American Journal of Plant Sciences, 2015, 6, 2385-2392
Published Online September 2015 in SciRes. http://www.scirp.org/journal/ajps
http://dx.doi.org/10.4236/ajps.2015.614241

Scientific Research Publishing

Clonal Fidelity of Micropropagated *Psidium guajava* L. Plants Using Microsatellite Markers

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Received 22 August 2015; accepted 20 September 2015; published 23 September 2015

Abstract

Micropropagation of *Psidium guajava* L. (guava) is a viable alternative to currently adopted techniques for large-scale plant propagation of commercial cultivars. Assessment of clonal fidelity in micropropagated plants is the first step towards ensuring genetic uniformity in mass production of planting material. In the present study, 31 plants of guava cultivar "Lucknow 49" regenerated by micropropagation were tested for genetic fidelity by comparing them to the mother plant from which explant material was obtained. Efficient rooting of *in vitro* proliferated shoots was obtained by culture on 1/2 strength MS medium supplemented with either 9.8 µM indole butyric acid (IBA) or 11.4 µM indole acetic acid (IAA). Leaf samples of 31 regenerated plants were compared to the mother plant using 17 simple sequence repeat (SSR) markers. While 16 SSRs detected the same allele, locus mPgCIR07 detected slight differences, where six micropropagated plants were 1 bp smaller (152 bp) than the parental genotype (153 bp). Differences in leaf tissues for anthocyanin pigmentation were also noted among micropropagated plants. Results of the study indicated efficient rooting of "Lucknow-49" cultivar for rapid propagation of planting material, and revealed that micropropagated plants were identical for 16 of the 17 loci examined. Although most mutations induced by tissue culture may not have an effect on phenotype, the possibility that novel phenotypes can be generated in a commercial setting exists.

Keywords

Genetic Stability, Guava, Simple Sequence Repeats, Tissue Culture

How to cite this paper: Rawls, B., Harris-Shultz, K., Dhekney, S., Forrester, I. and Sitther, V. (2015) Clonal Fidelity of Micropropagated *Psidium guajava* L. Plants Using Microsatellite Markers. *American Journal of Plant Sciences*, **6**, 2385-2392.
http://dx.doi.org/10.4236/ajps.2015.614241

1. Introduction

Guava (*Psidium guajava* L.), one of the most valued crop plants, is grown in tropical and subtropical regions all over the world, with Mexico, Brazil, India, and Thailand being the largest producers [1]. The fruit is an excellent source of ascorbic acid, vitamin A, dietary fiber, iron, phosphorus, calcium, thiamin, and niacin [2] [3]. An increasing awareness of the health benefits of guava has resulted in an increased demand for the fruit with a subsequent increase in acreage and production. For instance, guava production in Hawaii, the largest producer of guava in the United States, grew from 1.3 million pounds in 2010 to 1.9 million pounds in 2011 [4]. The demand in expanding guava cultivation has increased the need for production of uniform planting material with high yield, good fruit quality, disease resistance, long fruit shelf life, and high nutrient content.

Guava is commercially propagated by seed while vegetative/asexual propagation by cuttings and layering is practiced on a limited scale. Since guava is an open pollinated species, reaching up to 35% out-crossing [5], seeds that are not true-to-type are inevitably produced. Although clonal material can be obtained by vegetative propagation, these methods are not commercially viable due to the cumbersome nature of the processes and the absence of a taproot in vegetatively propagated plants [6]. Micropropagation serves as a viable alternative to seed propagation as it enables rapid propagation of elite stock cultivars in a relatively short period of time [7]. Genetic uniformity of micropropagated plants is a prerequisite for production of quality plant material. However, the use of plant growth regulators such as auxins induces somaclonal variation during the culture process ultimately leading to genetic variation in regenerated plants [8] [9]. Even at optimal levels, frequent transfers of cultures during micropropagation can result in genetic variation, thus questioning the clonal fidelity of regenerated plants. In order to make this technology commercially viable, it is important to verify that plants obtained by micropropagation are true-to-type to the parent plant from which they were derived.

Several molecular markers such as RAPD, ISSR and RFLP-ISSR have been used to detect genetic uniformity and identify any potential somaclonal variation in plants produced through micropropagation [10] [11]. Of these, simple sequence repeats (SSRs) have a distinctive advantage as they are co-dominant markers that produce reproducible results. Microsatellite markers for *P. guajava* ($2n = 2x = 22$) have been developed using genomic libraries of the species for the simple sequence repeats $(GA)_n$ and $(GT)_n$ [12]. These SSRs were very efficient in the determination of genetic diversity among guava cultivars [13] [14].

Among the commercial guava cultivars, “Lucknow-49”, commonly known as “Sardar”, is a high yielding cultivar with fruit that has few seeds and a sweet pleasant flavor. With high vitamin C and total soluble solids of juice at 12.5 percent, it is widely used for commercial cultivation [15]. Additionally, it is resistant to the guava fruit fly, thus making it an ideal selection for growers [16]. So far, *in vitro* rooting of micropropagated shoots and clonal fidelity of micropropagated plants for “Lucknow 49” have not been optimized. The objectives of the present study were to establish efficient rooting of shoot cultures for “Lucknow-49” and test genetic fidelity of regenerated plants using microsatellite markers.

2. Materials and Methods

2.1. Explant Material

Seed material of guava cultivar “Lucknow 49” was obtained from a seven year old tree growing in a greenhouse. Seeds were surface sterilized in 70% ethanol for 1 min, washed in 10% (v/v) commercial bleach solution for 15 min and then rinsed three times, 5 min each, in sterile distilled water. Seeds were then germinated on MS medium [17] supplemented with 3% sucrose, 2.5 $g \cdot L^{-1}$phytagel, 1.0 $ml \cdot L^{-1}$ plant preservation mixture (Plant Cell Technology, Inc., Washington DC) and 0.4 µM 6-benzylaminopurine (BA). Test tubes were incubated in the dark at 25˚C ± 2˚C for three weeks following which the seedlings were maintained under a 16 h photoperiod (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 5 - 6 weeks. Two nodal explants from a single seedling were transferred to MS medium supplemented with 8.8 µM BA and 9.3 µM kinetin (KIN) for shoot proliferation. Cultures were maintained as described previously [18]. After shoot establishment, explants were grown in G7 Magenta boxes (Magenta Corporation, Chicago, IL, USA.) containing 50 ml MS medium for two weeks prior to root induction.

2.2. Rooting of *in Vitro* Shoots

Two cm long shoots were excised and transferred into test tubes containing 10 mL 1/2 strength MS medium supplemented with varying levels of indole acetic acid (IAA), indolebutryic acid (IBA), and naphthaleneacetic

acid (NAA) to induce rooting (**Table 1**). Cultures were incubated in dark for one week and then transferred to light at 25˚C ± 2˚C with a 16 h photoperiod (50 µmol·m^{-2}·s^{-1}). Test tubes were arranged in a completely randomized design with ten replicates per treatment and the experiment was repeated three times. Destructive root count was performed after six weeks and the number of roots produced by each shoot was recorded. Incidence of callus and hyperhydricity occurrence in rooting cultures was also noted. Data were analyzed using a Mixed Model of the statistical analysis software [19]. Means were separated using Tukey's test and differences were considered significant at $p \leq 0.05$.

2.3. Acclimatization and Plant Regeneration

Thirty one *in vitro* generated plants with a well-developed shoot and root system were transferred to 8 cm dia. plastic pots containing PRO-MIX (Premier Horticulture Inc., Quakertown, PA). Plants were acclimatized by covering the pots with transparent plastic bags to maintain high relative humidity. After one week, bags were removed and plants were treated with a water soluble 20-20-20 (N-P-K) fertilizer at biweekly intervals. Survival percentage of plantlets was recorded. After four weeks of acclimation, plants were transferred to a greenhouse.

2.4. DNA Isolation and Quantification

Actively growing shoot apices of the stock plant and 31 plants produced through micropropagation were harvested and immediately frozen at −80˚C. Samples were ground in liquid nitrogen and homogenized in a tissue lyser (Qiagen, Valencia, CA) for 2 min at 30 Hz to disrupt leaf material. Total genomic DNA was extracted from 500 mg of leaf tissue using a DNeasy Plant Maxi Kit (Qiagen, Valencia, CA). DNA quality was checked on a 1% (w/v) agarose gel, and concentration was measured as equivalent of absorbance at 260 nm using a spectrophotometer (Nano Drop, Wilmington, DE). DNA was diluted to10 ng·µL^{-1} concentration using molecular grade water and stored at −80˚C prior to use in marker analyses.

2.5. PCR Amplification, Microsatellite Detection and Analysis

The stock plant and 31 micropropagated plants were tested for clonal fidelity using a set of seventeen microsatellite loci cloned and sequenced by Risterucci *et al*. [12] (**Table 2**). PCR amplifications were performed using WellRED fluorescent dye-labeled primers (Beckman Coulter, Inc., Fullerton, CA). Reactions were carried out in 25 µL volume containing 10 ng genomic DNA, 0.4 µM dNTPs, 0.1 µM fluorescent-labeled forward and reverse primers, 3.0 mM $MgCl_2$, and 0.1 U Taq polymerase mixed in reaction buffer (pH 8.5). After an initial denaturation at 94˚C for 5 min, PCR amplification was performed using a Bio-Rad iCycler ver. 1.259 system (Bio-Rad, Hercules, CA, USA) under the following conditions: 30 cycles consisting of 94˚C for 45 s, 55˚C for 60 s and 72˚C for 60 s with a final extension of 8 min at 72˚C. The amplified loci were separated by capillary electrophoresis and analyzed on a CEQ^{TM} 8000 eight-channel capillary genetic analysis system (Beckman Coulter, Fullerton, CA, USA). Fragment sizes were calculated to two decimal places by the CEQ^{TM} 8000 genetic analysis system, and assessed manually to remove spurious and stutter peaks. Fragment sizes were graphed and alleles were called based on a 1 bp separation. For the UPGMA cluster analysis, marker data was imported into NTSYSpc [20]. Genetic similarity between each pair of micropropagated plant was calculated using the SIMQUAL module using the DICE coefficient of similarity [21]. A dendrogram was generated from the similarity matrix by using the UPGMA procedure in the SAHN module of NTSYSpc. The Free Tree software program [22] was used to perform bootstrapping analysis with a total of 500 repetitions. Bootstrap values greater than 50% are depicted.

3. Results

3.1. Establishment of Explants and Root Induction

Shoot proliferation from nodal explants was observed within 4 weeks of culture initiation with a total of 31 shoots being produced from two nodes (**Figure 1(a)**). Root initiation was observed after three weeks of transfer to rooting media treatments. No root formation was observed in hormone free medium (control). The maximum number of roots (6 - 7 roots per shoot) were observed in shoots grown on half strength MS medium containing either 9.8 µM IBA or 11.4 µM IAA followed by 4.9 µM IBA and 10.6 µM NAA (**Table 1**). However, shoots grown on media treatments with higher auxin concentrations produced stunted roots (data not shown).

Table 1. Effect of plant growth regulators on root production of *in vitro* propagated shoots in "Lucknow 49" guava.

Medium Treatment (µM)	Number of Roots/Explant (Mean ± SE)
Control	0^{e}
1/2 MS + NAA (5.3)	2 ± 1^{d}
1/2 MS + IBA (4.9)	4 ± 1^{b}
1/2 MS + IAA (5.7)	2 ± 1^{cd}
1/2 MS + NAA (10.6)	4 ± 1^{b}
1/2 MS + IBA (9.8)	7 ± 1^{a}
1/2 MS + IAA (11.4)	6 ± 1^{a}
1/2 MS + NAA (21.2)	2 ± 1^{cd}
1/2 MS + IBA (19.6)	3 ± 1^{bcd}
1/2 MS + IAA (22.8)	3 ± 1^{bc}

Means followed by the same letters are not significantly different at $p \leq 0.05$.

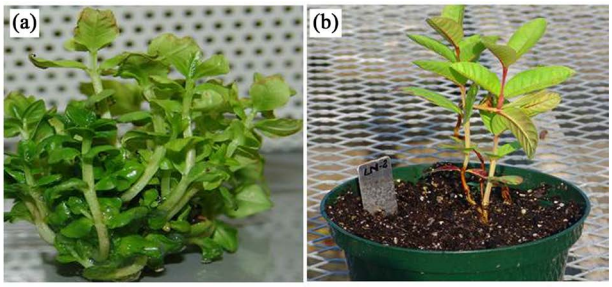

Figure 1. Establishment of micropropagated "Lucknow-49" *Psidium guajava*: (a) *In vitro* shoot proliferation from seedling explant on MS medium; (b) Two-month old rooted plant grown in the greenhouse.

3.2. Plant Regeneration

Production of new leaves was observed in plants produced through micropropagation after 3 weeks of transfer to potting mix and 100% survival rate was obtained (**Figure 1(b)**). Plants were labeled from P2 to P32, of which P27 to P32 exhibited anthocyanin pigmentation.

3.3. Microsatellite Analysis

All SSR primers generated amplicons in the 31 micropropagated plants and mother plant. Allele peaks were observed in the same size range as reported by Risterucci *et al.* [12]. Of the 17 SSRs tested, primers mPgCIR04, mPgCIR09 and mPgCIR16 generated two peaks indicating heterozygosity at these loci (**Table 2**). Of a total of 544 amplification profiles (32 samples × 17 primer pairs) scored in the study, 448 (82.4%) were homozygous while 96 (17.6%) were heterozygous. Comparison of the micropropagated plants to the mother plant showed the presence of the same allele for 16 of the 17 loci tested. However, primer mPgCIR07 generated two alleles among the 31 micropropagated plants. At this locus, six micropropagated plants had an allele that was 1 bp smaller (152 bp) than the parental genotype (153 bp) (**Figure 2**). The UPGMA dendrogram displayed differences between the micropropagated plants and the mother plant (**Figure 3**). Of these six plants with the smaller allele, three (P27, P28, and P30) exhibited mutations that affected the phenotype as manifested by anthocyanin pigmentation of the leaves.

4. Discussion

Guava (*Psidium guajava* L.) is commercially propagated by seed. However, average yield is lower than its potential due to the use of seedlings [23]. In addition, genetic purity is not maintained due to segregation and recombination of traits, which has given rise to several cultivars [13]. Clonal propagation offers a solution to this

Table 2. Characteristics of 17 simple sequence repeat markers, previously developed by Risterucci *et al*. (2005), used in the analysis of clonal fidelity in *Psidium guajava* cultivar Lucknow-49.

Primer	EMBL Accession no.	Repeat motif	Primer Sequences (5' - 3')		
			Forward	Reverse	Clone size (bp)
mPgCIR01	AJ639775	$(GA)_{17}$	TAGTGCTTTGGTTGCTT	GCAGGTGGATATAAGGTC	237
mPgCIR03	AJ639754	(GA)40	TTGTGGCTTGATTTCC	TCGTTTAGAGGACATTTCT	158
mPgCIR04	AJ639755	$(GA)_{25}$	TTCAGGGTCTATGGCTAC	CAACAAGATACAGCGAACT	148
mPgCIR05	AJ639756	$(GA)_{31}$	GCCTTTGAACCACATC	TCAATACGAGAGGCAATA	252
mPgCIR07	AJ639757	$(CA)_{13}AA(GAA)_3$	ATGGAGGTAGGTTGATG	CGTAGTAATCGAAGAAATG	149
mPgCIR08	AJ639758	$(GA)_{12}$	ACTTTCGGTCTCAACAAG	AGGCTTCCTACAAAAGTG	214
mPgCIR09	AJ639759	$(GA)_{19}$	GCGTGTCGTATTGTTTC	ATTTTCTTCTGCCTTGTC	173
mPgCIR10	AJ639760	$(CT)_{12}$	GTTGGCTCTTATTTTGGT	GCCCCATATCTAGGAAG	261
mPgCIR11	AJ639761	$(CT)_{17}$	TGAAAGACAACAAACGAG	TTACACCCACCTAAATAAGA	298
mPgCIR13	AJ639762	$(AC)_{12}(AT)_4G(GA)_2$	CCTTTTTCCCGACCATTACA	TCGCACTGAGATTTTGTGCT	245
mPgCIR15	AJ639764	$(GA)_8GG(GA)_9$	TCTAATCCCCTGAGTTTC	CCGATCATCTCTTTCTTT	147
mPgCIR16	AJ639765	$(TC)_{25}$	AATACCAGCAACACCAA	CATCCGTCTCTAAACCTC	292
mPgCIR17	AJ639766	$(CT)_{23}$	CCTTTCGTCATATTCACTT	CATTGGATGGTTGACAT	231
mPgCIR21	AJ639770	$(AG)_{15}GG(AG)_7$	TGCCCTTCTAAGTATAACAG	AGCTACAAACCTTCCTAAA	154
mPgCIR22	AJ639771	$(GT)_9(GA)_{14}$	CATAAGGACATTTGAGGAA	AATAAGAAAGCGAGCAGA	235
mPgCIR23	AJ639772	$(TA)_4(GT)_7$	GTCTATACCTAATGCTCTGG	CCCAGGAAAATCTATCAC	185
mPgCIR25	AJ639773	$(GA)_{24}$	GACAATCCAATCTCACTTT	TGTGTCAAGCATACCTTC	124

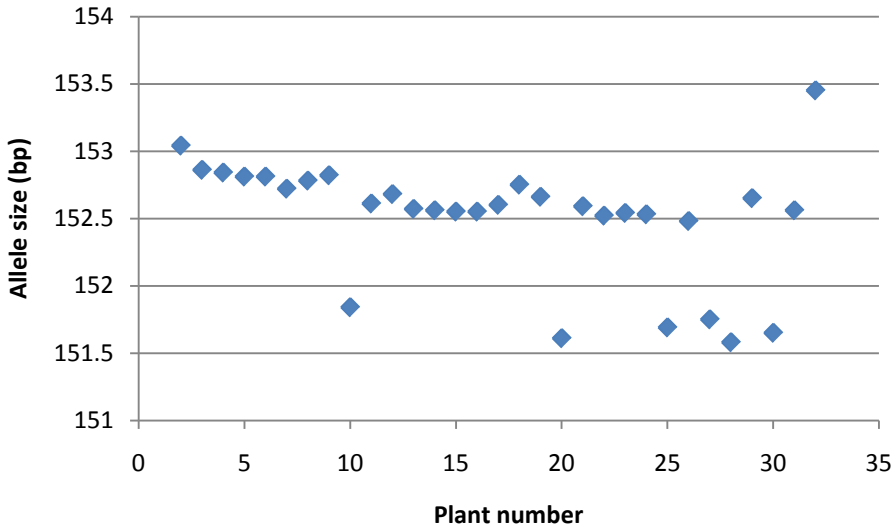

Figure 2. Allele sizes of "Lucknow-49" mother plant and 31 micropropagated *Psidium guajava* with the microsatellite loci mPgCIR07. Six of the 31 plants tested had an allele size of 152 bp.

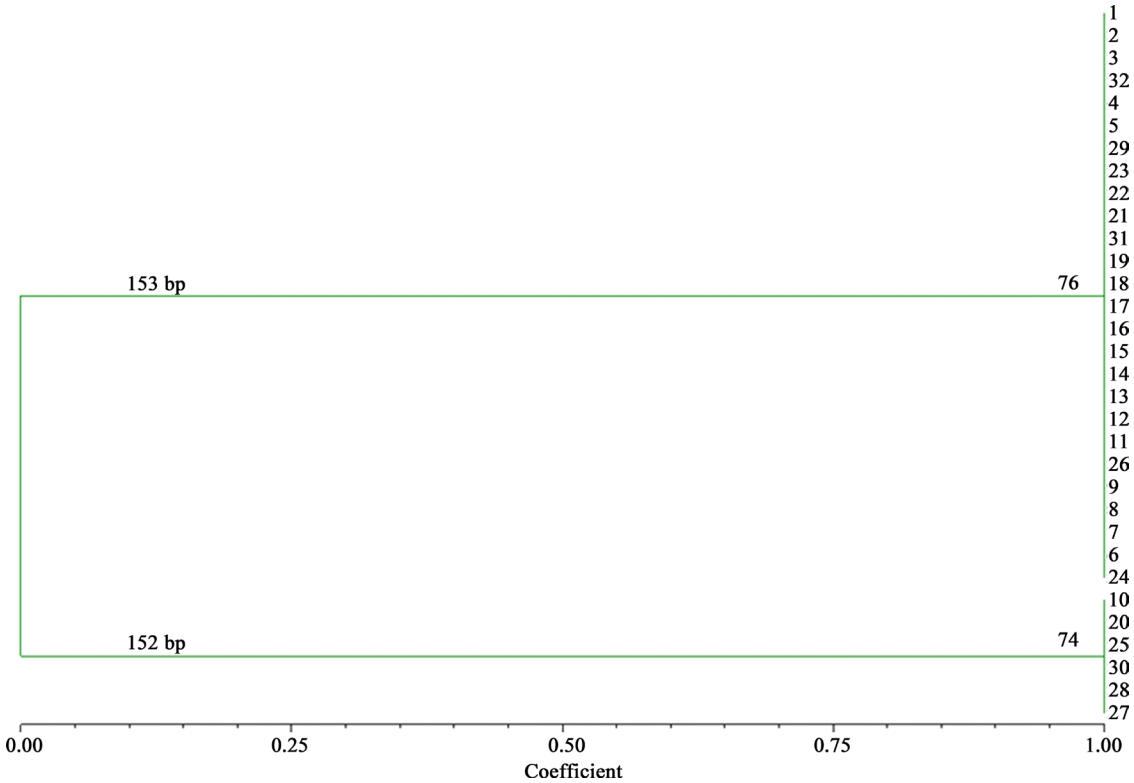

Figure 3. UPGMA dendrogram displaying the genetic relationships of 31 micropropagated *Psidium guajava* plants (#2 - 32) and the mother plant (#1) with the microsatellite loci mPgCIR07.

problem, where outstanding clones could be propagated for commercial plantings allowing uniformity in fruit characteristics, disease resistance, and increased yield within a limited time frame [24]. Successful rooting of *in vitro* produced shoots is an essential prerequisite for rapid plant production using micropropagation [25]. While root initiation has been tested by various researchers, differential response in various guava cultivars has been noted. Guava cultivars exhibit a differential response in their ability to produce roots from shoot cultures with some cultivars exhibiting poor rooting in medium containing auxins [26]. In our study, the highest number of

roots was produced from "Lucknow 49" shoots grown on MS media supplemented with IBA and IAA, which resulted in 100% survival of the plants. Thus, it is paramount to test individual cultivars for their rooting response using various sources and levels of auxins.

Of the 17 loci tested, 16 detected the same allele in plants obtained via micropropagation, while loci mPgCIR07 produced two alleles (**Figure 2**). Six micropropagated plants had an allele that was 1 bp smaller (152 bp) than the parental genotype (153 bp) at one locus (5.9%). This is similar to the rate of polymorphism (10%) detected among micropropagated guava plants from an unlisted cultivar using ISSR markers [1]. While 14 SSRs were homozygous (82.4%) for the 31 clones and mother plant, three primers (mPgCIR04, mPgCIR09 and mPgCIR16) were heterozygous (17.6%). In a previous study of genetic diversity assessment of 35 accessions in the US, these loci were shown to be heterozygous for "Lucknow 49" as well [14].

It should be noted that three of the six plants (P27, P28, and P30) that exhibited anthocyanin pigmentation had the smaller allele (152 bp) for loci mPgCIR07. It is possible that a mutation in the anthocyanin pathway could have resulted in enhanced anthocyanin pigmentation, which is a common phenomenon in plants derived from cell culture involving somatic cells. Somaclonal variation can create novel variants with desirable traits, however, may produce plants with diverse phenotypes. The cause of somaclonal variation has not been fully elucidated although plant growth regulators, number of transfers during the culture process and their duration, and stress during tissue culture may enhance the rate of somaclonal variation [27]. Phenotypic variation is genetic or epigenetic in origin [28] and could include differences in chromosome number and sequence changes such as translocations, deletions, insertions, inversions, duplications, and base pair changes [29]. Epigenetic modifications such as differences in DNA methylation, histone modification, and small RNA levels have been reported previously in plant cell cultures [28].

5. Conclusion

In this study, a protocol for efficient rooting of "Lucknow 49" shoots and plant regeneration was established, and SSR markers were used to assess the genetic fidelity of micropropagated plants. Information presented in the study indicated that although most micropropagated plants were identical to the mother plant (94.1%), low variation (5.9%) was detected. While most mutations have no effect on phenotype, those that do may be problematic if phenotypic uniformity of guava is required.

Acknowledgements

Facilities provided at Morgan State University and Fort Valley State University are greatly appreciated. The authors thank Ms. Donna Harris for technical help. S.A. Dhekney holds the E.A. Whitney Endowed Professorship in the Department of Plant Sciences.

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