Endogenous Production of Geosmin in Table Beet

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Additional index words. *Beta vulgaris*, *Streptomyces*, 16S rRNA

Abstract. The earthy flavor of table beet is due to an aromatic terpene derivative called geosmin. It has been hypothesized that geosmin presence in beet is due to geosmin-producing bacteria such as *Streptomyces* spp. that exist in the soil. However, recent findings suggest that beet may produce geosmin endogenously without microbial influence. The purpose of this study was to determine whether such endogenous production of geosmin occurred in beet by making use of an aseptic tissue culture (TC) environment to remove potential microbial influences on geosmin production. Four table beet accessions ('Bull's Blood', 'Touchstone Gold', 'W364B', and 'Pacemaker III') were grown in three separate TC experiments and in the greenhouse and measured for geosmin concentration via gas chromatography–mass spectrometry (GC-MS). Sequencing of 16S ribosomal RNA was used to identify potential microbial contaminants in TC. Operational taxa units (OTUs) classification resulted in RNA sequences with homology to bacterial RNA of either chloroplast (98%) or mitochondria (2%) origin. Other OTUs identified were considered within the range of sequencing error. In 15 of the 16 TC-grown samples used for the 16S rRNA aseptic validation and in all the greenhouse-grown plant samples, geosmin was detected. Geosmin concentrations from bulked beet tissue of each accession were higher in the TC environment than the greenhouse environment. The lack of microbial detection in the TC environment and the subsequent identification of geosmin from beets grown in the aseptic environment is a strong indication that geosmin is produced endogenously by beets. This finding raises several interesting questions about the functional significance of this molecule for *Beta vulgaris*.

The aromatic earthy compound geosmin has a characteristic flavor and scent to which humans are remarkably sensitive. The detection rate for geosmin and another musty-earthy compound, 2-methylisoborneol, is as low as 6–10 ng·L⁻¹ (Rashash et al., 1997). Many microbial species produce geosmin including most of the *Streptomyces*, cyanobacteria, and some fungal species (Jiang et al., 2006). The abundance of *Streptomyces* species in the soil is the primary cause of the quintessential earthy aroma of soil (Jones and Elliot, 2017). Geosmin-producing microbial species, including those in the genus *Streptomyces*, have been found as the source of geosmin contamination in wine, beer, and other foodstuffs, conferring an undesirable musty taste (Paterson et al., 2007). Also drawing consumer complaints are instances of musty- and earthy-tasting water caused by geosmin-producing microbes in water supplies. A large body of literature is devoted to the mitigation of geosmin and geosmin-producing microbes in drinking water supplies

(Jüttner and Watson, 2007). Although geosmin is often a negative quality in food, it can sometimes be a desirable characteristic for consumers seeking earthy flavors. The earthy taste of beet (*B. vulgaris* L.), caused by geosmin, elicits strong favorable and unfavorable reactions from consumers.

The interest in monitoring food and water quality because of volatiles such as geosmin led to the development of analytical protocols using GC-MS (Acree and Lee, 1976; Jiang et al., 2006; Lu et al., 2003b). Further refinements to the GC-MS protocol for analysis of geosmin in beet have used headspace solid-phase micro-extraction (HSPME) (Lu et al., 2003a, 2003b). A HSPME protocol to measure geosmin in table beet was adapted from Lu et al. (2003a, 2003b) by Freidig and Goldman (2014) and Maher and Goldman (2017) to analyze geosmin concentration in various beet cultivars across multiple environments.

To date, the presence of geosmin in root vegetables other than beet has not been recorded. However, geosmin does occur in other subspecies of *B. vulgaris* such as swiss chard and sugar beet (Freidig and Goldman, 2014; Maga, 1987). In addition, dehydrogeosmin, when in a particular configuration, is identical to geosmin and has been recorded as a floral volatile of some species of Cactaceae (Schlumberger et al., 2004). It is interesting to note that these species all belong to the order Caryophyllales.

The biosynthetic pathway for geosmin, first elucidated in *Streptomyces coelicolor*, is

an Mg²⁺ dependent cyclization reaction (Jiang et al., 2006). Geosmin synthase, a unique bifunctional sesquiterpene synthase, first converts farnesyl diphosphate into an 85:15 mix of germacranol and germacrene D via the N terminal unit. The C terminal then converts germacranol into geosmin (Jiang et al., 2007). The purpose of geosmin production in *Streptomyces* spp. is unknown, but many *Streptomyces* species produce geosmin, suggesting that the trait is conserved and has some importance (Seipke et al., 2012). Schöller et al. (2002) screened 26 different *Streptomyces* spp. and characterized 120 different volatile organic compounds (VOCs). They observed that geosmin and sporulation were strongly correlated. VOCs could potentially be used as communication signals between bacteria. A recent study by Jones and Elliot (2017) found that *Streptomyces* exploration, a novel form of growth and development by the bacteria when in a multispecies culture, was stimulated by the VOC trimethylamine.

The functional significance of geosmin in beet is unknown. It was initially hypothesized that the presence of geosmin may be due to the interaction between the beetroot and soil microbes over the course of the growing season (Buttery and Garibaldi, 1976). Geosmin levels are much higher in the beetroot epidermal layer than the inner hypocotyl layers (Lu et al., 2003a). Lu et al. (2003a) tested the possibility of geosmin accumulating in the beet epidermal layer by adding pure geosmin to Murashige and Skoog basal salt (MSBS) medium and testing the differences between beet seedlings grown on nonadjusted MSBS medium for 4–6 months. They found no significant difference in geosmin concentration between beet plants grown with and without exogenously applied geosmin.

An alternative explanation for geosmin production in beet could be the result of endophytic bacteria within the beetroot. Both pathogenic and symbiotic *Streptomyces* spp. are known to invade and colonize plant hosts (Seipke et al., 2012). A plant colonized by endophytic *Streptomyces* spp. may not show disease symptoms that are common with pathogenic *Streptomyces* spp. (Coombs and Franco, 2003). Known plant endophytic *Streptomyces* species can provide growth promotion via auxin production and increased nutrient assimilation. The prolific production of antimicrobial compounds by the endophytic *Streptomyces* species can also provide protection from phytopathogens to the host plant (Seipke et al., 2012). Actinomycete filaments were observed by Jacobs et al. (1985) and Lu et al. (2003a) in the inner cellular region and outer tissue of beets, but it was unclear if those actinomycetes were true endophytes or could produce geosmin.

Last, geosmin in beets could be the result of endogenous production. Recent work by Freidig and Goldman (2014) showed that geosmin concentration in beet is cultivar specific. They grew eight beet cultivars, three cultivars of close table beet relatives

Received for publication 8 Sept. 2017. Accepted for publication 25 Oct. 2017.

We thank the University of Wisconsin Biotechnology Center DNA Sequencing Facility for providing DNA extraction, library construction, and the bioinformatic pipeline services. The technical assistance and advice from Andy Witherell and Amy Charkowski are gratefully acknowledged.

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(mangel, sugar beet, and swiss chard) and one carrot cultivar over 2 years in three environments. The environments consisted of a greenhouse environment using autoclaved soil and non-autoclaved soil and a field environment. No significant differences in cultivar rank for geosmin concentration between years in the field environments and the greenhouse environments were found for beet and related cultivars. Although they identified repeatable geosmin signatures in beet cultivars, they were not able to determine whether this cultivar specificity was due to endogenous production in planta or associations between the host plant genotype and the geosmin-producing soil microbes. Maher and Goldman (2017) recently selected two table beet populations for high and low geosmin concentrations. They found significant responses to selection, suggesting either endogenous production of geosmin or selection for improved microbial associations with this host plant. Theirs was the first study to show that geosmin concentration could be manipulated by selection in beet.

Plant material is often grown in sterile culture to obtain disease-free material, but it is also a method to eliminate microbial influence. For example, Dai et al. (2016) examined the effects of endophytic bacteria *Bacillus* sp. on an invasive plant species *Wedelia trilobata* and its native congener *Wedelia chinensis* by eliminating all other bacterial influencing factors in an aseptic culture system. Through this system, they were able to conclude that the plant growth-promoting effects of the endophytic bacteria enhanced the growth of the invasive species, but not the native species. To address the question of whether geosmin levels in beet are due to microbial associations or endogenous production, Lu et al. (2003a) grew 'Round Red' beets aseptically on MSBS medium and reported geosmin concentration of $14 \mu\text{g}\cdot\text{kg}^{-1}$ of plant tissue at 4 months of growth. Lu et al. (2003a) also reported geosmin concentrations of $135 \pm 30.6 \mu\text{g}\cdot\text{kg}^{-1}$ and $65.2 \pm 25.9 \mu\text{g}\cdot\text{kg}^{-1}$ of plant tissue beet for cultivars Chioggia and Detroit Dark Red, respectively. A limitation of this study was the lack of a microbial assay beyond visual inspection to confirm sterility of the beet cultures. Bacterial populations that associate with plants may be present in the root zone (rhizosphere), the aboveground biomass (phyllosphere), and inside the plant (endophytes). The challenge for studying microbial communities in any of these locations is that as few as 1% of bacteria can be identified using traditional culturing techniques. Advances in sequencing technology allow for a greater understanding of the diversity of microbial communities in marine systems, soil, and agricultural environments (Jackson et al., 2015). Developments in metagenomic sequencing tools such as 16S ribosomal RNA gene (rRNA) sequencing provide a powerful method to characterize these microbial communities. 16S rRNA gene sequencing has been used to characterize the endophytic bacteria of sugar beets at various developmental periods

grown on Tianshan Mountain in China (Shi et al., 2014). The diversity of the bacterial communities varied with developmental period, generating the greatest number of OTUs during rosette and root growth of the beet. Alphaproteobacteria represented the largest taxonomic group identified in that study.

Results from the Maher and Goldman (2017) selection study showed that geosmin concentration is a trait that can be selected in beet, yet the exact targets of selection remain unknown. Further understanding of the nature of geosmin production in beet is essential for future breeding efforts. The objective for this study was to characterize geosmin concentrations in root and shoot tissues from *B. vulgaris* plants grown in sterile tissue culture (TC) and to verify whether these cultures contained geosmin-producing microbes using 16S rRNA sequencing.

Methods

Plant sterilization and propagation. Four beet accessions, 'Pacemaker III' (hybrid; Territorial Seed Company, Cottage Grove, OR), 'Touchstone Gold' (open pollinated; Johnny's Selected Seeds, Winslow, ME), 'Bull's Blood' (open pollinated; Johnny's Selected Seeds, Winslow, ME), and 'W364B' (inbred, University of Wisconsin-Madison, Madison, WI) were propagated in 25-mm glass tubes with 36 tubes per accession. All seeds were multigerm where multiple embryos exist within the corky seed ball because of two to five inflorescences clustered together. The seed sterilization protocol was adapted from Yildiz et al. (2007). The seeds were soaked in 100% concentrated Clorox® bleach (8.5% sodium hypochlorite) for 5 h while shaken at room temperature on a New Brunswick G10 gyrotory shaker® at low speed. The seeds were rinsed three to four times and soaked in Milli-Q water (Millipore, Bedford, MA) for 24 h. Before entry into TC, the seeds were treated with 70% ethanol and rinsed three to four times in Milli-Q water. The seeds were germinated on MSBS medium [medium mixed with vitamins and sucrose from Phytotechnology Laboratories (Lenexa, KS)] prepared as directed by the manufacturer. Beets were grown for 12 weeks (15 June–7 Sept. 2016) in a walk-in controlled culture chamber at 22 °C under cool fluorescent light (average $15.94 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 24 h of light. Vials were visually checked for sterility (cloudy medium and bacterial or fungal growth) and removed if contaminated. Leaf tissue was sampled for DNA extraction and sequencing and the remaining plant material frozen at –20 °C until processed for geosmin analysis. Beets were grown for 1 month in Promix HP potting soil in the greenhouse (Walnut Street Greenhouse, University of Wisconsin-Madison, Madison, WI) at 16-h daylength. Greenhouse-grown beet seedlings were identical in maturity to TC-grown beets when harvested. The level of maturity was the beet growth stage where the beet hypocotyl had elongated but minor swelling of the root had

yet to occur. Beet tissue for sampling included the leaf tissue and hypocotyl. Plant materials were frozen at –20 °C until processed for geosmin concentration analysis. These same beet accessions were also grown in 2014 and 2015 in TC and greenhouse environments and visually checked for contamination. The plant material produced in all 3 years was frozen at –20 °C until analyzed for geosmin concentration. Samples were prepared for bulk analysis as described in the following paragraphs. Sequencing of 16S ribosomal RNA was performed only once with plant materials from the final experiment.

DNA extraction, construction, and sequencing of V3–V4 16s metagenomic libraries. Six to 10 mg of lyophilized beet leaf tissue was used for DNA extraction. The CTAB method, described in Saghai-Maroo et al. (1984) with minimal modification, was used to extract the DNA. DNA was eluted and a final DNA cleaning step was carried out to remove any remaining inhibitory compounds with a 1.5:1 by volume ratio of Axygen Clean-Seq beads (Corning Life Sciences, Corning, NY). Quant-IT PicoGreen fluorescent dye (Thermo Fisher, Waltham, MA) was used for DNA quantification. Purified genomic DNA concentration was verified using the Qubit® dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA) and the samples were prepared by the University of Wisconsin-Madison Biotechnology Center for the 16S Metagenomic Sequencing Library Preparation Protocol, Part # 15044223 Rev. B (Illumina Inc., San Diego, CA). Modifications to the protocol included amplification of the 16S rRNA gene V3/V4 variable region with fusion primers (forward primer 341f: 5'-ACACTCTTTCCCTA-CACG ACGCTCTTCCGATCTCCTACG-GGNGGCWGCAG-3', reverse primer 805r: 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGACTACHVGGGTATCTAATCC-3') and region-specific primers, previously described in Klindworth et al. (2013) were modified by an addition of Illumina adapter overhang nucleotide sequences to gene-specific sequences. A 0.7× volume of AxyPrep Mag PCR clean-up beads (Axygen Biosciences, Union City, CA) was used to clean reactions after first amplification. Illumina dual indexes and sequencing adapters were added by primers (forward primer: 5'-AATGATACGCGACCAACGAGATCTACAC[55555555]ACACTCTTTCCCTA-CACGACGCTCTTCCGATCT-3', reverse primer: 5'-CAAGCAGAAGACGGCATACGAGAT[77777777]GTGACTGGAGTT CAG-ACGT GTGCTCTTCCGATCT-3', where bracketed sequences are equivalent to the Illumina Dual Index adapters D501-D508 and D701-D712) in a later polymerase chain reaction (PCR). Another 0.7× volume of AxyPrep Mag PCR clean-up beads (Axygen Biosciences) was used to clean the reactions after PCR. Finished libraries were assessed for quality with an Agilent DNA 1000 chip and quantity with a Qubit® dsDNA HS Assay Kit. Before sequencing, the libraries were

pooled after standardization to 2 nM. Illumina MiSeq Sequencer and a MiSeq. 500 bp (v2) sequencing cartridge were used for paired end, 250-bp sequencing. Images were analyzed using the standard Illumina Pipeline, version 1.8.2. OTU assignments and diversity plots were created using QIIME analysis pipeline (Caporaso et al., 2010).

Sample preparation for geosmin concentration analysis. Samples were prepared for two different analyses; single-plant geosmin concentration and bulk plant geosmin concentration. All plants were ground in liquid nitrogen. Single plants, TC-grown and greenhouse-grown, were ground and blended with 5 mL Milli-Q water and frozen at -20°C until analyzed. This deviation from a 1:1 ratio was to ensure adequate sample for HSPME protocol. A 1:1 ratio of tissue to water was used for the bulk plant geosmin concentration analysis. Four grams of tissue-cultured plants and greenhouse-grown plants were bulked by accession and environmental treatment and blended with 4 mL Milli-Q water and frozen at -20°C until analyzed. Four grams of beetroot was also ground with liquid nitrogen and blended with 4 mL Milli-Q water. About seven to nine young beets were used for each bulked sample.

Beet homogenate samples were thawed in a water bath and 5 g of homogenate was added to a glass vial with 1 g of NaCl and capped immediately with a screw cap containing polytetrafluoroethylene/silicone septum. Menthone [(1R,4S)-p-menthan-3-one], used as the internal standard, was added at a concentration of $2.82\text{ }\mu\text{g geosmin/kg root tissue}$. For the bulk tissue analysis, geosmin (2 β ,6 α -dimethylbicyclo [4.4.0]decan-1 β -ol) standards of 5, 10, and $21.6\text{ }\mu\text{g geosmin/kg root tissue}$ was added to Milli-Q water blanks. Menthone and geosmin standards were purchased from Sigma-Aldrich (St. Louis, MO). Relative recovery rates, determined by the calculation from Lu et al. (2003a), used beet leaf tissue spiked with 5, 10, 15, and $21.6\text{ }\mu\text{g geosmin/kg root tissue}$.

$$\text{Relative Recovery} = \frac{\mu\text{g geosmin total} - \mu\text{g geosmin originally in beet}}{\mu\text{g geosmin spiked}} \times 100\%$$

HSPME and GC-MS analysis methods were according to Maher and Goldman (2017).

Results

Beet growth in TC and greenhouse environments. Through preliminary studies, a basic, yet intensive bleach wash and treatment with ethanol of the multigerm seed ball was determined to be enough to create sterile beet seeds that preserved the seedcoat and retained a high germination rate. The treatment resulted in few vials with visible contamination. These were confirmed with regular visual checks. In comparison with methods used in other studies (Lu et al.,

2003a; Yildiz et al., 2007), we found that the removal of the corky seed ball exterior to expose the true seed was a difficult process that increased the potential of further ambient contamination and resulted in a greater likelihood of damaged embryos. Most of the germination occurred over an 11-d window beginning 5 d after introduction into culture. Overall, 0.018% of the vials with germinated seed were removed because of contamination using observed visual assessments. Minimal swelling of the hypocotyl into what would be considered a beet was observed over the 12 weeks in culture, but the entire root-hypocotyl axis was evaluated in our assays (Fig. 1). Limited space in the growing environment is a probable cause for this restricted root development in comparison with unconstrained beets grown in normal soil conditions. Greenhouse-grown beets were harvested after 4 weeks of growth. These beets were at similar maturity (no hypocotyl swelling) to the TC-grown beets. We observed fewer leaves on the greenhouse-grown beets, but the leaves

were much larger than their tissue-cultured counterparts.

16S amplicon sequencing analysis. Illumina sequencing of the V3–V4 region of the bacterial 16S rRNA gene for a selected subset of 16 beets grown in a TC environment resulted in a total of 553,372 amplicons across all samples with an average of 34,586 amplicons per sample after quality filtering. The median length of amplicons was 441 bp for each sample. Most of the OTUs were classified as chloroplast, classified by the QIIME pipeline as Stetophyta, ranging between 96.8% and 99.0% over the 16 samples (Table 1). The second largest group were mitochondria ranging between 1.0% and 3.0% over the 16 samples classified as *Rickettsiales*. Total percentage was 98% chloroplast and 2% mitochondria for all 16 beet samples.

After initial sequence processing, reads/OTUs were filtered to remove chloroplast and mitochondria reads. The remaining OTUs resulted in an average of 0.037% of

Fig. 1. Table beet accession ‘Touchstone Gold’ grown in tissue culture (TC) and greenhouse. (A) Example of TC beets in culture. (B) Germination of beet multigerm seed ball in TC. (C) Greenhouse-grown beet tissue rinsed for geosmin concentration analysis.

Table 1. Percentage and classification of operational taxa units (OTU)^a found in aseptically grown beet accessions.

Bacterial classification	Total (%)	Cultivar and replication															
		Bull's Blood				Pacemaker III				Touchstone Gold				W364			
		1	2	3	4	1	2	3	4 ^b	1 ^c	2	3	4	1 ^w	2 ^v	3	4
Cyanobacteria	98.0	96.8	98.7	97.1	97.8	98.6	97.9	98.1	97.2	98.6	98.0	97.3	99.0	97.5	98.7	98.1	98.4
Chloroplast																	
Streptophyta																	
Proteobacteria	2.0	3.1	1.3	2.9	2.2	1.4	2.1	1.9	2.7	1.4	2.0	2.7	1.0	2.4	1.2	1.8	1.6
Rickettsiales																	
Mitochondria																	
Unassigned	0.0	0.1	35 OTU	7 OTU	18 OTU	16 OTU	12 OTU	17 OTU	5 OTU	5 OTU	15 OTU	16 OTU	1 OTU	3 OTU	5 OTU	13 OTU	7 OTU

^aNumber of operational taxa units appear where percentage is 0.0 or 0.1%. These numbers were negligible in the overall percentage and are attributed to sequence error.

^bThe following are additional OTUs identified during QIIME analysis:

^cProteobacteria_Betaproteobacteria_Burkholderiales_Oxalobacteraceae (3 OTU); Proteobacteria_Gammaproteobacteria_Pseudomonadales_Pseudomonas (2 OTU); Proteobacteria_Xanthomonadales_Xanthomonadaceae_Luteimonas (2 OTU);

^wProteobacteria_Betaproteobacteria_Burkholderiales_Oxalobacteraceae (1 OTU);

^vProteobacteria_Alphaproteobacteria_Rhizobiales_Methylobacteriaceae (2 OTU);

^wFirmicutes_Bacilli_Lactobacillales_Streptococcaceae_Streptococcus (2 OTU).

the total OTUs. Subsequently, although these OTUs were classified, they did not contribute to the rounded percentages found in Table 1. The number of OTUs is reported for each of the negligible bacterial classifications in Table 1.

Geosmin concentrations. A total of 16 individual samples were used for the 16S rRNA analysis to verify the sterility of the beets grown in TC. Four individual beet plants from a total of four accessions, open-pollinated 'Bull's Blood' and 'Touchstone Gold', hybrid 'Pacemaker III', and an inbred from the University of Wisconsin-Madison, 'W364', were selected at random. All 16 plant samples returned negligible amounts of classifiable bacteria from the 16S V3–V4 rRNA. The lack of amplification indicated no endophytic bacterial presence. After the verification of sterility, the HSPME protocol outlined in the methods resulted in geosmin detection in 15 of the 16 beet samples. One sample, from the hybrid 'Pacemaker III', did not register geosmin presence (Table 2).

Geosmin concentration levels were compared for each of the accessions that were grown in both the TC and greenhouse environments using bulked beet leaf and hypocotyl tissue at pre-root development stage (Fig. 2). Geosmin levels were measured from tissue ground with liquid nitrogen, the same preparation used for the leaf and hypocotyl tissue. Beetroot tissue was from the low-geosmin population described in Maher and Goldman (2017). This was to insure the method of preparation for beet leaves, altered from Maher and Goldman (2017) which measured geosmin concentration in roots, was similar to the method used for roots. Relative recovery was 28.1%, within the range of previously reported recovery rates taken from beetroot samples (Maher and Goldman, 2017). The geosmin concentrations for the greenhouse-grown beets for each of the four accessions was lower than their TC-grown counterparts (Fig. 2). Interestingly, the 'Bull's Blood' grown in TC had a substantially higher geosmin concentration with a mean of 61.8 $\mu\text{g}\cdot\text{kg}^{-1}$ beet tissue as compared with the greenhouse-grown 'Bull's Blood' which had a mean of 8.35 $\mu\text{g}\cdot\text{kg}^{-1}$ beet tissue. The greenhouse-grown 'Bull's Blood' was also slightly lower than the TC-grown hybrid 'Pacemaker' with a mean of 10.89 $\mu\text{g}\cdot\text{kg}^{-1}$ beet tissue and inbred 'W364B' with a mean of 9.98 $\mu\text{g}\cdot\text{kg}^{-1}$ beet tissue. Overall 'Bull's Blood' grown in TC had a much higher mean than any of the other beets grown both in TC and the greenhouse. Geosmin concentrations from plants grown in 2016 were within the range of geosmin concentrations measured in tissue-cultured and greenhouse-grown plants for these same accessions in 2014 and 2015 (data not shown).

Discussion

The earthy flavor of beet, caused by the aromatic compound geosmin, would be a desirable breeding target because of its defining

flavor in the beet taste profile (Bach et al., 2014). However, the degree to which this trait is under genetic control is presently unknown. Three potential explanations for geosmin production in beet have been proposed. First, beets could produce geosmin endogenously. Support for this explanation may be found in the studies by Lu et al. (2003a), Freidig and Goldman (2014), and Maher and Goldman (2017). Each of these studies pointed to geosmin levels that were either seemingly independent of microbial associations or repeatable with particular cultivars and environments. In addition, Maher and Goldman (2017) demonstrated that geosmin concentration responded to bidirectional selection, strongly suggesting a heritable component for this trait.

The second two explanations for geosmin presence in beet pertain to the interaction between microbes and beets. Geosmin production could be due in part or in whole to an association between soil microbes and beets over the course of a growing season or, alternatively, the result of endophytic geosmin-producing bacteria within the beetroot. Disentangling the potential contribution or association by microbial species requires the verification of the sterility of the beets grown in TC. Lu et al. (2003a) grew 'Round Red', 'Chioggia', and 'Detroit Dark Red' in aseptic conditions and successfully measured geosmin from all cultivars. However, a limitation to their study was an assessment for microbial presence beyond a visual assay.

Our negative results from the 16S rRNA sequencing of the bacterial V3–V4 region and the subsequent geosmin detection in beet tissue present a strong claim for endogenous production of geosmin in beet. Most of the amplicons were classified as chloroplast (grouped in the family *Streptophyta*) or mitochondria (grouped in the family *Rickettsiales*). We attribute the chloroplast and mitochondrial classification to the amplification of beet chloroplast and mitochondrial 16S rRNA. Evolutionarily, chloroplasts are derived from bacteria and thus the homology of bacterial 16S rRNA and plant chloroplast 16S rRNA result in the amplification of the nonbacterial sequences (Hanshew et al., 2013).

After the chloroplast and mitochondria OTUs were filtered from the data set, the remaining OTUs had an average of 0.037% percentage of the total. Sequencing error for Illumina has been reported to be less than 0.1% and this can vary depending on the methods used (Glenn, 2011). Dominant sources of Illumina error come from substitution-like miscalls (Schirmer et al., 2015). In addition, microbial growth in the vials was minute at 0.018% confirmed by visual inspection. The lack of classification for the amplified OTUs beyond chloroplast and mitochondria is an important verification of the aseptic environment in which the beets were grown. An added benefit of sequencing DNA from tissue-cultured beet plants was that should microbial detection have occurred, the species identification would have been

used to implement an appropriate application of antibiotics into the TC medium in an attempt to create an aseptic environment. For this study, it was not necessary as the TCs proved to be a sterile environment.

Other interesting observations from this study include the high level of geosmin in TC-grown beets as compared with greenhouse-grown beets measured at similar developmental growth stages. Secondary metabolites have been shown to be higher in TC environments in both beet (betalain production) and other plants (berberine, nicotine, etc.) than in field environments (Neelwarne, 2013). In commercial settings, increasing secondary metabolite production will sometimes use elicitors that are biological, chemical, or physical applications to the plant growth environment. One such abiotic factor used to increase betalain production in beet was the addition of calcium chloride (Savitha et al., 2006). The type of medium can also affect the growth rate and betalain production in beet (Thimmaraju et al., 2003). Medium, pH, light regiment, and limited space may

have effected geosmin concentration differential found between the bulk greenhouse and TC-grown beets. The limiting space constraints of the TC tube and perhaps some yet unknown other environmental factors caused slower than normal developmental growth in the beet and lack of hypocotyl swelling that would be expected after 3 months of growth. To prepare samples of similar tissue developmental stage for geosmin concentration, beets grown in the greenhouse environment were harvested after 1 month. Geosmin concentrations found in this study were lower than the Lu et al. (2003a) study, but different growth times and cultivars may have contributed to this difference.

In conclusion, verification of the sterility of the TC environment provides an important clue to understanding geosmin production in beet. We showed that beets grown in an aseptic environment produced geosmin and demonstrated a lack of microbial contamination in beets grown in this environment. This result challenges the long-held presupposition that geosmin production in beet is due to microbial interaction between soil microbes and the root vegetable. Our findings point toward the high likelihood that beets produce geosmin endogenously. In addition, the determination that table beet produces geosmin offers a new flavor breeding target in beet. This breeding work has recently begun in the University of Wisconsin-Madison's table beet breeding program. Finally, this study lays the groundwork for future genetic studies of geosmin production in table beet. The lack of geosmin detection in other root vegetables raises the interesting question of the functional significance of geosmin production in beet. One possibility for the presence of this trait could be horizontal gene

transfer from geosmin-producing bacteria. An evaluation of both the family Amaranthaceae and order Caryophyllales, of which the beet belongs, for geosmin production would be an area for further research to determine if and where horizontal gene transfer might have occurred.

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Table 2. Geosmin presence (+) or absence (-) in beet tissue^a grown in tissue culture in single 25-mm test tubes. Four accessions replicated four times (R1-R4).

Cultivar	Sample			
	R1	R2	R3	R4
W364B	+	+	+	+
Pacemaker III	+	+	-	+
Touchstone Gold	+	+	+	+
Bull's Blood	+	+	+	+

^aGeosmin concentration was measured on beet leaf and hypocotyl tissue. (+) or (-) represents the tissue taken from one 25-mm test tube that contained two multigerm beet seeds.

Fig. 2. Geosmin concentration ($\mu\text{g.kg}^{-1}$) of bulked beet leaf tissue ($n=3$) of open pollinated ('Bull's Blood' and 'Touchstone Gold'), hybrid ('Pacemaker'), and inbred ('W364') beets grown in either aseptic tissue culture (TC) or greenhouse conditions (GH). LGC Root is beetroot tissue from a low-geosmin beet population and prepared for analysis under the same conditions as beet accession leaf tissue.

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