophilia,* one of the candidate bacteria for strain H7R (Alonso and Martinez 1997). Before antibiotic treatment, broth culture containing bacteria were Gram-stained to check for bacteria presence and to confirm monocultures. One bacterial isolate, H7S, belonging to the genus *Luteibacter*, was not included due to colony death. After inoculation of antibiotic media and subsequent bacterial growth, the strain H7R, belonging to the *Stenotrophomonas, Pseudomonas,* or *Lysobacter* genera, was also removed from this portion of the study due to lack of growth in the control tubes after three weeks.

Potential reasons for non-culturability: Isolates H7R and H7S may have ceased growing in culture for a number of reasons. In vitro conditions are often inadequate for culturing microbes, particularly endophytes, due to the fact that these bacteria

often have fastidious growth requirements that may not be met by the artificial medium (Wade 2002; Vartoukian et al. 2010; Sharma et al. 2005). If the medium lacks the specific nutrients or pH conditions, or if the oxygen levels, carbon dioxide levels, or incubation temperatures are not optimal, bacteria may not grow (Vartoukian et al. 2010; Stewart 2012). It is possible that strains H7R and H7S showed initial growth in culture due to storage of a vital nutrient obtained directly from the plant tissue. Upon isolation and initial growth, the amount stored may have become inadequate for growth. It is unclear whether the lack of growth was due to colony mortality or dormancy.

Additionally, endophytic microorganisms often form biofilms inside of plant tissues, such as *Paenibacillus polymyxa* (Timmusk et al. 2005) and *Methylobacterium* sp. (Podolich et al. 2009). Biofilms allow bacteria to cooperate with one another to resist external stresses. For example, *Streptococcus mutans* has been shown to produce lactic acid, which is the primary source of carbon utilized by *Veillonella spp.* in the same biofilm (Vartoukian et al. 2010). Without the mutualistic benefits derived from these complex formations, bacteria often struggle to proliferate in monoculture (Vartoukian et al. 2010). For example, *Methylobacterium*, an endophyte found to form biofilms in potato roots was unculturable in monoculture, but was induced to grow after inoculation with *Pseudomonas fluorescens,* indicating the dependence of this bacterium on other organisms for growth (Podolich et al. 2009).

Bactericidal results: 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 single treatments and combinational treatments including this antibiotic at 12.5 µg/mL or greater showing bactericidal properties against isolated colonies of *Paenibacillus* sp. H7G (Figure 1.5). Results of these preliminary tests are shown in Table 1.2. Combinations of antibiotics are often useful in micropropagation because some bacteria can develop tolerance to antimicrobials following prolonged exposure. Combinations of antibiotics, on the other hand, can overcome this problem due to the difficulty of acquiring resistance to more than one antibiotic (Falkiner et al. 1997). Short-term treatments are typically best to avoid prolonged exposure that could lead to resistance (Tanprasert and Reed 1997).

Minimal bactericidal concentrations for plant treatment: MBCs were used to determine plant treatments. Since only treatments containing at least 12.5 µg/mL of gentamicin sulfate were bactericidal, only these treatments were included in the remainder of the experiment. Two single treatments, gentamicin sulfate at 12.5 or 50 µg/mL, and four combinational treatments, gentamicin sulfate (12.5 or 25 µg/mL) + streptomycin sulfate (125 or 250 µg/mL), and gentamicin sulfate (12.5 or 50 µg/mL) + cefotaxime (62.5 or 125 µg/mL) were checked for phytotoxicity and effective elimination of bacteria from plant material.

Phytotoxicity: 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 were given a ranking of 0 if they had no phytotoxicity symptoms. Plantlets showing phytotoxicity symptoms were judged on the presence of chlorosis, tissue browning, softening of tissue, and morphological changes. Plantlets exhibiting one minor symptom were given a ranking of 1. Plantlets were assigned to the ranking 2 if they showed one of the above symptoms severely. Plantlets given a ranking of 3 showed tissue blackening and one additional symptom. Plantlets affected the most severely, showing three severe symptoms and/or death, were given a ranking of 4. Rankings can be visualized in Figure 1.6. 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 gentamicin sulfate. Plantlets treated with 25 µg/mL gentamicin sulfate and 125 µg/mL cefotaxime or 25 µg/mL gentamicin sulfate and 250 µg/mL streptomycin sulfate showed more severe phytotoxicity, with shoots showing tissue browning and tissue softening. Plantlets in the 50 µg/mL 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 treatment and 33% of plantlets from the 25 µg/mL gentamicin sulfate and 250 µg/mL 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. Results are summarized in Table 1.3.

Antibiotic effectiveness on plant tissue: Initial experiments indicated that the 12-day antibiotic treatments of all single and combinational treatments containing 12.5 μg/mL gentamicin were ineffective for eliminating bacteria from culture, with all treatments showing the growth of at least one species (Figure 1.7). 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). In higher concentration treatments, including concentrations of gentamicin twice or four-times the MBCs, up to 44% of plantlets were clean after the first 4-week cycle following treatment. However, after the second cycle, bacterial growth resumed on all plantlets, indicating that initial success was due to a slowing of bacterial growth rather than colony death (Table 1.4).

CONCLUSIONS

The establishment of aseptic culture is one of the major challenges associated with micropropagation of hellebore species, including *H. ×nigercors. Paenibacillus, Luteibacter,* and *Stenotrophomonas, Lysobacter,* or *Pseudomonas* are major genera found as endogenous bacteria in *H. ×nigercors*. Antibiotic treatment was unsuccessful at eliminating these contaminants due to 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.

Bacteria were assumed to be endophytic organisms due to their resistance to surface sterilization and persistence in culture. Although the bacteria were not visualized within the plant tissue, their failure to be eliminated from plants using concentrations adequate to kill them in isolation indicates that they likely dwell within the plant tissues. If the bacteria were simply epiphytic organisms, one could presume that the MBCs would be sufficient to sterilize the surface of plant tissue. 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 to understand their impact on performance of micropropagated plantlets of *H.* ×*nigercors.*

Table 1.1. Endophytic bacteria isolates identified using Gram reaction and cell morphology and 16S rRNA sequencing analysis.

Isolate	Gram Reaction and Cell Morphology	Colony color (grown on tryptic soy agar)	16S Bacteria Identification (>97% top identity matches)[a]	16S rRNA/ nucleotide database % match [b]
Isolate 1 (H7G)	Negative rod	Grey	*Paenibacillus xylanexedens*	98%, 99%
			Paenibacillus tundrae	99%, 99%
			Paenibacillus amylolyticus	98%, 98%
			Paenibacillus taichungensis	98%, N/A
Isolate 2 (H7S)	Negative rod	Yellow	*Luteibacter rhizovicinus*	98%, 98%
			Luteibacter anthropi	98%, 98%
			Luteibacter yeojuensis	97%, N/A
Isolate 3 (H7R)	Negative rod	Beige	*Stenotrophomonas maltophilia*	100%,100%
			Stenotrophomonas pavanii	100%, N/A
			Stenotrophomonas chelatinphaga	100%, N/A
			Stenotrophomonas humi	100%, N/A
			Stenotrophomonas terrae	99%, N/A

Table 1.1 continued. Endophytic bacteria isolates identified using Gram reaction and cell morphology and 16S rRNA sequencing analysis.

Isolate	Gram Reaction and Cell Morphology	Colony color	16S Bacteria Identification (>97% top identity matches)[a]	16S rRNA/ nucleotide database % match [b]
Isolate 3 (H7R)	Negative rod	Beige	*Stenotrophomonas nitritireducens*	99%, N/A
			Stenotrophomonas panacihumi	99%, N/A
			Stenotrophomonas ginsengisoli	99%, N/A
			Stenotrophomonas rhizophila	99%, N/A
			Stenotrophomonas daejeonensis	99%, N/A
			Stenotrophomonas acidiminiphilia	99%, N/A
			Stenotrophomonas koreensis	98%, N/A
			Pseudomonas geniculata	100%, 100%
			Pseudomonas hibiscicola	99%, 100%
			Pseudomonas pictorum	99%, 100%
			Lysobacter enzymogens	99%, N/A
			Lysobacter soli	98%, N/A

Table 1.1 continued. Endophytic bacteria isolates identified using Gram reaction and cell morphology and 16S rRNA sequencing analysis.

Isolate	Gram Reaction and Cell Morphology	Colony color	16S Bacteria Identification (>97% top identity matches)[a]	16S rRNA/ nucleotide database % match [b]
Isolate 3 (H7R)	Negative rod	Beige	*Lysobacter rushenii*	98%, N/A
			Lysobacter oryzae	98%, N/A
			Lysobacter yangpyeongensis	98%, N/A

[a] Bacterial candidates not fitting the gram reaction and morphology observed were not included in the top identity matches

[b] Percentages from 16S rRNA database listed first, followed by percent matches from the nucleotide database, if applicable

Table 1.2. Effectiveness of antibiotic treatments against isolated *Paenibacillus* sp. H7G colonies after 10 days growth in half-strength MS medium.

Antibiotic Type	Concentration (µg/mL)	Effect on *Paenibacillus* sp. H7G
Streptomycin sulfate	1000	No effect on growth
Streptomycin sulfate	500	No effect on growth
Streptomycin sulfate	250	No effect on growth
Streptomycin sulfate	125	No effect on growth
Cefotaxime	500	No effect on growth
Cefotaxime	250	No effect on growth
Cefotaxime	125	No effect on growth
Cefotaxime	62.5	No effect on growth
Gentamicin sulfate	50	Bactericidal [a]
Gentamicin sulfate	25	Bactericidal
Gentamicin sulfate	12.5 [c]	Bactericidal
Gentamicin sulfate	6.25	Bacteriostatic [b]
Streptomycin sulfate + cefotaxime	250 + 125	No effect on growth
Streptomycin sulfate + cefotaxime	250 + 62.5	No effect on growth
Streptomycin sulfate + cefotaxime	125 + 125	No effect on growth
Streptomycin sulfate + cefotaxime	125 + 62.5	No effect on growth
Streptomycin sulfate + gentamicin	250 + 12.5	Bactericidal
Streptomycin sulfate + gentamicin	250 + 6.25	Bacteriostatic
Streptomycin sulfate + gentamicin	125 + 12.5 [c]	Bactericidal
Streptomycin sulfate + gentamicin	125 + 6.25	Bacteriostatic
Cefotaxime + gentamicin sulfate	125 + 12.5	Bactericidal
Cefotaxime + gentamicin sulfate	125 + 6.25	Bacteriostatic
Cefotaxime + gentamicin sulfate	62.5 + 12.5 [c]	Bactericidal

Table 1.2 continued. Effectiveness of antibiotic treatments against isolated *Paenibacillus* sp. H7G colonies after 10 days growth in half-strength MS medium.

Antibiotic Type	Concentration (µg/mL)	Effect on *Paenibacillus* sp. H7G
Cefotaxime + gentamicin sulfate	62.5 + 6.25	Bacteriostatic

[a] Bactericidal treatments killed all bacterial isolates, even after transfer to media containing no antibiotics

[b] Bacteriostatic treatments halted the growth of bacterial isolates, but growth resumed once isolates were transferred to media containing no antibiotics

[c] Minimum Bactericidal Concentrations (MBCs) for gentamicin sulfate single and combinational treatments.

Table 1.3. Subjective 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 [a]	Control	G(12.5)	G(50)	G(12.5) + C(62.5)	G(25) + C(125)	G(12.5) + S(125)	G(25) + S(250)
Average Phytotoxicity ranking [b]	1	1.67	3.67	1.67	2.0	1.67	3
Survival percentage [c]	100%	100%	0%	100%	100%	100%	67%

[a] Antibiotic concentrations in µg/mL.

[b] 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.

[c] Results taken after the second cycle as a percentage of the original number of plantlets placed in treatment. Original treatments had 3 Magenta GA7 boxes containing 3 plantlets per box.

Table 1.4. 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.

Cycle	Bacteria-free plants/treated plants					
	G(12.5) [a]	G(50)	G(12.5) + C(62.5)	G(25) + C(125)	G(12.5) + S(125)	G(25) + S(125)
Cycle 1 [b]	0/9	0/9	0/9	4/9	0/9	3/9
Cycle 2	--	--	--	0/9	--	0/9

[a] Antibiotic concentrations in µg/mL.

[b] Cycle 1 was only cycle completed for treatments containing 12.5 or 50 µg/mL of gentamicin.

Figure 1.1. Contaminated plantlets of *Helleborus* ×*nigercors* in liquid and gellan systems. (A) Bacterial contaminants visible on the surface of micropropagated plantlets, (B) visible contamination and clouding of liquid medium, and (C) bacterial colonies growing on surface of gellan medium.

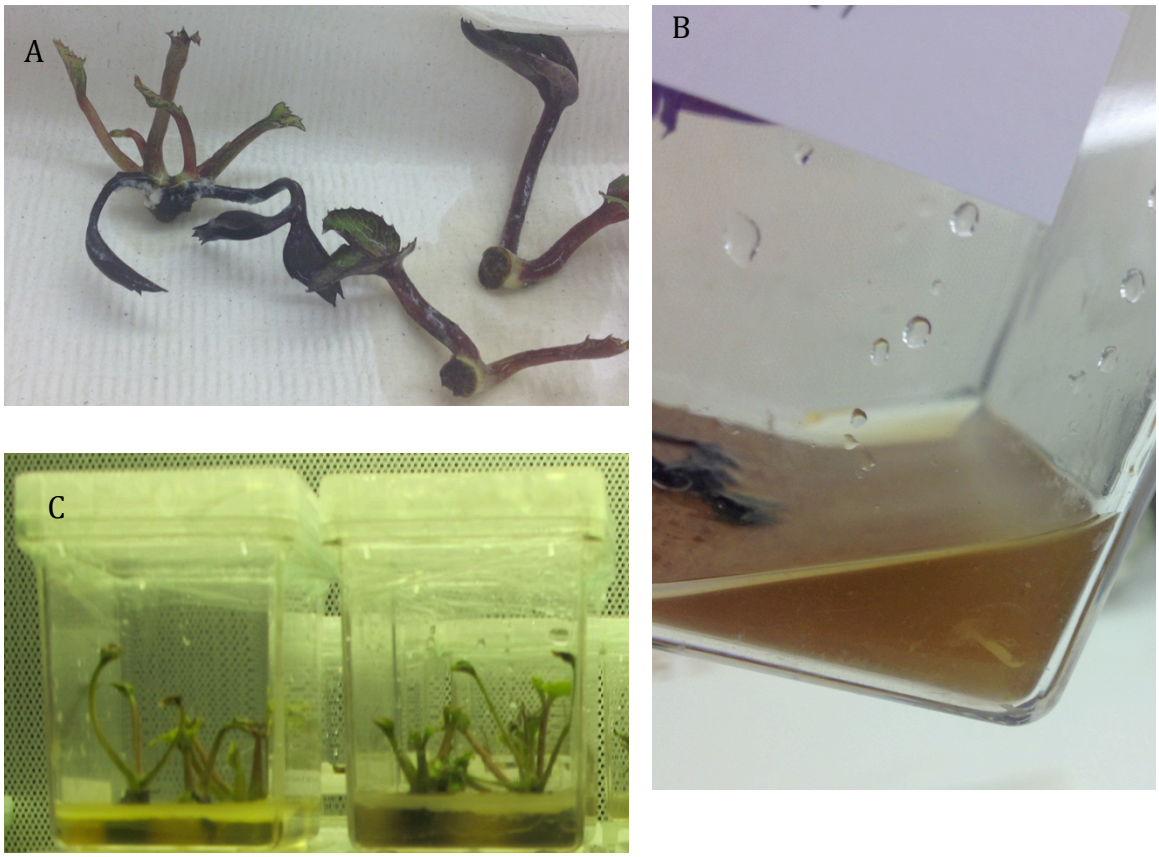


Figure 1.2. Plantlets submerged in ½ strength MS (Murashige and Skoog 1962) media containing 3% sucrose and antibiotic treatments.

Figure 1.3. Isolates of (A) round (H7R), (B) smooth (H7S), and (C) gray (H7G) bacteria grown on TSA.

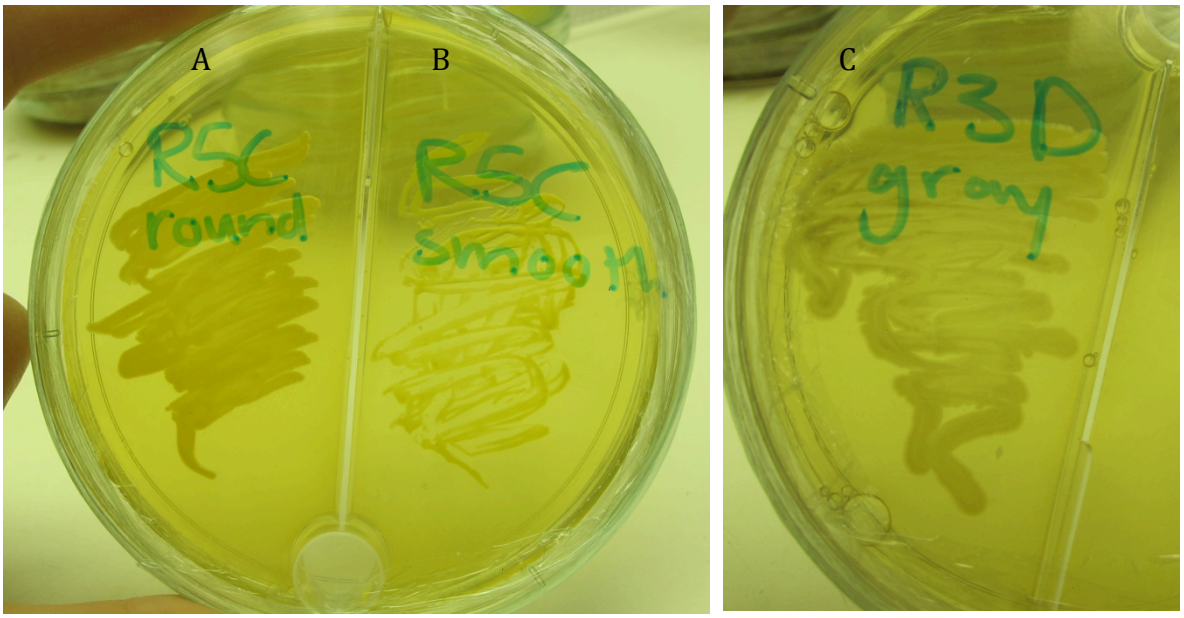
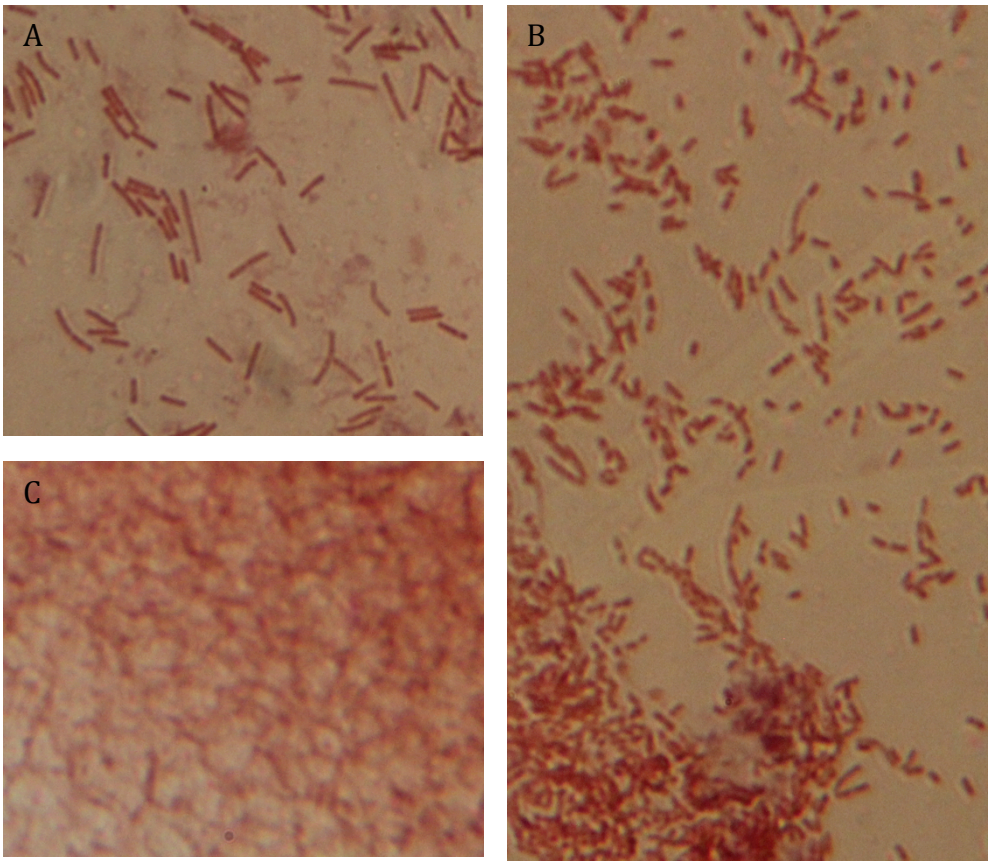
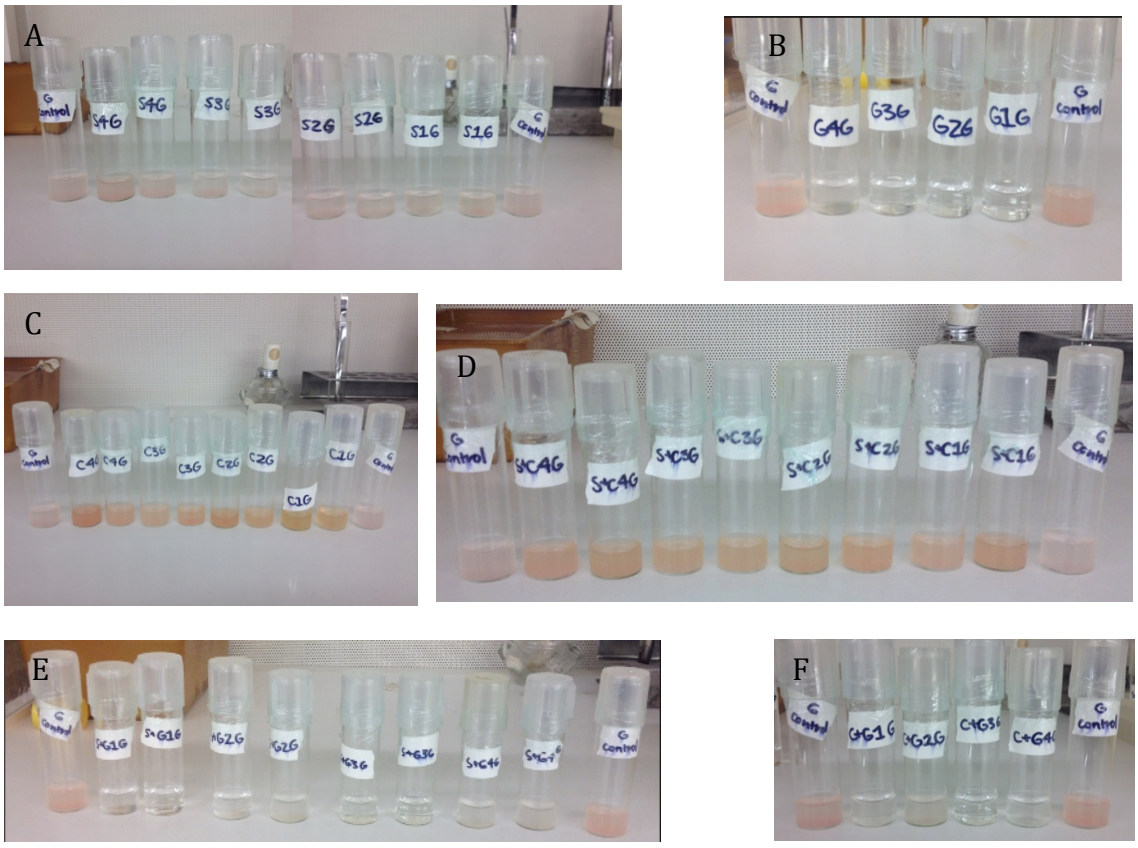


Figure 1.4. Gram-negative stains of *Paenibacillus* sp. H7G (A), isolate H7R [a] (B), and *Luteibacter* sp. H7S (C).



[a] Isolate H7R showed high DNA sequence similarity to the *Stenotrophomonas, Pseudomonas,* and *Lysobacter* genera.

Figure 1.5. Isolated colonies of *Paenibacillus* sp. H7G treated with antibiotics listed in Table 1.2. Bacterial growth in (A) single streptomycin sulfates treatments, (B) single gentamicin sulfate treatments [a], (C) single cefotaxime treatments, (D) streptomycin sulfate + cefotaxime treatments, (E) streptomycin sulfate + gentamicin sulfate treatments [b], and (F) cefotaxime + gentamicin sulfate treatments [c].



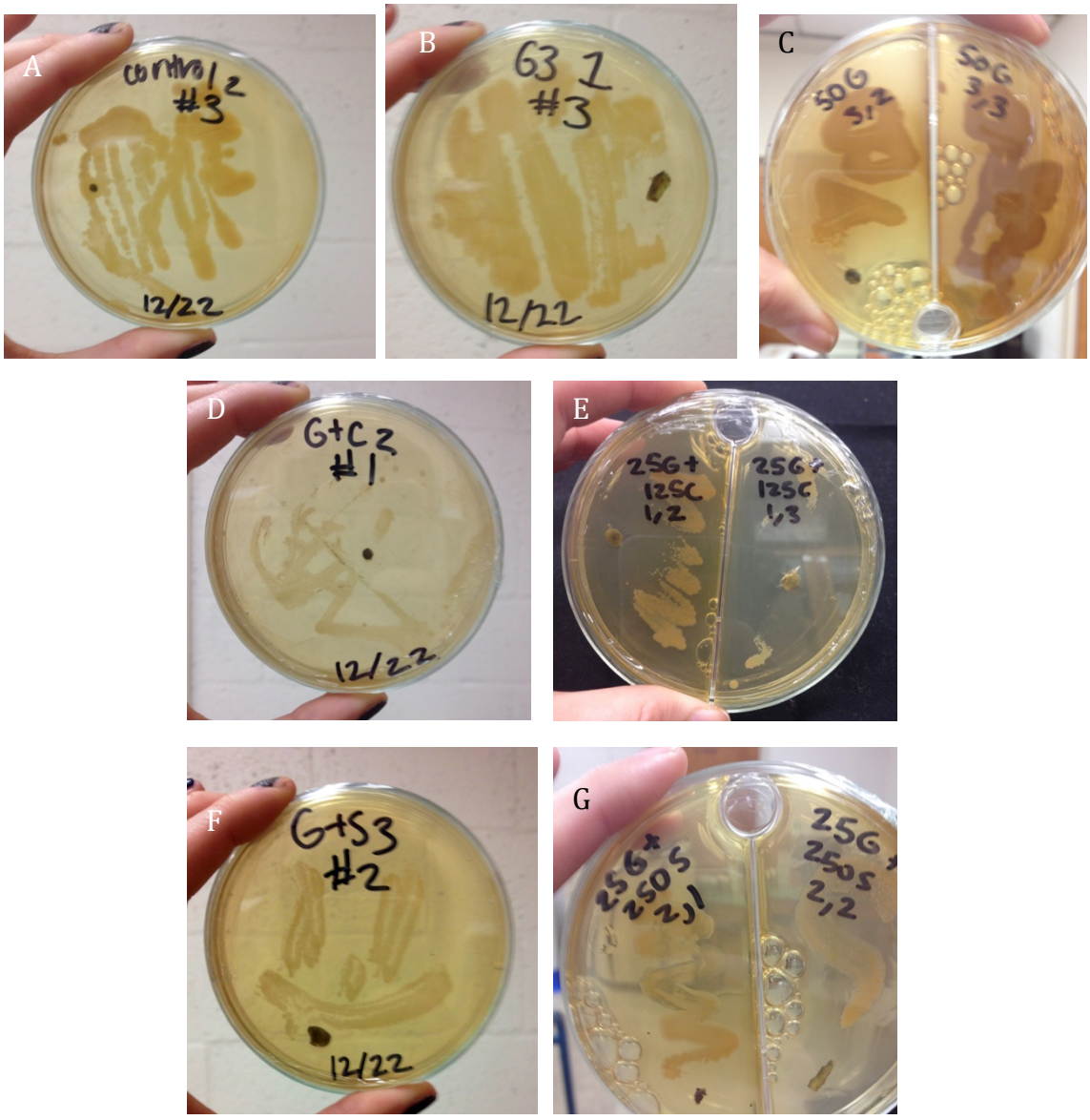
[a] Picture shown is from after colony transfer into media not containing antibiotics. Only the low treatment, gentamicin sulfate at 6.25 µg/mL was considered bacteriostatic due to cloudiness of media only after removal of antibiotics. All other treatments (12.5, 25, and 50 µg/mL gentamicin sulfate) were found to be bactericidal against *Paenibacillus* sp. H7G.

[b] Colonies pictured after transfer into media not containing antibiotics. Treatments S+G 1 (250 + 12.5 µg/mL) and S+G 3 (125 + 12.5 µg/mL) were shown to be bactericidal, and treatments S+G 2 (250 + 6.25 µg/mL) and S+G 4 (125 + 6.25 µg/mL) were found to be bacteriostatic.

[c] Pictured after transfer out of antibiotic-containing media. Treatments C+G 1 (125 + 12.5 µg/mL) and C+G 3 (62.5 + 12.5 µg/mL) were found to be bactericidal against *Paenibacillus* sp. H7G, while C+G 2 (125 + 6.25 µg/mL) and C+G 4 (62.5 + 6.25 µg/mL) were found to be bacteriostatic.

Figure 1.6. Visualization of phytotoxicity rankings. (A) Control plant showing no phytotoxicity symptoms, given a ranking of 0, (B) plantlet treated with 12.5 µg/mL gentamicin sulfate and 62.5 µg/mL cefotaxime, ranked 1, (C) plant treated with 12.5 µg/mL gentamicin sulfate and 125 µg/mL streptomycin sulfate, given a ranking of 2, (D) plant treated with 25 µg/mL gentamicin sulfate and 125 µg/mL cefotaxime, assigned to ranking 3, and (E) plant assigned to 50 µg/mL gentamicin sulfate treatment, showing severe phytotoxicity symptoms, given a ranking of 4.

Figure 1.7. Bacterial growth after streaking TSA plates with callus from antibiotic-treated plantlets of *Helleborus* ×*nigercors*. (A) control, (B) 12.5 µg/mL gentamicin sulfate, (C) 50 µg/mL gentamicin sulfate, (D) 12.5 µg/mL gentamicin sulfate and 62.5 µg/mL cefotaxime, (E) 25 µg/mL gentamicin sulfate and 125 µg/mL cefotaxime, (F) 12.5 µg/mL gentamicin sulfate and 125 µg/mL streptomycin sulfate, and (G) 25 µg/mL gentamicin sulfate and 250 µg/mL streptomycin sulfate.



CHAPTER 2: USING A MULTIVARIATE APPROACH FOR IMPROVING RESPONSES OF *HELLEBORUS* SPP. *IN VITRO*

ABSTRACT

To improve performance of hellebores (*Helleborus* spp.) in vitro, the influence of sucrose [1-3%], phosphate (PO_4^{3-}) [1.25-6.25mM], nitrate (NO_3^-) [39.8-69.8 mM], ammonium (NH_4^+) [5-35 mM], plant density, micronutrient dilution (from Woody Plant Medium; WPM), and thiadiazuron (TDZ) [1-9 µM] on multiplication ratios, plant quality, and callus development were evaluated. Each factor was tested across a range of concentrations, and individual treatments were chosen using D-optimal criteria. Two hellebore genotypes (*Helleborus* ×*nigercors* and *Helleborus* ×*ballardiae* 'Raulston Remembered') were propagated in each treatment. Analysis of variance (ANOVA) indicated that sucrose, NO_3^-, and the sucrose × PO_4^{3-} interaction had the largest positive effects on plant multiplication. Responses to nutrient factors were consistent across genotypes. Nitrate levels were within the range tested. Multiplication was highest at maximum concentrations of phosphate and sucrose, indicating that ideal concentrations may be greater than the range tested. A 3x3 factorial study extending the range of sucrose and phosphorus levels was conducted, and found that the interaction was not significant in gelled medium. The D-optimal response surface approach was successful for defining factors of interest to improve micropropagation of these hellebore genotypes.

Keywords: *Helleborus* spp, D-optimization, Multifactor, Response surface design, Micropropagation, Mineral nutrition

INTRODUCTION

The *Helleborus* genus, a member of the *Ranunculaceae* family, is comprised of 20 species of herbaceous perennial flowering plants. Hellebores are popular in ornamental trade, but commercialization of certain cultivars is dependent on successful vegetative propagation of these genotypes. Since *Helleborus* species are slow to divide in nature, propagation rates may be improved by using a suitable tissue culture method. Several studies were completed on members of this genus, but multiplication rates have remained relatively low (Beruto et al. 2013; Lim and Kitto 1995; Seyring 2002). Genotype appears to have a large effect on the efficiency of micropropagation, and consequently, genotype-specific media may be important in increasing the efficiency of tissue culture systems.

Two hellebore hybrids, *Helleborus* ×*ballardiae* and *Helleborus* ×*nigercors* showed low division ratios and poor growth in Stage II tissue culture (elsewhere in this volume). Plant quality was consistently low, and plantlets often displayed physiological abnormalities such as engorged petioles, excessive callus development, and shoot tip necrosis. Multiplication rates were negligible. Some of this poor performance was possibly due to endophytic contamination of the plant tissue, as discussed in the previous chapter.

Micropropagation has allowed for the production of large quantities of disease-indexed, genetically identical plants. Micropropagation can be applied to a number of ornamental species, including hellebores. About 80% of species grown in tissue culture are micropropagated on Murashige and Skoog (MS; 1962) basal

nutrient medium (George and De Klerk 2009), which is considered a high salt medium. Murashige and Skoog (1962) developed this high-salt medium for maximizing tobacco callus growth. This medium was formulated by altering nutrients one factor at a time (OFAT) by altering concentrations of the inorganic nutrient salts. OFAT analysis was used to create other early media formulations, such as those developed for soybean (Gamborg et al. 1968; Schenk and Hilderbrandt 1972), epiphytic orchids (Knudson 1946; Vacin and Went 1949), protoplasts, (Kao and Michayluk 1975), woody shrubs (Anderson 1980; Lloyd and McCown 1980), nut and hardwood trees (Driver and Kuniyuku 1984), and microspores (Nitsch and Nitsch 1969; Chu et al. 1975).

Creating individualized growth media for specific cultivars is complicated because of complex interactions between chemical nutrient components (Reed et al. 2013a). Because of this, many tissue culture protocols will simply alter standard medium formulations. Although these media are sufficient for the multiplication of a wide range of crops, they may not be suited to all species. Because of the wide range of genotype-specific growth practices, media better optimized to suit the needs of a particular genotype may exist. Although some media optimization experiments have been conducted on other species, only pre-formulated media salts have been used in the micropropagation of hellebores (Beruto et al. 2013; Seyring 2002). Nutrient solutes have not been formulated in a medium to optimize in vitro growth of hellebore genotypes.

The effects of nutrient supply are species-specific and are related to plant uptake, stage of differentiation (Kintzios et al. 2004), and nutrient concentration (Reed et al. 2013a). A poorly optimized medium can result in many physiological abnormalities such as engorged petioles, extreme callus development, and shoot tip necrosis (Reed et al. 2013b). In addition to reduced plant quality, poorly optimized media can contribute to low multiplication rates and even plant death. The specific mechanisms inducing these consequences are poorly understood, and in order to overcome them and elucidate their causes, species-specific and even cultivar-specific media optimization experiments should be conducted.

Response surface designs in multi-factor experiments can be used to optimize micropropagation media by accounting for interactive effects among ion pairs (Niedz and Evens 2007; Niedz et al. 2007). Design of Experiments (DOE) is a branch of applied statistics that uses algorithms to reduce experimental costs and treatment points by carefully selecting treatment points that can sample a multidimensional design space (Anderson and Whitcomb 2007). One DOE approach is D-optimization, which allows for the analysis of multiple factors when standard factorial designs would require a prohibitive number of samples. Using DOE methods such as D-optimization, combinations of nutrients as well as other factors within the vessel can be replicated and analyzed simultaneously.

D-optimization has been helpful in elucidating ideal nutrient salt concentrations of pear (Reed et al. 2013a; Reed et al. 2013b) and turmeric (Adelberg et al. 2013). In the first of two tandem experiments conducted by Reed et

al. (2013a), five mineral nutrient factors were tested and their effects on multiplication ratio and plant quality were determined. Phosphorus and calcium led to improved shoot growth, and calcium, magnesium, and mesonutrients ($CaCl_2$*$2H_2O$—KH_2PO_4—$MgSO_4$) improved leaf quality (Reed et al. 2013a). In a related experiment, physiological abnormalities in pear were analyzed in different media in an attempt to formulate media to maximize plant quality (Reed et al. 2013b). Adelberg et al. (2013) included non-nutrient factors in the experiment, such as plant density and media volume, demonstrating that process affects optimal concentrations of nutrient ions. High volumes of media containing high sucrose and macronutrient concentration with no ammonium allowed for the production of high-quality turmeric plants in high density vessels (Adelberg et al. 2013).

This study was designed to identify potential main and interactive effects among nutritional factors influencing performance of *Helleborus* ×*nigercors* and *Helleborus* ×*ballardiae* 'Raulston Remembered' in micropropagation. In this study, the effects of three plant nutrients (NH_4^+, NO_3-, and PO_4^{3-}), sucrose concentration, plant density, WPM dilution (taking into account the effects of other meso- and micronutrients not included as individual factors), and TDZ concentration were evaluated based on growth and multiplication of plantlets. Results from the initial D-optimized Response Surface design were used to frame a validation study was conducted with *H.* ×*ballardiae* 'Raulston Remembered.' The validation study was designed to determine if the newly formulated medium led to improved

multiplication ratios and plant quality when compared with the standard MS medium formulation in gelled medium, as is used in the micropropagation trade.

MATERIALS AND METHODS

Plant Material

Genotypes tested were *Helleborus* ×*ballardiae* 'Raulston Remembered' and *Helleborus* ×*nigercors*. The Institute for Sustainable and Renewable Resources (ISRR; Danville, Virginia) initiated the shoot tips acquired from Pine Knot Farms in Clarksville, Virginia. Source plants were rinsed under running tap water to remove visible debris before outer leaf and root removal. Plants were then placed under running tap water, following which they were exposed to two drops of surfactant detergent per liter to remove waxes on the plant surface inhibiting penetration of bleach. Following this, plants were rinsed three times with sterile deionized water, and soaked in 80% ethanol for 30 seconds. Plants were then rinsed with deionized water in three, five-minute intervals under a laminar flow hood. Plants were placed in a 1.65% bleach solution (20% dilution of Clorox ™, 8.25% sodium hypochlorite solution) and agitated for 20 minutes. Axillary buds were removed, and the remaining meristem-containing portion was divided into pieces and plated for entry into micropropagation. Initiated plantlets were originally maintained at Dan River Plants Laboratory in Danville, Virginia.

Stage II plantlets were maintained in Magenta GA7 Boxes (Magenta Corp., Chicago, IL) containing 50 mL of medium at pH 5.7 containing 3% sucrose, WPM

salts (Lloyd and McCown 1980), 9 µM TDZ, and either 0 g or 3.5 g gellan gum per liter. Cultures were maintained at 12 °C with a 16 hour photoperiod under 28µmol red+blue monochromatic LEDs (33.3% blue wavelength, 66.7% red wavelength) and transferred into new vessels every 6-8 weeks.

Experimental Design

The software application JMP® Pro 10.0.0 (SAS Institute Inc. 2012) was used for D-optimal design, evaluation, and analyses. The experiment was a 7-factor response surface design. A complete factorial design would require 2,187 treatments (3^7), but the D-optimized response surface design only required a subset of 44 treatments per clone to sample the same design space. Combinations of the seven factors were assigned to treatments using D-optimal criteria included the mineral nutrient factors (NH_4^+, NO_3^-, and PO_4^{3-}) defined in relation to WPM medium (Lloyd and McCown 1980). Other factors studied, including plant density (1-5 plantlets per Magenta box), WPM dilution (0.333, 0.667, and 1x WPM, expressed as a dilution of all meso- and micronutrients not included as individual factors), TDZ concentration (1, 5, and 9 µM), and sucrose concentration (1, 2, and 3%), were defined in relation to the standard media protocol from Dan River Plants (Table 2.1). Levels of phosphate (1.25, 3.75, and 6.25 mM) were obtained by adding NaH_2PO_4 salts, levels of ammonium (5, 20, and 35 mM) raised using NH_4NO_3, and levels of nitrate (39.8, 54.8, and 69.8 mM) achieved using a combination of NH_4NO_3 and KNO_3. Ranges of potassium varied in each treatment (9.9-80 mM) because it was used to charge balance nitrate (Table A8). Dan River Plants' standard WPM and

MS recipes, with 3% sucrose and 9 μM TDZ, were included as controls. All treatments were grown in Magenta GA7 boxes (Magenta Corp., Chicago, IL) in 12 mL of liquid medium at a pH of 5.7 containing a 5 x 5 cm square of germination paper (Anchor Paper Company, Saint Paul, MN). A list of the selected treatments can be found in Table 2.2.

Uniform plantlets were transferred into treatments at random, and boxes were arranged randomly on the growth room shelves under 28 μmol red+blue monochromatic LED lights. The growth room temperature ranged from 10-13 °C. Shoots were transferred into the same assigned treatment at 6-week intervals, and harvested after three repeated cycles. If plant density was greater than 5 plantlets per vessel, shoots were divided and subcultured in two separate vessels.

Average plant quality per vessel was defined on a subjective numerical scale (-2 = very poor quality, -1 = poor quality, 0 = acceptable quality, 1 = good quality, 2 = very good quality). Plant quality was defined in reference to the average plant quality of original plantlets obtained from Dan River Plants. Plantlets matching this reference condition were given a score of 0. Plantlets were given a score of -1 if they showed one or two of the following conditions: shoot tip necrosis, chlorosis, hyperhydricity, engorged petioles, or adventitious bud growth. Plantlets containing three or more negative attributes were assigned a score of -2. A score of 1 was assigned to plantlets with one or two of the following positive characteristics: thickened petioles, large leaves, dark green color, or abundant shoot branching. Plants were assigned a score of 2 if they contained three or more positive features.

Rankings can be visualized in Figure 2.1. Callus development was also ranked subjectively (-1 = excessive callus, 0 = acceptable callus, 1 = no callus), as was visible contamination (-2 = excessive contamination characterized by growth on the surface of the medium, -1 = minor visible contamination, visualized by a gray halo around the base of the plant, 0 = no visible contamination). Shoot multiplication was determined by dividing the total number of buds at the end of a cycle by the number of buds originally placed in the vessel.

Plant response at each treatment point was scored based on plant performance in each vessel during three culture cycles. The first culture cycle was omitted from analysis to allow for plantlets to acclimatize to treatment conditions. Response surface model terms were developed using data from the second and third cycles. Data were subjected to stepwise forward selection, and all possible linear, quadratic, and second-order interactions were evaluated. Potassium and visible contamination were considered as covariates. Any factor with a main-effect or second-order *P*-value less than 0.2 was included in the model, and other terms were eliminated. Following stepwise elimination, the individual responses and second-order interactions were analyzed at the 0.025 significance level by analysis of variance (ANOVA; SAS Institute Inc. 2012). In addition to *P*-value assessment, the R^2 value was used to evaluate the efficacy of the final model. Using data collected from tested points, projected responses at untested points could be estimated.

Validation Study

A complete factorial analysis containing phosphorus and sucrose as factors was completed. All treatments were grown in Magenta GA7 boxes (Magenta Corp., Chicago, IL) in 50mL of media containing MS salts (Murashige and Skoog 1962), 9 μM TDZ, and 3.5 g/L gellan. Although the multivariate experiment was conducted in liquid culture, micropropagation laboratories typically use gelling agents such as agar or gellan. In an attempt to optimize based on industry standards, gellan was included as a media component. Three plantlets were grown in each box. Levels of phosphorus were 1.25, 6.25, or 11.3 mM using NaH_2PO_4. Sucrose was added to be 3, 4, or 5% (g/L). Treatments are outlined in Table 2.3. Plantlets were randomly assigned to treatments and kept at 12 °C under 28 μmol red+blue monochromatic LED lights (66.7% red, 33.3% blue) and transferred every 6 weeks. The multiplication ratio and subjective numerical response of plant quality were determined upon transfer as in the prior experiment.

RESULTS AND DISCUSSION

Multivariate Response Surface Design Experiment

Multiplication Ratios:

Multiple factors and second-order interactions included in this study affected the multiplication ratios of both *Helleborus* ×*nigercors* and *Helleborus* ×*ballardiae* 'Raulston Remembered' (R^2=0.379, p<0.0001; Table 2.4). Sucrose (1-3%) was the most significant factor, with multiplication ratios increasing at higher concentrations (p=0.0001). Phosphorus concentration (1.25-6.25 mM) was not

significant as a main effect, but interacted highly with sucrose (p=0.0044). At low sucrose, high phosphorus prohibited successful division, but multiplication ratios were highest when both sucrose percentage and phosphorus concentration were highest (Figure 2.2). The model predicted that the optimal sucrose and phosphorus concentrations might be beyond the ranges tested in this experiment.

Nitrate concentration also significantly influenced multiplication ratios, and both linear and quadratic model terms were significant (p=0.0123, p=0.0212, respectively). Optimal concentrations of nitrate were within the range tested, suggesting that MS levels of nitrate (40 mM) are near ideal. Nitrate was supplied by both ammonium nitrate and potassium nitrate, indicating that potassium could be contributing to the desired result. When potassium was considered as a covariate, however, it was not found to be statistically significant. Although *Helleborus* ×*ballardiae* 'Raulston Remembered' performed significantly better in terms of multiplication, no interactions between genotype and nutrient factors were determined, indicating that responses were consistent across genotypes. Multiplication ratios were highest at the maximum tested concentration of sucrose (3%) and phosphorus (6.25 mM), and moderate levels of nitrate (40-50 mM).

Based on the D-optimized Response Surface model, the highest multiplication ratio predicted was 2.62 (2.02-3.23, 95% confidence interval) for *Helleborus* ×*ballardiae* 'Raulston Remembered' and 2.31 (1.71-2.92, 95% confidence interval) for *Helleborus* ×*nigercors.* Multiplication ratios were highest at a 0.75 dilution of the other meso-and micronutrients (boric acid, calcium chloride, calcium

nitrate, cupric sulfate, $Na_2EDTA \times 2H_2O$, ferrous sulfate, magnesium sulfate, manganese sulfate, molybdic acid, potassium sulfate, zinc sulfate, glycine, myo-inositol, nicotinic acid, pyridoxine x HCl, and thiamine x HCl), 40-50 mM of nitrate, and highest levels all other factors. Since plant density did not influence multiplication ratios, maximizing the output of plants per vessel was achieved with the highest initial bud density (5 plants per vessel).

Plant Quality:

Many model terms influenced plant quality ratings for both genotypes (R^2=0.445, p<0.0001) (Table 2.5). Nitrate concentration was the most significant factor, and was included as a positive quadratic (p<0.0001) and a positive linear (p=0.0229) term. Moderate levels of nitrate (40-50 mM) produced the highest quality plants. Plant density also contributed to plant quality, and treatments with 3 shoots per vessel produced the highest quality plants. This density is lower than that required to maximize plant output, but high quality plants can still be produced using a higher plant density per vessel. Sucrose and phosphorus, the main factors contributing to higher multiplication ratios, were not contributing factors in the plant quality model. However, plant quality responses predicted at treatment points leading to optimal multiplication ratios were among the highest predicted. Model predictions based on these treatment points provided a plant quality rating estimate of 2.02 (1.08-2.97, 95% confidence interval) (Figure 2.3), indicating that media may be formulated to maximize multiplication and still produce high quality plantlets.

Callus Development:

Model parameters affected callus development (R^2=0.43, p<0.0001) (Table 2.6). Callus formation was ranked on a subjective numerical scale from -2 to 0, with -2 being assigned to plants with prohibitively large calluses and 0 being assigned to plants with no visible callus. Ammonium concentration was the most pertinent factor, with higher concentrations of ammonium leading to smaller calluses (p=0.0006). Significant model terms included the following factors: TDZ concentration, genotype, phosphorus, and nitrate concentration. Although *Helleborus* ×*nigercors* had larger calluses than *Helleborus* ×*ballardiae* 'Raulston Remembered', and TDZ and phosphorus concentration showed interactive effects, the medium best formulated to maximize multiplication ratios (dilution = 0.75 ×WPM, [TDZ] = 9 µM, $[PO_4^{3-}]$=6.25 mM, $[NH_4^+]$=35 mM, $[NO_3^-]$=40 mM, and sucrose = 3%) also produced plantlets with acceptable calluses. The model prediction set to these treatment points for *Helleborus* ×*ballardiae* 'Raulston Remembered' was a rating of -0.011 (-0.14 to 0.12, 95% confidence interval), and the prediction for *Helleborus* ×*nigercors* was -0.32 (-0.46 to -0.18, 95% confidence interval). Media can be formulated to maximize multiplication ratios without compromising plants in terms of callus development.

Sucrose and Phosphorus Optimization Study

When in gelled media, there was no effect of increased sucrose or phosphorus on multiplication ratios of *Helleborus* ×*ballardiae* 'Raulston Remembered' (R^2=0.042, p=0.18). Increased phosphate concentration slightly

improved plant quality (R^2=0.02, p=0.048). Callus development was not affected by model terms (R^2=0.02, p=0.56).

CONCLUSIONS

Media formulation using D-optimal response surface criteria was valuable for identifying factors of importance related to multiplication ratios of hellebores in liquid media. Micropropagation research often focuses on the effects of growth hormones on plant multiplication, but this experiment indicates that nutrient supply may be more important. In the multivariate portion of this study, sucrose was found to be the most important factor, with main and second-order interactive effects.

Proton-pump ATPases have been found in plasma membranes of companion cells in *Arabidopsis thaliana* and in transfer cells of *Vicia faba* (Taiz et al. 2014). These sucrose transport systems are ATP-dependent, which could explain why higher levels of sucrose had a stronger beneficial influence on division rates in hellebores when combined with high levels of phosphate. Ransom-Hodgkins et al. (2003) found that protein phosphorylation influenced regulation of sucrose uptake in *Beta vulgaris* L. The proton-sucrose symporter *BvSUT1* found in phloem is controlled in part by a protein phosphorylation pathway, and inhibitors of protein phosphatase caused lowered rates of sucrose transport (Ransom-Hodgkins et al. 2003).

The data obtained in the multivariate experiment support the idea that sucrose uptake into plant cells is phosphate-dependent. However, in gelled media, the sugar and phosphate interaction was no longer significant. The limited water

availability in gelled medium could have reduced the ability for *Helleborus* plantlets to take up phosphate and sucrose effectively, and thus a true comparison could not be made. Preliminary experiments indicated that plant quality and multiplication ratios decrease in liquid media, so the benefits of increased phosphorus and sucrose may not be effectively realized in micropropagation of hellebores, especially since the industry standards use gelled rather than liquid media. Future studies to determine if the negative consequences of liquid media can be overcome by nutrient supply should be conducted when attempting to optimize micropropagation protocols for *Helleborus* spp.

Understanding how nutrients interact within plant tissue may illuminate enzymatic influences on plant growth and division. The partitioning and transport of nutrients is imperative to plant development and can have drastic effects on plant productivity (Vaughn et al. 2002). The results of this study suggest that mechanisms related to nutrient transport may provide insight into how to design media to maximize enzymatic efficiency. Shifting focus away from hormonal regulation and towards the effects of nutrient supply on cellular functions could allow for improvements in the micropropagation of difficult plants. Future studies using response surface methods may elucidate important aspects of mineral nutrition.

Table 2.1. The seven factors used to construct the seven-dimensional factorial space, their component Woody Plant Medium (WPM) salts, and concentration range expressed as compared to ×WPM levels, if applicable.

Factors	WPM salts	Range
Ammonium (NH_4)	NH_4NO_3	1-7×
Nitrate (NO_3)	NH_4NO_3	4-7×
	KNO_3	
Phosphate (PO_4)	NaH_2PO_4	1-5×
Dilution (other meso- and micronutrients contained in WPM media)	Boric acid	0.333–1×
	Calcium chloride	
	Calcium nitrate	
	Cupric sulfate * 5H20	
	Na2EDTA*2H20	
	Ferrous sulfate * 7H20	
	Magnesium sulfate	
	Manganese sulfate	
	Molybdic acid	
	Potassium sulfate	
	Zinc sulfate	
	Glycine	
	Myo-inositol	
	Nicotinic acid	
	Pyridoxine*HCl	
	Thiamine*HCl	
Thiadiazuron (TDZ)	n/a	3-9 μM
Sucrose	n/a	1-3%
Plant density	n/a	1-5

Table 2.2. Seven-factor treatment breakdown, including MS medium (45) and WPM medium (46) for error estimation.

Treatment	NH_4 (mM)	NO_3 (mM)	PO_4 (mM)	Dilution (×WPM)	TDZ (μM)	Sucrose (%)	Density	K [a] (mM)
1	5	39.8	1.25	0.333	1	3	1	39.9
2	5	69.8	1.25	0.333	1	1	5	69.9
3	35	39.8	1.25	0.333	1	1	1	9.9
4	35	39.8	1.25	0.333	1	3	5	9.9
5	35	69.8	1.25	0.333	1	3	1	39.9
6	5	39.8	6.25	0.333	1	3	5	39.9
7	20	69.8	6.25	0.333	1	1	1	54.9
8	35	39.8	6.25	0.333	1	3	1	9.9
9	35	69.8	6.25	0.333	1	2	5	39.9
10	5	39.8	1.25	0.333	9	3	5	39.9
11	5	69.8	1.25	0.333	9	1	1	69.9
12	35	39.8	1.25	0.333	9	3	1	9.9
13	35	54.8	1.25	0.333	9	1	5	24.9
14*	35	69.8	3.75	0.333	9	3	5	39.9
15*	35	69.8	3.75	0.333	9	3	5	39.9
16	5	39.8	6.25	0.333	9	1	1	39.9
17	5	69.8	6.25	0.333	9	3	1	69.9
18	35	39.8	6.25	0.333	9	1	5	9.9
19	5	54.8	6.25	0.667	1	1	1	59.9
20*	35	39.8	6.25	0.667	1	1	5	14.9
21*	35	39.8	6.25	0.667	1	1	5	14.9
22	20	54.8	3.75	0.667	5	2	3	44.9
23*	5	69.8	3.75	0.667	9	1	5	74.9
24*	5	69.8	3.75	0.667	9	1	5	74.9
25	35	69.8	6.25	0.667	9	1	1	44.9
26	5	39.8	1.25	1	1	1	5	50
27	5	69.8	1.25	1	1	1	1	80
28	35	39.8	1.25	1	1	3	1	20
29	5	69.8	3.75	1	1	3	5	80
30	35	54.8	3.75	1	1	1	3	35
31	20	39.8	6.25	1	1	2	1	35
32	35	69.8	6.25	1	1	3	3	50
33	35	69.8	1.25	1	5	1	5	50
34	5	39.8	3.75	1	5	3	1	50
35	5	69.8	6.25	1	5	1	5	80
36	35	39.8	6.25	1	5	1	1	20
37	5	69.8	1.25	1	9	3	3	80
38	20	39.8	1.25	1	9	1	1	35

Table 2.2 continued. Seven-factor treatment breakdown, including MS medium (45) and WPM medium (46) for error estimation.

Treatment	NH_4 (mM)	NO_3 (mM)	PO_4 (mM)	Dilution (×WPM)	TDZ (μM)	Sucrose (%)	Density	K [a] (mM)
39	20	39.8	1.25	1	9	1	1	35
40	35	39.8	1.25	1	9	2	5	20
41*	35	69.8	3.75	1	9	3	1	50
42*	35	69.8	3.75	1	9	3	1	50
43	5	39.8	6.25	1	9	3	3	50
44	20	54.8	6.25	1	9	3	5	50
45	5	10	1.25	1	9	3	3	20
46	19.4	40	1.25	1	9	3	3	20

[a] Potassium is considered a covariate and was used along with ammonium nitrate to bring levels of nitrate to desired treatment conditions and to maintain charge balance.
* Treatments that were part of true replications.

Table 2.3. Treatments for the 3x3 factorial study to confirm influence of sucrose and phosphorus concentrations on multiplication and plant quality of micropropagated *Helleborus* ×*ballardiae* 'Raulston Remembered' plantlets.[a]

Treatment	Phosphorus concentration (mM)	Sucrose concentration (%)
1	1.25	3
2	1.25	4
3	1.25	5
4	6.25	3
5	6.25	4
6	6.25	5
7	11.3	3
8	11.3	4
9	11.3	5

[a] All treatments contained 50 mL of media containing 1M MS salts and 9 μM TDZ, and a plant density of 3 plants per vessel at a temperature of 12 °C

Table 2.4. Multiplication ratio of hellebore following three 54-day cycles (model terms: percent sucrose, WPM dilution, and concentrations of phosphorus, nitrogen, ammonium, and TDZ). The final model had an R^2=0.379 and p<0.0001.

Model Terms	Parameter estimate [a]	P-value [b]	Mean Square
Sucrose	0.270 ± 0.068	0.0001*	6.86
Sucrose x PO_4^{3-}	0.085 ± 0.029	0.004*	3.67
NO_3^-	-0.010 ± 0.004	0.012*	2.82
Genotype	0.133 ± 0.054	0.015*	2.67
$(NO_3^-)^2$	-0.000 ± 0.000	0.021*	2.38
PO_4^{3-} x NH_4^-	0.004 ± 0.002	0.027	2.21
(WPM dilution)^2	-3.62 ± 1.63	0.028	2.17
Cycle	-0.110 ± 0.053	0.041	1.86
WPM dilution x PO_4^{3-}	-0.159 ± 0.083	0.057	1.62
TDZ	0.030 ± 0.015	0.058	1.60
Genotype x Cycle	-0.097 ± 0.053	0.072	1.44
NO_3^- x NH_4^+	-0.000 ± 0.000	0.118	1.08
WPM dilution x Cycle	-0.258 ± 0.178	0.149	0.921
NH_4^+ x Cycle	0.006 ± 0.004	0.150	0.918
NH_4^+ x Genotype	-0.005 ± 0.004	0.163	0.862
WPM dilution x TDZ	0.068 ± 0.050	0.175	0.815
WPM dilution x sucrose	0.263 ± 0.201	0.192	0.753
NH_4^+	-0.005 ± 0.004	0.199	0.730
PO_4^{3-}	0.008 ± 0.028	0.766	0.039
WPM dilution	0.063 ± 0.214	0.768	0.038

[a] Parameter estimates and mean square values are shown with a maximum of 3 significant figures.
[b] *P* value of *T* test
* Significance assigned at the 0.025 significance level

Table 2.5. Plant quality of hellebore following three 54-day (model terms: nitrate, phosphorus, ammonium, sucrose, and TDZ concentrations, plant density, WPM dilution, and visible contamination). Responses were consistent across genotypes. Whole model $R^2 = 0.445$ and $p<0.0001$. [a]

Model Terms	Parameter estimate [b]	P-value [c]	Mean Square
$(NO_3^-)^2$	-0.002 ± 0.000	<0.0001*	25.4
Density	0.295 ± 0.074	0.0001*	17.5
PO_4^{3-} x mM NH_4^-	0.012 ± 0.003	0.0003*	14.8
WPM dilution	0.975 ± 0.308	0.002*	11.0
WPM dilution x TDZ	0.259 ± 0.082	0.002*	10.8
Visible Contamination [d]	0.424 ± 0.140	0.003*	10.1
TDZ	0.067 ± 0.025	0.010*	7.59
Density x sucrose	0.016 ± 0.064	0.015*	6.68
WPM dilution x sucrose	0.796 ± 0.323	0.015*	6.67
Cycle	0.214 ± 0.088	0.016*	6.55
NH_4^+ x sucrose	0.018 ± 0.008	0.021*	5.97
NO_3^-	-0.014 ± 0.006	0.023*	5.81
$(Density)^2$	-0.013 ± 0.058	0.025	5.63
TDZ x sucrose	-0.049 ± 0.024	0.047	4.41
WPM dilution x Cycle	-0.532 ± 0.281	0.060	3.95
$(sucrose)^2$	-0.630 ± 0.336	0.062	3.87
TDZ x Cycle	-0.033 ± 0.022	0.145	2.36
PO_4^{3-}	0.054 ± 0.041	0.187	1.93
$(NH_4^+)^2$	0.002 ± 0.001	0.197	1.84
NH_4^+ x Density	-0.005 ± 0.004	0.236	1.56
sucrose	0.108 ± 0.100	0.281	1.29
NH_4^+	0.000 ± 0.007	0.989	0.0002

[a] Plant quality was assessed on a subjective numerical scale, with the best quality plants being assigned a rating of 2, and the worst quality plants being assigned -2.
[b] Parameter estimates and mean square values are shown with a maximum of 3 significant figures.
[c] *P* value of *T* test
[d] Visible contamination was included as a covariate, and was ranked from -2 (very contaminated) to zero (no visible contamination)
* Significance assigned at the 0.025 significance level

Table 2.6. Callus development on hellebore plantlets following three 54-day cycles (model terms: ammonium, TDZ, and phosphorus concentrations and genotype). Whole model $R^2 = 0.426$ and $p<0.0001$. [a]

Model Terms	Parameter estimate [b]	P-value [c]	Mean Square
NH_4^+	0.003 ± 0.001	0.0006*	0.304
TDZ x Genotype	0.012 ± 0.003	0.0008*	0.291
PO_4^{3-} x Genotype	0.019 ± 0.006	0.001*	0.265
TDZ x PO_4^{3-}	-0.005 ± 0.002	0.004*	0.219
TDZ	-0.011 ± 0.004	0.004*	0.210
$(NO_3^-)^2$	0.000 ± 0.000	0.012*	0.159
PO_4^{3-}	-0.015 ± 0.007	0.033	0.114
Genotype	0.028 ± 0.013	0.035	0.112
Cycle	0.026 ± 0.013	0.040	0.106
NH_4^+ x Cycle	-0.002 ± 0.001	0.044	0.102
Sucrose x Genotype	0.025 ± 0.013	0.063	0.087
NH_4^+ x Genotype	-0.002 ± 0.001	0.065	0.086
Sucrose	-0.027 ± 0.015	0.076	0.079
WPM dilution x Cycle	-0.071 ± 0.042	0.094	0.071
WPM dilution x PO_4^{3-}	-0.032 ± 0.020	0.114	0.063
TDZ x NH_4^+	0.001 ± 0.000	0.120	0.061
$(TDZ)^2$	-0.005 ± 0.003	0.129	0.058
NH_4^+ x NO_3^-	-0.001 ± 0.000	0.140	0.054
Genotype x Cycle	-0.018 ± 0.013	0.157	0.050
NO_3^- x Genotype	-0.001 ± 0.001	0.212	0.039
TDZ x NO_3^-	0.000 ± 0.000	0.245	0.034
NO_3^-	0.001 ± 0.001	0.324	0.024
Visible Contamination [d]	0.018 ± 0.021	0.377	0.019
$(PO_4^{3-})^2$	-0.005 ± 0.006	0.432	0.015
WPM dilution	-0.004 ± 0.045	0.935	0.0002

[a] Callus was defined on a scale from -2 (excessive callus development) to 0 (normal callus development).
[b] Parameter estimates and mean square values are shown with a maximum of 3 significant figures.
[c] *P* value of *T* test
[d] Visible contamination was included as a covariate, and was ranked from -2 (very contaminated) to zero (no visible contamination)
* Significance assigned at the 0.025 significance level

Figure 2.1. Visualization of plant quality rankings. (A) *Helleborus* ×*ballardiae* plantlet from treatment 38, given a ranking of 2, (B) *H.* ×*ballardiae* plantlet from treatment 44, given a ranking of 1, (C) *H.* ×*ballardiae* plantlet from WPM control, given a ranking of 0, (D) *H.* ×*ballardiae* plantlet from treatment 15 given a ranking of -1, and (E) *H.* ×*ballardiae* microcutting from treatment 2, given a ranking of -2.

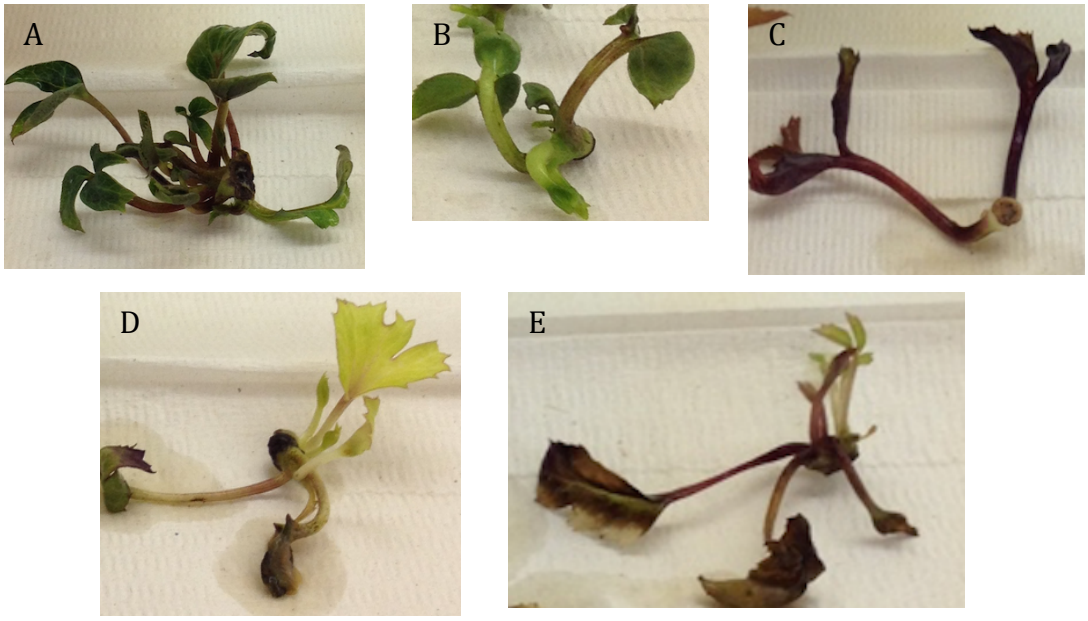


Figure 2.2. Response surface plot illustrating the interaction between % sucrose and [PO_4^{3-}] noted during the multivariate analysis: response of (A) *Helleborus* ×*nigercors* and (B) *Helleborus* ×*ballardiae* 'Raulston Remembered' with WPM dilution set to 0.667, [TDZ] to 9 µM, [NH_4^+] to 20 mM, and [NO_3^-] to 54.8 mM.

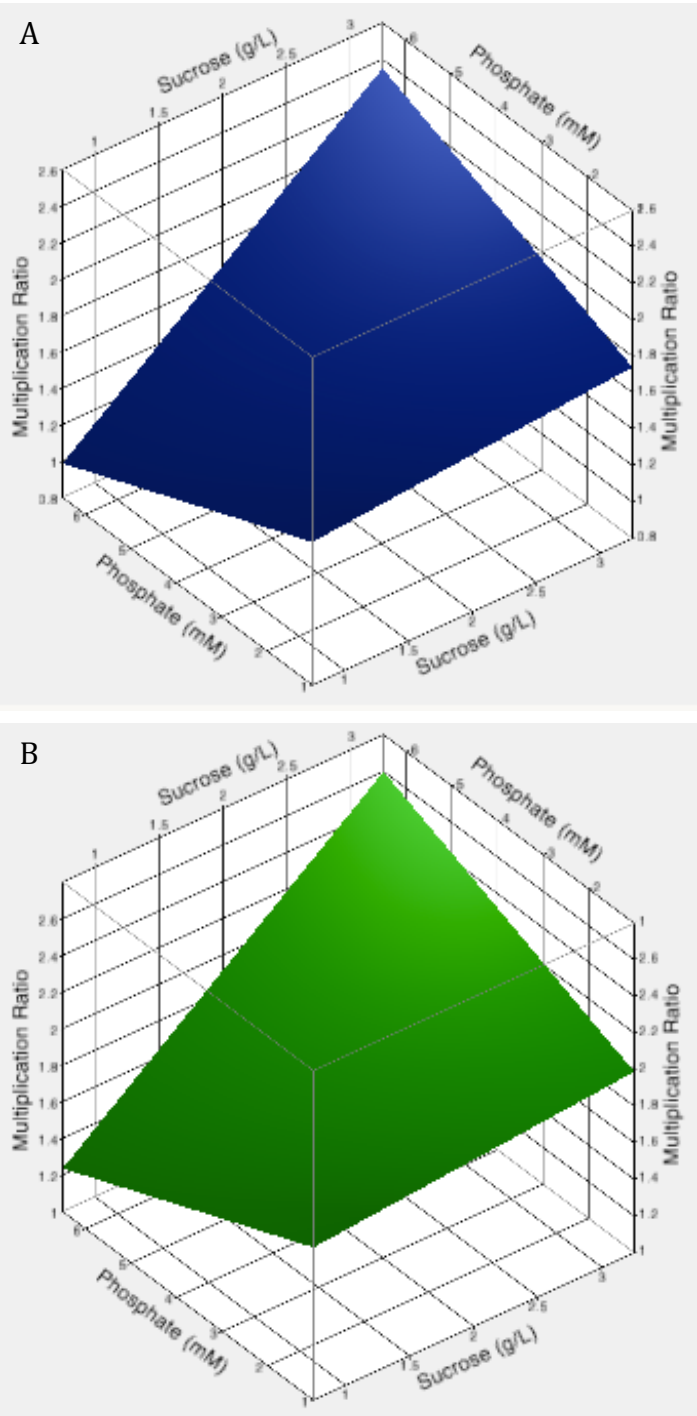
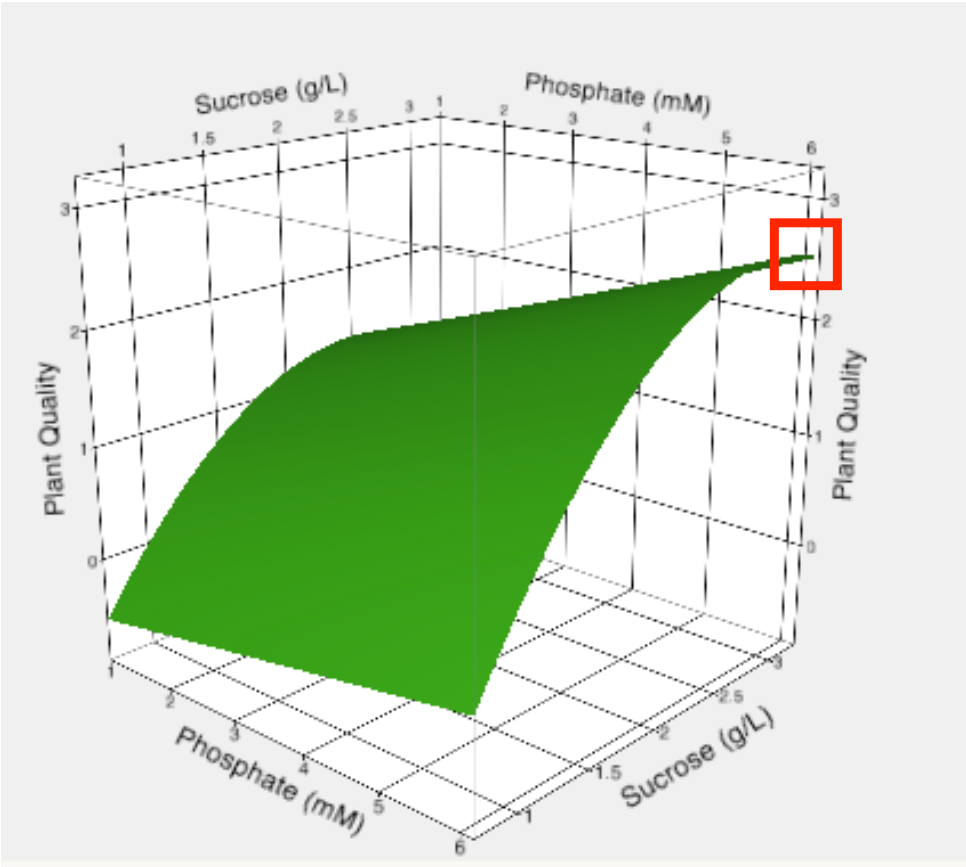


Figure 2.3. Response surface plot illustrating plant quality ratings modeled at factor points yielding highest multiplication ratios: WPM dilution at 0.75, [TDZ] at 9 µM, [NH_4^+] to 35 mM, [NO_3^-] to 40 mM. and density at 5 plants per vessel. The point outlined in red maximizes multiplication ratios while still producing very high quality plantlets.



APPENDIX

Table A1. The four clones included in one or more experiments of this study, their parental species, and hardiness zones

Clone	Parentage	Hardiness Zones
Helleborus ×*nigercors*	*H. niger* × *H. argutifolius*	Zones 6-9
Helleborus ×*ballardiae* 'Raulston Remembered'	*H. niger* × *H. lividus*	Zone 7 [a]
Helleborus ×*ashwoodensis* 'Briar Rose'	*H. niger* × *H. vesicarius*	Zones unknown [b]
Helleborus ×*hybridus*	*Helleborus* spp. [c]	Zones 4-9

[a] Full extent of hardiness range is not yet known, but plants flourish in Zone 7 (Burrell and Tyler 2006)
[b] Currently, 'Briar Rose' is used almost exclusively as a container plant, and its performance in garden landscapes and across hardiness zones is unknown (Burrell and Tyler 2006)
[c] Because of the ease of hybridization among *Helleborus* species, many modern ornamentals are complex hybrids involving many species (Burrell and Tyler 2006)

Table A2. Multiplication responses of three hybrid clones to changes in temperature and media type. [a, b]

Clone	Temperature [c]	Media State [d]	Average Multiplication Ratio (buds out/in)[e]	Confidence Interval
H. ×*ballardiae*	High	Gellan	0.71	0.19-1.22
H. ×*ballardiae*	High	Liquid	0.79	0.34-1.24
H. ×*ballardiae*	Medium	Gellan	1.69	1.16-2.21
H. ×*ballardiae*	Medium	Liquid	1.48	0.94-2.00
H. ×*ballardiae*	Low	Gellan	2.00	1.55-2.46
H. ×*ballardiae*	Low	Liquid	1.22	0.81-1.63
H. ×*nigercors*	High	Gellan	0.98	0.57-1.40
H. ×*nigercors*	High	Liquid	1.12	0.71-1.53
H. ×*nigercors*	Medium	Gellan	1.51	1.10-1.92
H. ×*nigercors*	Medium	Liquid	1.50	1.09-1.91
H. ×*nigercors*	Low	Gellan	1.44	0.99-1.89
H. ×*nigercors*	Low	Liquid	1.77	1.36-2.18
H. ×*hybridus*	High	Gellan	0.90	0.39-1.42
H. ×*hybridus*	High	Liquid	1.02	0.60-1.44
H. ×*hybridus*	Medium	Gellan	1.30	0.85-1.75
H. ×*hybridus*	Medium	Liquid	1.35	0.93-1.76
H. ×*hybridus*	Low	Gellan	1.41	1.00-1.82
H. ×*hybridus*	Low	Liquid	1.45	0.99-1.90

[a] Cultures were maintained under a 16-hour photoeriod under 28 µmol red + blue monochromatic LEDs (33.3% blue wavelength, 66.7% red wavelength) and transferred every 6-8 weeks. Plants were grown in Magenta boxes containing 50 mL of medium at pH 5.7, 3% sucrose, 1M WPM (Lloyd and McCown 1980), and 9 µm thiadiazuron (TDZ). Plants were randomly assigned to high temperature (18-22 °C), medium temperature (15-17 °C), and low temperature treatments (10-13 °C) and to gellan (3.5 g/L) or no-gellan treatments.

[b] Overall ANOVA results indicate that model terms significantly correlated with multiplication ratio (R^2=0.61, p=0.0008)

[c] Temperature had a significant overall effect on multiplication rate (p<0.0001). Plants of all genotypes and in all media types had significantly lower multiplication ratios (p<0.0001), and plants at low and medium temperatures had significantly higher multiplication ratios (p=0.0021, p=0.045). Medium and low temperature treatments were not significantly different from each other.

[d] Media state did not have a significant effect on multiplication ratio (p=0.7855)

[e] Average multiplication ratios calculated across a 54 day cycle.

Table A3. Multiplication responses of three hybrid clones to changes in light quality and media type. [a, b]

Clone [c]	Light Quality [d]	Media State [e]	Multiplication Ratio (buds out/in) [f]	Confidence Interval
H. ×*ballardiae*	R [g]	Gellan	1.58	1.35-1.81
H. ×*ballardiae*	R	Liquid	1.19	0.93-1.44
H. ×*ballardiae*	B [g]	Gellan	1.14	0.88-1.39
H. ×*ballardiae*	B	Liquid	1.04	0.78-1.31
H. ×*ballardiae*	RRB [g]	Gellan	1.32	1.03-1.61
H. ×*ballardiae*	RRB	Liquid	1.26	1.01-1.51
H. ×*ballardiae*	BBR [g]	Gellan	1.05	0.79-1.31
H. ×*ballardiae*	BBR	Liquid	0.97	0.72-1.22
H. ×*nigercors*	R	Gellan	1.28	0.95-1.61
H. ×*nigercors*	R	Liquid	0.98	0.70-1.25
H. ×*nigercors*	B	Gellan	1.20	0.95-1.44
H. ×*nigercors*	B	Liquid	1.20	0.91-1.48
H. ×*nigercors*	RRB	Gellan	1.03	0.71-1.34
H. ×*nigercors*	RRB	Liquid	1.06	0.71-1.41
H. ×*nigercors*	BBR	Gellan	1.17	0.91-1.43
H. ×*nigercors*	BBR	Liquid	1.18	0.94-1.42
H. ×*hybridus*	R	Gellan	1.08	0.83-1.34
H. ×*hybridus*	R	Liquid	0.67	0.35-0.99
H. ×*hybridus*	B	Gellan	0.87	0.60-1.13
H. ×*hybridus*	B	Liquid	0.76	0.47-1.05
H. ×*hybridus*	RRB	Gellan	1.10	0.76-1.44
H. ×*hybridus*	RRB	Liquid	1.02	0.71-1.33
H. ×*hybridus*	BBR	Gellan	0.84	0.55-1.12
H. ×*hybridus*	BBR	Liquid	0.74	0.50-0.99

[a] Cultures were maintained under a 16-hour photoperiod and transferred every 6-8 weeks. Plants were grown at 12 °C in Magenta boxes containing 50 mL of medium at pH 5.7, 3% sucrose, 1M WPM (Lloyd and McCown 1980), and 9 µm thiadiazuron (TDZ). Plants were randomly assigned to one of four light quality treatments (100% red, 100% blue, 33.3% red +66.7% blue, and 66.7% red + 33.3% blue monochromatic LEDs) and to gellan (3.5 g/L) or no-gellan treatments.

[b] Overall ANOVA results show significant model terms for multiplication ratios ($R^2 = 0.43$, $p<0.0001$)

[c] *H.* ×*hybridus* showed lower multiplication rates than other clones in this experiment ($p<0.0001$).

[d] Light Quality did not have a significant effect on multiplication ratio ($p=0.505$)

[e] Media state had a significant effect on multiplication ratios ($p=0.0047$), with plants grown on gellan media having significantly higher ratios

[f] Average multiplication ratios calculated across a 54 day cycle

[g] Plants were assigned to treatments R (100% red light), B (100% blue light), RRB (66.7% red, 33.3% blue), or BBR (66.7% blue, 33.3% red).

Table A4. Rooting responses of two hybrid clones to changes in light quality and media type. Cultures were maintained under a 16-hour photoperiod and transferred every 6-8 weeks. [a, b]

Clone	Light Quality [c]	Media State [d]	Average Rooting Percentage (buds out/in) [e]	Confidence Interval
H. ×*ballardiae*	R [f]	Gellan	86%	61-100%
H. ×*ballardiae*	R	Liquid	19%	1-39%
H. ×*ballardiae*	B [f]	Gellan	70%	48-92%
H. ×*ballardiae*	B	Liquid	6.7%	0-32%
H. ×*ballardiae*	RRB [f]	Gellan	68%	43-93%
H. ×*ballardiae*	RRB	Liquid	21%	0-43%
H. ×*ballardiae*	BBR [f]	Gellan	96%	74-100%
H. ×*ballardiae*	BBR	Liquid	22%	0-47%
H. ×*nigercors*	R	Gellan	95%	75-100%
H. ×*nigercors*	R	Liquid	39%	14-64%
H. ×*nigercors*	B	Gellan	61%	36-86%
H. ×*nigercors*	B	Liquid	0%	0-25%
H. ×*nigercors*	RRB	Gellan	66%	40-91%
H. ×*nigercors*	RRB	Liquid	14%	0-36%
H. ×*nigercors*	BBR	Gellan	89%	67-100%
H. ×*nigercors*	BBR	Liquid	21%	0-43%

[a] Plants were grown at 12 °C in Magenta boxes containing 50 mL of medium at pH 5.7, 3% sucrose, 1M MS (Murashige and Skoog 1962), and 5.4 µm napthalic acid anhydride(NAA). Plants were randomly assigned to one of four light quality treatments (100% red, 100% blue, 33.3% red +66.7% blue, and 66.7% red + 33.3% blue monochromatic LEDs) and to gellan (3.5 g/L) or no-gellan treatments.

[b] ANOVA results indicate that the model terms are correlated with rooting percentage (R^2=0.71, p<0.0001).

[c] Light Quality did not have a significant effect on rooting percentage (p=0.09)

[d] Media state had a significant effect on rooting ability (p<0.0001), with plants grown on gellan showing significantly higher rooting percentages

[e] Average rooting percentage calculated over a 55 day cycle

[f] Plants were assigned to treatments R (100% red light), B (100% blue light), RRB (66.7% red, 33.3% blue), or BBR (66.7% blue, 33.3% red)

Table A5. Multiplication ratios of two hybrid clones to changes in growth hormone type and concentration, salt type, and media state. [a, b]

Clone [c]	Growth Hormone Type and Concentration [d, e]	Salt Type [f]	Media State [g]	Multiplication Ratio (buds out/in) [h]	Confidence Interval
6 [i]	High BA	MS	Gellan	1.15	1.02-1.28
6	High BA	MS	Liquid	1.16	1.03-1.28
6	High BA	WPM	Gellan	1.11	0.99-1.24
6	High BA	WPM	Liquid	1.12	1.00-1.25
6	Low BA	MS	Gellan	1.23	1.11-1.35
6 *	Low BA	MS	Liquid	1.24	1.11-1.36
6	Low BA	WPM	Gellan	1.20	1.08-1.31
6 *	Low BA	WPM	Liquid	1.20	1.09-1.32
6	High BA + IAA [j]	MS	Gellan	1.10	0.97-1.23
6	High BA + IAA	MS	Liquid	1.11	0.98-1.24
6	High BA + IAA	WPM	Gellan	1.07	0.94-1.19
6 *	High BA + IAA	WPM	Liquid	1.07	0.95-1.20
6	Low BA + IAA	MS	Gellan	1.11	0.98-1.24
6 *	Low BA + IAA	MS	Liquid	1.12	0.99-1.25
6	Low BA + IAA	WPM	Gellan	1.08	0.95-1.21
6	Low BA + IAA	WPM	Liquid	1.09	0.96-1.21
6	High BA + 2ip [j]	MS	Gellan	1.19	1.06-1.32
6 *	High BA + 2ip	MS	Liquid	1.20	1.08-1.33
6	High BA + 2ip	WPM	Gellan	1.16	1.04-1.28
6 *	High BA + 2ip	WPM	Liquid	1.17	1.05-1.29
6	Low BA + 2ip	MS	Gellan	1.07	0.94-1.20
6	Low BA + 2ip	MS	Liquid	1.08	0.94-1.21
6	Low BA + 2ip	WPM	Gellan	1.16	1.04-1.28
6	Low BA + 2ip	WPM	Liquid	1.04	0.91-1.17

Table A5 continued. Multiplication ratios of two hybrid clones to changes in growth hormone type and concentration, salt type, and media state. [a, b]

Clone [c]	Growth Hormone Type and Concentration [d,e]	Salt Type [f]	Media State [g]	Multiplication Ratio (buds out/in) [h]	Confidence Interval
6	High TDZ	MS	Gellan	1.22	1.09-1.34
6 *	High TDZ	MS	Liquid	1.23	1.10-1.35
6	High TDZ	WPM	Gellan	1.18	1.06-1.31
6	High TDZ	MS	Gellan	1.22	1.09-1.34
6	Low TDZ	MS	Gellan	1.24	1.13-1.37
6	Low TDZ	MS	Liquid	1.26	1.14-1.38
6	Low TDZ	WPM	Gellan	1.21	1.09-1.34
6 *	Low TDZ	WPM	Liquid	1.22	1.10-1.35
6	High Kin + IAA	MS	Gellan	1.15	1.01-1.28
6	High Kin + IAA	MS	Liquid	1.16	1.02-1.29
6	High Kin + IAA	WPM	Gellan	1.11	0.98-1.25
6 *	High Kin + IAA	WPM	Liquid	1.12	0.99-1.25
6	Low Kin + IAA	MS	Gellan	1.08	0.94-1.21
6	Low Kin + IAA	MS	Liquid	1.08	0.95-1.22
6	Low Kin + IAA	WPM	Gellan	1.04	0.91-1.18
6	Low Kin + IAA	WPM	Liquid	1.05	0.92-1.18
7 [i]	High BA	MS	Gellan	1.09	0.96-1.22
7 *	High BA	MS	Liquid	1.10	0.97-1.22
7	High BA	WPM	Gellan	1.06	0.92-1.19
7	High BA	WPM	Liquid	1.06	0.94-1.19
7	Low BA	MS	Gellan	1.14	1.02-1.27
7 *	Low BA	MS	Liquid	1.14	1.02-1.27
7	Low BA	WPM	Gellan	1.14	1.01-1.26
7	Low BA	WPM	Liquid	1.14	0.02-1.27
7	High BA + IAA [j]	MS	Gellan	1.04	0.92-1.17

Table A5 continued. Multiplication ratios of two hybrid clones to changes in growth hormone type and concentration, salt type, and media state. [a, b]

Clone [c]	Growth Hormone Type and Concentration [d, e]	Salt Type [f]	Media State [g]	Multiplication Ratio (buds out/in) [h]	Confidence Interval
7	High BA + IAA	MS	Liquid	1.05	0.92-1.18
7	High BA + IAA	WPM	Gellan	1.01	0.88-1.13
7	High BA + IAA	WPM	Liquid	1.01	0.89-1.14
7	Low BA + IAA	MS	Gellan	1.05	0.93-1.18
7 *	Low BA + IAA	MS	Liquid	1.06	0.93-1.19
7	Low BA + IAA	WPM	Gellan	1.02	0.89-1.15
7	High BA + 2ip [j]	MS	Gellan	1.13	1.00-1.26
7	High BA + 2ip	MS	Liquid	1.14	1.01-1.27
7	High BA + 2ip	WPM	Gellan	1.10	0.97-1.23
7	High BA + 2ip	WPM	Liquid	1.11	0.98-1.24
7	Low BA + 2ip	MS	Gellan	1.01	0.88-1.14
7	Low BA + 2ip	MS	Liquid	1.02	0.89-1.15
7	Low BA + 2ip	WPM	Gellan	0.98	0.84-1.11
7	Low BA + 2ip	WPM	Liquid	0.98	0.85-1.12
7	High TDZ	MS	Gellan	1.16	1.04-1.28
7 *	High TDZ	MS	Liquid	1.17	1.04-1.29
7	High TDZ	WPM	Gellan	1.12	1.00-1.25
7	High TDZ	WPM	Liquid	1.13	1.00-1.26
7	Low TDZ	MS	Gellan	1.19	1.06-1.31
7	Low TDZ	MS	Liquid	1.19	1.07-1.32
7	Low TDZ	WPM	Gellan	1.15	1.02-1.29
7	Low TDZ	WPM	Liquid	1.16	1.03-1.29
7	High Kin + IAA	MS	Gellan	1.09	0.95-1.22
7	High Kin + IAA	MS	Liquid	1.10	0.96-1.23
7	High Kin + IAA	WPM	Gellan	1.05	0.92-1.19

Table A5 continued. Multiplication ratios of two hybrid clones to changes in growth hormone type and concentration, salt type, and media state. [a, b]

Clone [c]	Growth Hormone Type and Concentration [d, e]	Salt Type [f]	Media State [g]	Multiplication Ratio (buds out/in) [h]	Confidence Interval
7	High Kin + IAA	WPM	Liquid	1.06	0.93-1.20
7	Low Kin + IAA	MS	Gellan	1.01	0.88-1.15
7	Low Kin + IAA	MS	Liquid	1.02	0.89-1.16
7	Low Kin + IAA	WPM	Gellan	1.05	0.92-1.19
7	Low Kin + IAA	WPM	Liquid	1.06	0.93-1.20

[a] Cultures were maintained under a 16-hour photoperiod and transferred every 6-8 weeks. Plants were grown at 12° C in Magenta boxes containing 12 mL of medium at pH 5.7, and 3% sucrose and were randomly assigned to one of ten growth hormone combinations and concentrations, one of two salt types (1M MS or 1M WPM salts), and to gellan (3.5 g/L) or no gellan groups.

[b] ANOVA results indicate that there is a no significant correlation between model terms and multiplication ratios (R^2=0.02, p<0.2384).

[c] There is no significant difference between multiplication ratios of different clones included in this study (p=0.1022)

[d] High concentrations for all growth hormones (excluding IAA and 2ip) were set to 9 µM, and low concentrations were set to 1 µM.

[e] There is no significant difference between multiplication ratios of plants grown on different hormone treatments (p=0.175)

[f] There is no significant difference between salt types (p=0.3528)

[g] There was no difference in multiplication ratios between plants grown with or without gellan (p=0.8353)

[h] average multiplication ratios calculated over a 50 day cycle

[i] *Helleborus* ×*ballardiae* was designated Clone 6, and *Helleborus* ×*nigercors* designated Clone 7

[j] IAA and 2ip were set to 10 µM for all treatments in which they are included

* high-performing liquid treatments marked with an asterisk (*) were chosen at random to be included in the spent media analysis outlined in Table A8

Table A6. Callus development of two hybrid clones to changes in growth hormone type and concentration, salt type, and media state. [a, b]

Clone [c]	Growth Hormone Type and Concentration [d ,e]	Salt Type [f]	Media State [g]	Average Callus Development [h]	Confidence Interval
6 [i]	High BA	MS	Gellan	-0.19	-0.30 to -0.07
6	High BA	MS	Liquid	-0.16	-0.27 to -0.05
6	High BA	WPM	Gellan	-0.04	-0.15 to 0.07
6	High BA	WPM	Liquid	-0.02	-0.13 to 0.09
6	Low BA	MS	Gellan	-0.16	-0.27 to -0.06
6	Low BA	MS	Liquid	-0.14	-0.25 to -0.04
6	Low BA	WPM	Gellan	-0.02	-0.12 to 0.08
6	Low BA	WPM	Liquid	0.003	-0.1 to 0.11
6	High BA + IAA [j]	MS	Gellan	-0.32	-0.43 to -0.20
6	High BA + IAA	MS	Liquid	-0.29	-0.41 to -0.18
6	High BA + IAA	WPM	Gellan	-0.17	-0.28 to -0.06
6	High BA + IAA	WPM	Liquid	-0.15	-0.26 to -0.04
6	Low BA + IAA	MS	Gellan	-0.16	-0.28 to -0.05
6	Low BA + IAA	MS	Liquid	-0.14	-0.25 to -0.03
6	Low BA + IAA	WPM	Gellan	-0.02	-0.13 to -0.09
6	Low BA + IAA	WPM	Liquid	0.004	-0.12 to 0.12
6	High BA + 2ip [j]	MS	Gellan	-0.06	-0.17 to 0.04
6	High BA + 2ip	MS	Liquid	-0.04	-0.15 to 0.07
6	High BA + 2ip	WPM	Gellan	0.08	-0.02 to 0.19
6	High BA + 2ip	WPM	Liquid	0.10	-0.002 to 0.21
6	Low BA + 2ip	MS	Gellan	0.03	-0.09 to 0.14
6	Low BA + 2ip	MS	Liquid	0.05	-0.07 to 0.16
6	Low BA + 2ip	WPM	Gellan	0.17	0.06 to 0.29
6	Low BA + 2ip	WPM	Liquid	0.19	0.08 to 0.31

Table A6 continued. Callus development of two hybrid clones to changes in growth hormone type and concentration, salt type, and media state. [a,b]

Clone [c]	Growth Hormone Type and Concentration [d,e]	Salt Type [f]	Media State [g]	Average Callus Development [h]	Confidence Interval
6	High TDZ	MS	Gellan	-0.24	-0.34 to -0.13
6	High TDZ	MS	Liquid	-0.22	-0.32 to -0.11
6	High TDZ	WPM	Gellan	-0.09	-0.20 to 0.02
6	High TDZ	WPM	Liquid	-0.07	-0.18 to 0.04
6	Low TDZ	MS	Gellan	-0.09	-0.19 to 0.02
6	Low TDZ	MS	Liquid	-0.07	-0.17 to 0.04
6	Low TDZ	WPM	Gellan	0.06	-0.05 to 0.17
6	Low TDZ	WPM	Liquid	0.08	-0.03 to 0.19
6	High Kin + IAA	MS	Gellan	-0.43	-0.55 to -0.31
6	High Kin + IAA	MS	Liquid	-0.41	-0.53 to -0.29
6	High Kin + IAA	WPM	Gellan	-0.29	-0.40 to -0.17
6	High Kin + IAA	WPM	Liquid	-0.26	-0.38 to -0.15
6	Low Kin + IAA	MS	Gellan	-0.32	-0.44 to 0.21
6	Low Kin + IAA	MS	Liquid	-0.30	-0.42 to -0.18
6	Low Kin + IAA	WPM	Gellan	-0.18	-0.30 to -0.06
6	Low Kin + IAA	WPM	Liquid	-0.16	-0.27 to -0.04
7 [i]	High BA	MS	Gellan	-0.22	-0.33 to -0.11
7	High BA	MS	Liquid	-0.20	-0.31 to -0.09
7	High BA	WPM	Gellan	-0.08	-0.19 to 0.04
7	High BA	WPM	Liquid	-0.05	-0.17 to 0.06
7	Low BA	MS	Gellan	-0.20	-0.31 to -0.09
7	Low BA	MS	Liquid	-0.18	-0.29 to -0.07
7	Low BA	WPM	Gellan	-0.06	-0.16 to 0.05
7	Low BA	WPM	Liquid	-0.03	-0.14 to 0.07
7	High BA + IAA [j]	MS	Gellan	-0.35	-0.46 to -0.24

Table A6 continued. Callus development of two hybrid clones to changes in growth hormone type and concentration, salt type, and media state. [a, b]

Clone [c]	Growth Hormone Type and Concentration [d,e]	Salt Type [f]	Media State [g]	Average Callus Development [h]	Confidence Interval
7	High BA + IAA	MS	Liquid	-0.33	-0.44 to -0.22
7	High BA + IAA	WPM	Gellan	-0.21	-0.32 to -0.10
7	High BA + IAA	WPM	Liquid	-0.18	-0.30 to -0.07
7	Low BA + IAA	MS	Gellan	-0.20	-0.31 to -0.09
7	Low BA + IAA	MS	Liquid	-0.18	-0.29 to -0.07
7	Low BA + IAA	WPM	Gellan	-0.05	-0.17 to -0.06
7	Low BA + IAA	WPM	Liquid	-0.03	-0.14 to -0.08
7	High BA + 2ip [j]	MS	Gellan	-0.10	-0.21 to 0.01
7	High BA + 2ip	MS	Liquid	-0.08	-0.19 to 0.03
7	High BA + 2ip	WPM	Gellan	0.05	-0.07 to 0.16
7	High BA + 2ip	WPM	Liquid	0.07	-0.05 to 0.18
7	Low BA + 2ip	MS	Gellan	-0.01	-0.12 to 0.10
7	Low BA + 2ip	MS	Liquid	-0.01	-0.10 to 0.13
7	Low BA + 2ip	WPM	Gellan	0.14	0.02 to 0.25
7	Low BA + 2ip	WPM	Liquid	0.16	0.04 to 0.28
7	High TDZ	MS	Gellan	-0.27	-0.38 to -0.17
7	High TDZ	MS	Liquid	-0.25	-0.36 to -0.15
7	High TDZ	WPM	Gellan	-0.13	-0.24 to -0.02
7	High TDZ	WPM	Liquid	-0.11	-0.22 to 0.005
7	Low TDZ	MS	Gellan	-0.12	-0.23 to -0.02
7	Low TDZ	MS	Liquid	-0.10	-0.21 to 0.005
7	Low TDZ	WPM	Gellan	0.02	-0.09 to 0.14
7	Low TDZ	WPM	Liquid	0.04	-0.07 to 0.16
7	High Kin + IAA	MS	Gellan	-0.47	-0.59 to -0.35
7	High Kin + IAA	MS	Liquid	-0.45	-0.56 to -0.33
7	High Kin + IAA	WPM	Gellan	-0.32	-0.44 to -0.20

Table A6 continued. Callus development of two hybrid clones to changes in growth hormone type and concentration, salt type, and media state. [a, b]

Clone [c]	Growth Hormone Type and Concentration [d, e]	Salt Type [f]	Media State [g]	Average Callus Development [h]	Confidence Interval
7	High Kin + IAA	WPM	Liquid	-0.30	-0.42 to -0.18
7	Low Kin + IAA	MS	Gellan	-0.36	-0.48 to -0.24
7	Low Kin + IAA	MS	Liquid	-0.34	-0.45 to -0.22
7	Low Kin + IAA	WPM	Gellan	-0.21	-0.33 to -0.10
7	Low Kin + IAA	WPM	Liquid	-0.19	-0.31 to -0.07

[a] Cultures were maintained under a 16-hour photoperiod and transferred every 6-8 weeks. Plants were grown at 12 °C in Magenta boxes containing 12 mL of medium at pH 5.7, and 3% sucrose. Plants were randomly assigned to one of ten growth hormone combinations and concentrations, one of two salt types (1M MS or 1M WPM salts), and to gellan (3.5 g/L) or no gellan groups.
[b] ANOVA results show that there is a significant correlation between model terms and callus development (R^2=0.12, p<0.0001)
[c] Clones 6 and 7 were not significantly different in terms of callus development (p=0.234)
[d] High concentrations for all growth hormones (excluding IAA and 2ip) were set to 9 µM, and low concentrations were set to 1 µM.
[e] There is a significant difference between hormone treatment groups and callus development, with high and low Kin + IAA treatments producing larger calluses (p<0.0001 for both), and the high and low Ba + 2ip producing smaller calluses (p<0.0001, p=0.0001, respectively).
[f] There is a significant difference in callus development between plants treated with different pre-formulated salt types, with MS salt treatments producing plantlets with more callus (p<0.0001)
[g] There is no significant correlation between media state and callus development (p=0.4627)
[h] Callus formation was subjectively determined visually on a scale from -1 to 1, with lower numbers being associated with high callus formation, and high numbers being associated with no callus
[i] *Helleborus* ×*ballardiae* was designated Clone 6, and *Helleborus* ×*nigercors* designated Clone 7
[j] IAA and 2ip were set to 10 µM for all treatments in which they are included

Table A7. Plant Quality responses of two hybrid clones to changes in growth hormone type and concentration, salt type, and media state. Cultures were maintained under a 16-hour photoperiod and transferred every 6-8 weeks. Plants were grown at 12 °C in Magenta boxes containing 12 mL of medium at pH 5.7, and 3% sucrose. Plants were randomly assigned to one of ten growth hormone combinations and concentrations, one of two salt types (1M MS or 1M WPM salts), and to gellan (3.5 g/L) or no gellan groups.[a]

Clone [b]	Growth Hormone Type and Concentration [c, d]	Salt Type [e]	Media State [f]	Average Plant Quality [g]	Confidence Interval
6 [h]	High BA	MS	Gellan	-0.07	-0.31 to 0.16
6	High BA	MS	Liquid	0.10	-0.13 to 0.33
6	High BA	WPM	Gellan	-0.30	-0.53 to -0.07
6	High BA	WPM	Liquid	-0.12	-0.35 to 0.10
6	Low BA	MS	Gellan	0.20	-0.02 to 0.42
6	Low BA	MS	Liquid	0.38	0.16 to 0.60
6	Low BA	WPM	Gellan	-0.03	-0.24 to 0.19
6	Low BA	WPM	Liquid	0.15	-0.06 to 0.36
6	High BA + IAA [i]	MS	Gellan	-0.06	-0.30 to 0.17
6	High BA + IAA	MS	Liquid	0.11	-0.12 to 0.35
6	High BA + IAA	WPM	Gellan	-0.29	-0.52 to -0.06
6	High BA + IAA	WPM	Liquid	-0.11	-0.35 to 0.12
6	Low BA + IAA	MS	Gellan	0.03	-0.21 to 0.26
6	Low BA + IAA	MS	Liquid	0.20	-0.04 to 0.44
6	Low BA + IAA	WPM	Gellan	-0.20	-0.43 to 0.03
6	Low BA + IAA	WPM	Liquid	-0.02	-0.26 to 0.21
6	High BA + 2ip [i]	MS	Gellan	-0.05	-0.28 to 0.17
6	High BA + 2ip	MS	Liquid	0.12	-0.12 to 0.35
6	High BA + 2ip	WPM	Gellan	-0.28	-0.50 to -0.06
6	High BA + 2ip	WPM	Liquid	-0.10	-0.32 to 0.12
6	Low BA + 2ip	MS	Gellan	-0.12	-0.35 to 0.12
6	Low BA + 2ip	MS	Liquid	0.06	-0.18 to 0.30
6	Low BA + 2ip	WPM	Gellan	-0.34	-0.58 to -0.10
6	Low BA + 2ip	WPM	Liquid	-0.17	-0.41 to 0.07
6	High TDZ	MS	Gellan	0.01	-0.21 to 0.23

Table A7 continued. Plant Quality responses of two hybrid clones to changes in growth hormone type and concentration, salt type, and media state. [a, b]

Clone [c]	Growth Hormone Type and Concentration [d, e]	Salt Type [f]	Media State [g]	Average Plant Quality [h]	Confidence Interval
6	High TDZ	MS	Liquid	0.19	-0.04 to 0.41
6	High TDZ	WPM	Gellan	-0.22	-0.44 to 0.01
6	High TDZ	WPM	Liquid	-0.04	-0.27 to 0.19
6	Low TDZ	MS	Gellan	0.14	-0.08 to 0.36
6	Low TDZ	MS	Liquid	0.32	0.10 to 0.54
6	Low TDZ	WPM	Gellan	-0.09	-0.31 to 0.14
6	Low TDZ	WPM	Liquid	0.09	-0.13 to 0.31
6	High Kin + IAA	MS	Gellan	-0.33	-0.57 to -0.08
6	High Kin + IAA	MS	Liquid	-0.15	-0.39 to 0.09
6	High Kin + IAA	WPM	Gellan	-0.55	-0.79 to -0.31
6	High Kin + IAA	WPM	Liquid	-0.38	-0.62 to -0.14
6	Low Kin + IAA	MS	Gellan	-0.56	-0.81 to -0.31
6	Low Kin + IAA	MS	Liquid	-0.38	-0.63 to -0.14
6	Low Kin + IAA	WPM	Gellan	-0.78	-1.03 to -0.54
6	Low Kin + IAA	WPM	Liquid	-0.61	-0.85 to -0.37
7 [i]	High BA	MS	Gellan	-0.25	-0.48 to -0.01
7	High BA	MS	Liquid	-0.07	-0.30 to 0.16
7	High BA	WPM	Gellan	-0.47	-0.72 to -0.23
7	High BA	WPM	Liquid	-0.30	-0.53 to -0.07
7	Low BA	MS	Gellan	0.03	-0.20 to 0.25
7	Low BA	MS	Liquid	0.20	-0.02 to 0.43
7	Low BA	WPM	Gellan	-0.20	-0.43 to 0.03
7	Low BA	WPM	Liquid	-0.02	-0.25 to 0.20
7	High BA + IAA [j]	MS	Gellan	-0.24	-0.46 to -0.01
7	High BA + IAA	MS	Liquid	-0.06	-0.29 to 0.17

Table A7 continued. Plant Quality responses of two hybrid clones to changes in growth hormone type and concentration, salt type, and media state. [a, b]

Clone [c]	Growth Hormone Type and Concentration [d, e]	Salt Type [f]	Media State [g]	Average Plant Quality [h]	Confidence Interval
7	High BA + IAA	WPM	Gellan	-0.46	-0.70 to -0.23
7	High BA + IAA	WPM	Liquid	-0.29	-0.52 to -0.05
7	Low BA + IAA	MS	Gellan	-0.25	-0.38 to 0.09
7	Low BA + IAA	MS	Liquid	0.03	-0.21 to 0.26
7	Low BA + IAA	WPM	Gellan	-0.37	-0.61 to -0.14
7	Low BA + IAA	WPM	Liquid	-0.20	-0.42 to 0.04
7	High BA + 2ip [j]	MS	Gellan	-0.23	-0.46 to 0.008
7	High BA + 2ip	MS	Liquid	-0.05	-0.29 to 0.18
7	High BA + 2ip	WPM	Gellan	-0.45	-0.69 to -0.22
7	High BA + 2ip	WPM	Liquid	-0.28	-0.51 to -0.04
7	Low BA + 2ip	MS	Gellan	-0.29	-0.52 to -0.05
7	Low BA + 2ip	MS	Liquid	-0.11	-0.35 to 0.12
7	Low BA + 2ip	WPM	Gellan	-0.52	-0.76 to -0.27
7	Low BA + 2ip	WPM	Liquid	-0.34	-0.59 to -0.09
7	High TDZ	MS	Gellan	-0.16	-0.39 to 0.06
7	High TDZ	MS	Liquid	0.01	-0.21 to 0.24
7	High TDZ	WPM	Gellan	-0.39	-0.62 to -0.15
7	High TDZ	WPM	Liquid	-0.21	-0.45 to 0.02
7	Low TDZ	MS	Gellan	-0.03	-0.26 to 0.19
7	Low TDZ	MS	Liquid	0.14	-0.08 to 0.37
7	Low TDZ	WPM	Gellan	-0.26	-0.50 to -0.02
7	Low TDZ	WPM	Liquid	-0.08	-0.32 to 0.15
7	High Kin + IAA	MS	Gellan	-0.50	-0.75 to -0.25
7	High Kin + IAA	MS	Liquid	-0.32	-0.57 to -0.08
7	High Kin + IAA	WPM	Gellan	-0.73	-0.97 to -0.48

Table A7 continued. Plant Quality responses of two hybrid clones to changes in growth hormone type and concentration, salt type, and media state. [a, b]

Clone [c]	Growth Hormone Type and Concentration [d,e]	Salt Type [f]	Media State [g]	Average Plant Quality [h]	Confidence Interval
7	High Kin + IAA	WPM	Liquid	-0.55	-0.80 to -0.30
7	Low Kin + IAA	MS	Gellan	-0.73	-0.98 to -0.49
7	Low Kin + IAA	MS	Liquid	-0.56	-0.80 to -0.31
7	Low Kin + IAA	WPM	Gellan	-0.96	-1.21 to -0.71
7	Low Kin + IAA	WPM	Liquid	-0.78	-1.03 to -0.54

[a] Cultures were maintained under a 16-hour photoperiod and transferred every 6-8 weeks. Plants were grown at 12 °C in Magenta boxes containing 12 mL of medium at pH 5.7, and 3% sucrose. Plants were randomly assigned to one of ten growth hormone combinations and concentrations, one of two salt types (1M MS or 1M WPM salts), and to gellan (3.5 g/L) or no gellan groups.
[b] ANOVA results show that there is a significant correlation between the model terms and plant quality ($R^2 = 0.09$, $p<0.0001$)
[c] Clone 6, *Helleborus* ×*ballardiae* had significantly higher plant quality than Clone 7, *Helleborus* ×*nigercors* ($p=0.0065$)
[d] High concentrations for all growth hormones (excluding IAA and 2ip) were set to 9 µM, and low concentrations were set to 1 µM.
[e] growth hormone and concentration had a significant effect on plant quality ($p<0.0001$), with plants grown on high and low concentration Kinetin + IAA showing lower plant quality ($p=0.0155$, $p<0.0001$, respectively), and plants grown on low BA showing higher quality ($p=0.002$)
[f] Plants grown on MS media had significantly higher quality than those grown on WPM ($p=0.0004$)
[g] Plants grown on gellan media had significantly higher quality than those grown in liquid ($p=0.0052$)
[h] Average plant quality was determined based on a subjective numerical scale (-2=very poor quality, -1=poor quality, 0=acceptable quality, 1=good quality, 2=very good quality)
[i] *Helleborus* ×*ballardiae* was designated Clone 6, and *Helleborus* ×*nigercors* designated Clone 7
[j] IAA and 2ip were set to 10 µM for all treatments in which they are included

Table A8. Media analysis of spent liquid cultures following Cycle 1 of the media trial shown in Tables A5-A7. Nutrient levels showing low remaining percentages were chosen for further analysis.

Nutrient	Average percent remaining (*H.* ×*nigercors*)	Range (*H.* ×*nigercors)*	Average percent remaining (*H.* ×*ballardiae*)	Range (*H.* ×*ballardiae*)
Nitrate (NO_3^-) [a]	19.8%	3.4 – 35%	31.0%	0.3 - 64%
Ammonium (NH_4^+) [b]	30.3%	6.0 - 43%	54.9%	6.0 - 100%
Phosphate (PO_4^{3-}) [c]	5.80%	4.0 – 8.5%	24.5%	3.7 – 50%
Potassium (K^+)	65.0%	47 – 82 %	70.1%	46 – 95%
Calcium (Ca^{2+})	84.3%	63 – 100%	98.4%	84 – 100%
Magnesium (Mg^{2+})	76.5%	58 – 99%	93.0%	67 – 100%
Zinc (Zn^{2+})	39.5%	32 – 46%	44.3%	18 – 67%
Manganese (Mn^{2+})	83.5%	68 – 100%	90.8%	71.6 -100%
Iron (Fe^{3+}, Fe^{2+})	67.5%	47 – 88%	72.0%	53 – 82%
Sulfate (SO_4^{2-})	62.3%	47 – 100%	73.9%	60 – 100%
Sodium (Na^+) [d]	407%	353 –433%	478%	288 – 637%
Boron (B^{3+})	83.3%	75 – 91%	77.4%	61 – 93%
Chloride (Cl^-)	70.5%	51 – 88%	62.0%	33 – 93 %

[a] Low average percentages of remaining NO_3^- indicated it is a factor of interest for further analysis, with some plants utilizing >99% of NO_3^- contained in the media.

[b] Despite moderate average utilization percentages by plants, NH_4^+ was chosen for further analysis because certain plants utilized >94% of the NH_4^+ contained in the media

[c] Percentages remaining of PO_4^{3-} were consistently low, with some plants utilizing >95% of the total phosphate contained in the media, indicating phosphorus is a factor of interest

[d] Percentages remaining of Na^+ are skewed due to the use of sodium hydroxide (NaOH) as a buffering agent to maintain a pH of 5.7 in the media

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