Plant PCR Inhibitor Release as a Function of Sample Dissociation Method in Different Tissue Types of *Ocimum basilicum*.

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Introduction

The polymerase chain reaction (PCR) is an invaluable technique used in a wide range of molecular biology applications across multiple disciplines ranging from medical diagnosis [1,2] and forensic analysis [3,4] to botanical research [5,6]. Since PCR is an enzymatic reaction, it is sensitive to inhibitors that originate from the sample or through the extraction/purification process. PCR inhibitors may interfere with multiple steps in the PCR process leading to reduced sensitivity or even false negatives through multiple modes of inhibition including:

- Enzyme binding – competitive binding of inhibitor to template preventing the enzymatic reaction.
- Nucleic acid cross-linking/interaction – changing the chemical properties thus hampering nucleic acid isolation.
- Inhibition of DNA polymerase or reverse transcriptase activity – magnesium cofactor depletion leading to decreased enzyme activity.

For plant studies, PCR inhibition has been demonstrated to occur from contamination by:

- Polysaccharides including cellulose, starch, dextran sulfate and pectin.
- Polyphenols including flavanol, gallic acid, resveratrol, and secoisolariciresinol.

While PCR inhibition has been demonstrated for multiple plant-based compounds and procedural approaches have been recommended for removal of these inhibitors, to our knowledge, there has not been a study to compare the release of these compounds and the subsequent inhibitory effect based on different methods of sample dissociation.

Objectives

- Extract DNA from Ocimum basilicum (sweet) basil tissues using three dissociation techniques - Bead milling, Cryo-grinding and Rotor-stator
- Quantify polysaccharide, phenol, and nucleic acid content
- Determine PCR inhibition as a function of inhibitor concentration
- Evaluate PCR inhibitor removal approaches

Methods

Sample Dissociation

Briefly, 50 mg samples (leaf, stalk, roots and seeds) were homogenized by different methods as described in Table 1 in 1 mL H_2O to be used for polysaccharide quantification, 1 mL of 95% MeOH to be used for phenol quantification, or 0.5 mL CTAB buffer from Plant DNA extraction kit.

Table 1: Sample Dissociation Methods

Sample Description	Homogenization	Parameters
Basil homogenate with CTAB and chloroform/isoamyl alcohol as negative control	Cryo-grinding in mortar & pestle with liquid nitrogen	Grind to a fine powder
Basil homogenate without chloroform/isoamyl alcohol separation	Cryo-grinding in mortar & pestle with liquid nitrogen	Grind to a fine powder
Basil homogenate without chloroform/isoamyl alcohol separation	Rotor stator homogenizer with 7 mm Omni Tip plastic generator probe	20,000 rpm for 15 seconds
Basil homogenate without chloroform/isoamyl alcohol separation	Bead mill homogenizer with 2 mL tubes pre-filled with 2.8 mm ceramic beads	5 m/s for 30 seconds *Seeds took two cycles*

Polysaccharide Quantification

Total polysaccharide content was quantified using a similar protocol from Mohammed et al [7] with minor modifications. 100 µL of each extract was diluted in 400 µL of H_2O. 500 µL of Benedicts solution was added to each sample and boiled for 5 minutes. Absorbance was measured at 490 nm on the Biotek ELX808IU. Concentrations were determined by comparing absorbance values to those obtained from a serial dilution of glucose as shown in figure 1.

Phenol Quantification

Total phenol content was quantified as described in Ainsworth and Gillespie [8]. 100 µL of each extract was mixed with 200 µL 10% (vol/vol) F-C reagent and 800 µL 700mM Na_2CO_3. Samples were incubated for 2 hours at room temperature. Absorbance readings were taken at 765 nm on the NanoDrop 2000 (Thermo Scientific). Concentrations were determined by comparing absorbance values to those obtained from a serial dilution of gallic acid as shown in figure 2.

DNA Purification

DNA was purified using the E.Z.N.A HP Plant DNA Mini Kit (Omega-Biotek, Cat# D2485-01) following the manufacturer's protocol henceforth with a few exceptions. The E.Z.N.A purification method includes a high salt CTAB extraction buffer coupled with a chloroform/isoamyl alcohol phase separation step that removes polysaccharides and polyphenols. A negative control was created by performing an extraction per the manufacturers protocol. All other samples neglected the chloroform/isoamyl alcohol phase separation step. DNA was eluted in 100 µL of EB buffer and concentration was determined on the NanoDrop 2000 (Thermo Scientific) as shown in table 2.

Table 2: Average DNA Concentrations

Sample	Concentration	A_{260}/A_{280}	A_{260}/A_{230}
Leaf Negative Control (Neg cont)	19.8 ng/µL	1.96	1.88
Leaf M&P liquid nitrogen (LN2)	5.6 ng/µL	2.52	1.61
Leaf Rotor stator homogenizer (RS)	22.7 ng/µL	1.93	2.24
Leaf Bead mill homogenizer (BR)	20.5 ng/µL	1.94	2.34
Stalk Negative Control (Neg cont)	8.1ng/µL	1.93	1.60
Stalk M&P liquid nitrogen (LN2)	3.9 ng/µL	2.03	3.27
Stalk Rotor Stator homogenizer (RS)	4.9 ng/µL	2.22	1.43
Stalk Bead Mill homogenizer (BR)	6.5 ng/µL	1.98	2.27

Primer Design and qPCR

Primers were designed for the amplification of [GenBank: Z37424] Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Rubisco) and Universal 18S rRNA gene as shown in Table 4. qPCR was performed by creating 20 µL reaction mixtures containing 25 pg of DNA along with 1X iTaq Universal SYBR Green Supermix (Bio-Rad), 0.5 µM of each primer, and water. Amplification was carried out using the CFX Connect Real-Time PCR Detection System (Bio-Rad) as per the settings in Table 3. For spiked inhibitor studies the negative control DNA was spiked with increasing concentrations of gallic acid and glucose. Average Cq values were determined as shown in figures 3-7.

Table 3: PCR Primers and qPCR settings

	Rubisco gene Primers Forward: 5′ TGT CGA TAA TTC GCG CAG GT 3′ Reverse: 5′ CCA GTG CTG CTG GGG ATA AT 3′		18S gene Primers Forward: 5′ CAG CAG CCG CGG TAA TTC C 3′ Reverse: 5′ CCC GTG TTG AGT CAA ATT AAG C 3′	
	Temperature	Time	Temperature	Time
Hot Start/Denaturation	95°C	2 min	95°C	3 min
35 cycles	95°C	20 sec	95°C	15 sec
	55°C	20 sec	51°C	15 sec
	68°C	50 sec	72°C	30 sec
Final Extension	68°C	5 min	72°C	5 min

Results – Polysaccharide and polyphenol concentrations from basil tissues

Basil samples were dissociated using three methods. Mortar and pestle cryo-grinding with liquid nitrogen, rotor-stator homogenization and bead mill homogenization with the exception of seeds because rotor stator was not able to dissociate them. DNA was purified without the removal of polysaccharides or polyphenols. Polysaccharide and polyphenol content was quantified as shown in Figures 1-2. It was observed that as mechanical dissociation forces were increased, disruption efficiency was also increased. This increased lysis resulted in an increase in polyphenol and polysaccharide content. Mechanical sample dissociation methods were shown to release 30% and 57% more polysaccharides and 116% and 96% more phenols respectively than traditional cryo-grinding in a mortar and pestle. DNA yields also increased proportionally as shown in table 2. Total phenolic content was performed on eluted DNA samples and results showed nearly zero absorption indicating that phenols were successfully removed through all samples (not shown).

Figure 1: Polysaccharide Sample Concentration before DNA Purification

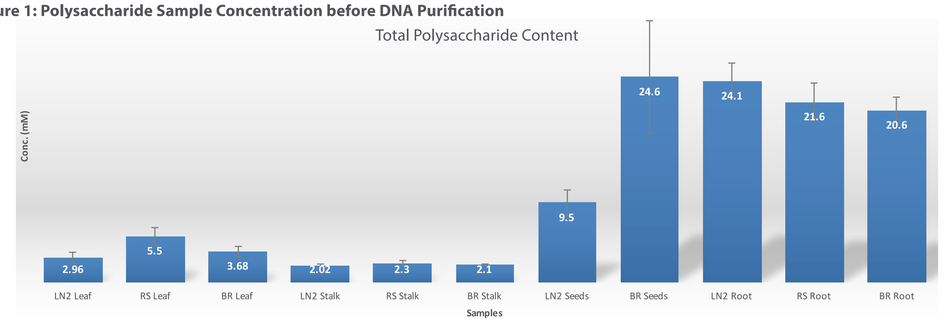
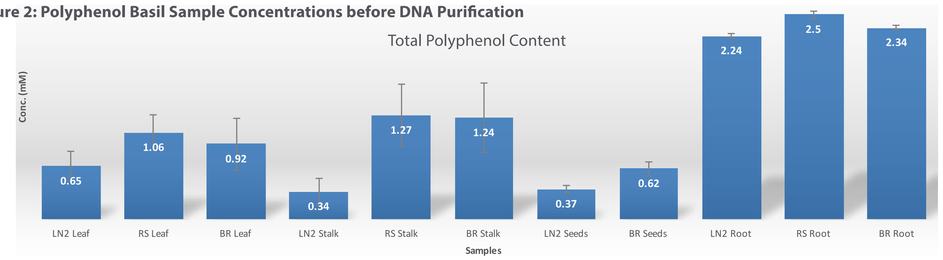


Figure 2: Polyphenol Basil Sample Concentrations before DNA Purification



Results – Inhibitor concentrations and PCR inhibition

DNA purified from the negative control was spiked with increasing quantities of dextran sufate and gallic acid prior to qPCR of the Rubisco and 18S gene. Dextran sulfate was chosen for qPCR standard curve polysaccharide inhibition because dextran sulfate has shown to inhibit PCR already [9]. Gallic acid was chosen for qPCR standard curve phenol inhibition as it is a naturally occurring phenol in plants and animals. It was found that over 27.5 pM of dextran sulfate and 2.75 µM gallic acid starts to cause inhibition of amplification of Rubisco as seen in Figures 3 - 6.

Figure 3: Average Cq Values from Dextran Sulfate Inhibition of Rubisco gene

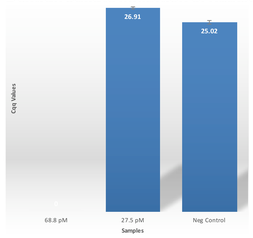


Figure 4: Average Cq Values of Gallic Acid Inhibition of Rubisco gene

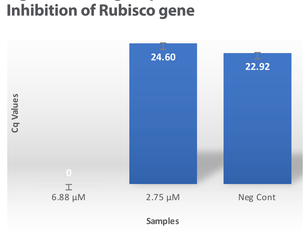


Figure 7: Average Cq Values of Dextran Inhibition of 18S gene

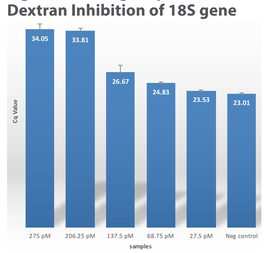


Figure 5: Amplification profiles of Rubisco gene resulting from qPCR of negative control DNA spiked with increasing concentrations of dextran sulfate.

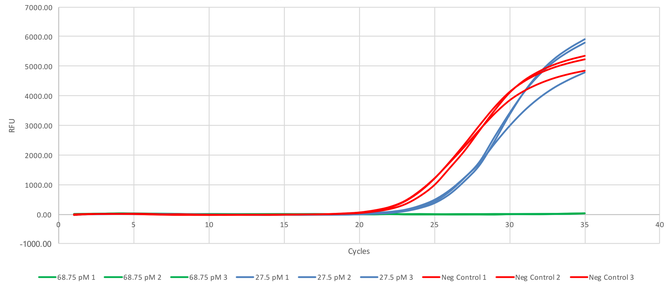


Figure 6: Amplification profiles of Rubisco gene resulting from qPCR of negative control DNA spiked with increasing concentrations of gallic acid.

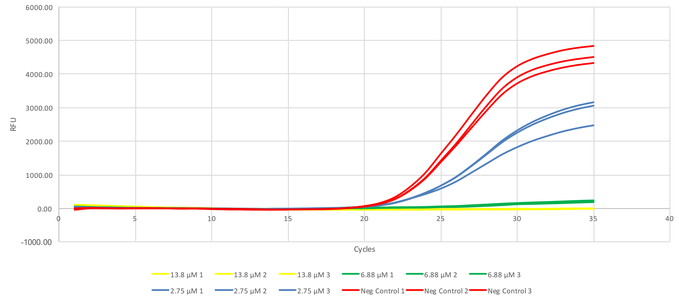
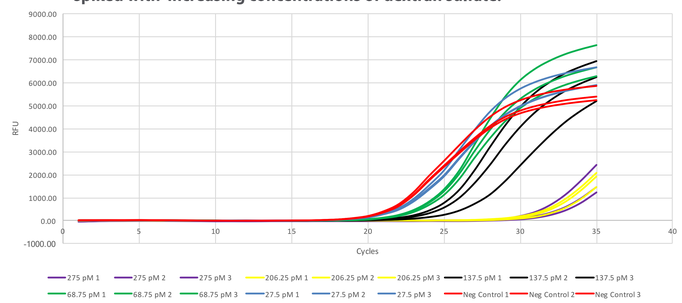


Figure 8: Amplification profiles of 18S gene resulting from qPCR of negative control DNA spiked with increasing concentrations of dextran sulfate.



DNA was extracted from most basil samples dissociated using three homogenization methods. Purification was performed in which the chloroform/isoamyl polysaccharide and polyphenol removal step was performed. However, attempts to extract DNA from basil seeds and roots failed due to spin column being clogged. This indicated that polysaccharides and/or polyphenols were not removed sufficiently since they were most likely binding to the column. As indicated in total polysaccharide and total polyphenol content, those samples had high levels compared to sample types where DNA was extracted successfully.

Conclusions and Next Steps

- The more aggressive the homogenization method, the more polysaccharides and phenols are released in basil tissues.
- PCR amplification of Rubisco is more sensitive to dextran sulfate presence than phenol presence as it takes 100-fold less polysaccharides to cause PCR inhibition than gallic acid.
- DNA extraction kit was effective at removing phenols and polysaccharides from only leaf and stalk tissues even though the chloroform/isoamyl step was removed from the protocol.

References

1.Templeton, K. E., et al. "Rapid and Sensitive Method Using Multiplex Real-Time PCR for Diagnosis of Infections by Influenza A and Influenza B Viruses, Respiratory Syncytial Virus, and Parainfluenza Viruses 1, 2, 3, and 4." *Journal of Clinical Microbiology*, vol. 42, no. 4, Jan. 2004, pp. 1564–1569.
2. Chakravorty, S., et al. "Diagnosis of Extrapulmonary Tuberculosis by Smear, Culture, and PCR Using Universal Sample Processing Technology." *Journal of Clinical Microbiology*, vol. 43, no. 9, Sept. 2005, pp. 4357–4362.
3. Nakanishi, Hiroaki, et al. "A Novel Method for the Identification of Saliva by Detecting Oral Streptococci Using PCR." *Forensic Science International*, vol. 183, no. 1-3, Jan. 2009, pp. 20–23.
4. Alonso, A., et al. "Real-Time PCR Designs to Estimate Nuclear and Mitochondrial DNA Copy Number in Forensic and Ancient DNA Studies." *Forensic Science International*, vol. 139, no. 2-3, Jan. 2004, pp. 141–149.
5. Appleby, Jm, et al. "Polymerase Chain Reaction Amplification of the RuBisCo Small Subunit Genes and Their Novel Application to Plant Tissue Identification." *Heredity*, vol. 79, no. 6, Dec. 1997, pp. 557–563.
6. Narzary, Diganta, et al. "Analysis of Genetic Diversity among Wild Pomegranates in Western Himalayas, Using PCR Methods." *Scientia Horticulturae*, vol. 121, no. 2, June 2009, pp. 237–242.
7. Mohammed, Zainab Yaseen, et al. "Quatitative Analysis of Total Polysaccharides and Total Carotene from Lycium Barbarum Fruit." *International Journal of Modern Biology and Medicine*, vol. 4, no. 3, pp. 204–215.
8. Ainsworth, Elizabeth A, and Kelly M Gillespie. "Estimation of Total Phenolic Content and Other Oxidation Substrates in Plant Tissues Using Folin–Ciocalteu Reagent." *Nature Protocols*, vol. 2, no. 4, 2007, pp. 875–877.
9. Demeke, Tigst, and Robert P. Adams. "The Effects of Plant Polysaccharides and Buffer Additives on PCR." *BioTechniques*, vol. 12, no. 3, 1992, pp. 332–334.