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APPLICATION OF HIGH FIDELITY PCR IN THE PLANT DISEASE DIAGNOSTIC LAB

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ABSTRACT: To compare the sensitivity of High-Fidelity (Hi-Fi) and standard PCR in detecting plant pathogens in symptomatic host plant tissue, four DNA extraction methods were tested in conjunction with a standard and two Hi-Fi PCR protocols. The DNA extraction methods were: 1) Extract-N-Amp Plant Kit (Sigma-Aldrich); 2) DNeasy Plant Mini Kit (Qiagen), 3) CTAB buffer, and 4) lithium chloride Shorty buffer. Symptomatic tissues (i.e. leaf, petiole and root tissue) from selected diagnostic samples were submitted to each extraction method. DNA samples were then used for each PCR protocol applying species-specific primers: 1) Standard PCR; 2) Hi-Fi PCR using LongAmp enzyme; and 3) Hi-Fi PCR Taq+Accuzyme. DNA quantification using spectrophotometry indicated Extract-N-Amp and Shorty methods yielded the highest DNA amounts with lower purity. Both Hi-Fi PCR protocols were more sensitive than standard PCR. The Accuzyme protocol detected targeted plant pathogens in all samples using the DNeasy and Extract-n-Amp methods, whereas the standard protocol detected the pathogen only in leaf samples by using the DNeasy kit. This study demonstrates that Hi-Fi PCR provides a highly sensitive tool for molecular diagnostics *in planta*, and that the DNA extraction method influences PCR sensitivity.

Keywords: molecular diagnostic techniques, PCR, Hi-Fidelity PCR, pathogen detection

INTRODUCTION

The introduction of significant numbers of plant pathogens into Florida and Puerto Rico go unrecognized due to limitations in visual and current molecular protocols to identify regulated species. This year the plant diagnostic clinic at the University of Puerto Rico received on average 100 samples from various crops every month. Only 5% of those samples were sequenced for species identification. Another 10% of the samples were diagnosed as unknown etiology and remain unidentified. In addition, numerous plant diagnostic samples processed by clinics within the Florida Plant Diagnostic Network are diagnosed as "no pathogens found" (Palmateer, personal communication). Some of these samples may in fact be infected by a pathogen, thus emphasizing the need for more sensitive and effective measures for plant diagnostics.

Although a plant may exhibit symptoms that indicate the presence of a bacterial, fungal, or viral pathogen, in many cases no such symptoms exist, even though the pathogen may still be present in a latent state and go undetected by plant inspectors and diagnosticians. Environmental factors such as temperature and moisture are extremely critical in the disease process and more often than not pathogens remain dormant in plant tissue (Swanson et al., 2007). During this phase of dormancy it can be very difficult to isolate the pathogen using conventional diagnostic methods (i.e. tissue plating on artificial media), which may lead to a misidentification due to the presence of secondary fungi and bacteria or false negatives, either of which could be catastrophic in situations involving select agents. In addition, many pathogens are obligate parasites and cannot

be cultured on artificial media, thus requiring molecular diagnostic techniques like the Polymerase Chain Reaction (PCR) for confirmation. However, plant cellular contents (organic and inorganic compounds), including host genomic DNA can interfere with the efficiency of standard PCR (Vickers and Graham 1996; Vincelli and Tisserat 2008; Wilson 1997), making the diagnosis of plant disease directly from plant tissue using the PCR is extremely difficult and one of the most common limiting factors for obtaining accurate results.

The polymerase chain reaction (PCR) has become widely used since the discovery (Chien et al. 1976) and subsequent use of heat stable DNA polymerase for *in vitro* replication of DNA (Saiki et al. 1988). This alleviated the tedious task of adding fresh DNA polymerase at the beginning of each PCR cycle (Mullis & Faloona 1987), which led to the automation of the procedure through the production of programmable thermocyclers. The PCR is now used routinely to amplify DNA for phylogenetic studies (Chaverri et al 2005; Dettman et al. 2006; Stewart et al. 1999; Crous et al. 2001; Palmateer et al. 2003), genomic analysis (Arneson et al. 2008; Nadeau et al. 1992; Lashkari et al. 1997), plant disease diagnosis (Yokomi et al. 2008; Trout et al. 1997), and is used to examine genetic diversity within populations of plant pathogens (Zhang et al. 2005; Urena-Padilla et al. 2002; Winton et al. 2006). However, the Standard PCR is not efficient at producing long sequences, and is generally not able to produce sequences of more than 5 kb (Barnes 1994). The High-fidelity PCR (=Long PCR), which incorporates a second heat-stable DNA polymerase with 3'-exonuclease activity, has been shown to produce longer sequences than standard PCR, with a product size of up to 35-kb (Barnes 1994). The addition of the proofreading enzyme to the reaction containing an n-terminal deletion mutant of *Taq* polymerase was shown by Barnes (1994) to remove mismatched base pairs, allowing strand synthesis to proceed. The use of the proofreading enzyme alone did not amplify the target DNA, which may have occurred because of the degradation of the primers by the 3'-exonuclease activity of the enzyme when used in excessive amounts (Barnes 1994).

In addition to producing longer sequences than the Standard PCR, the High-fidelity PCR has been shown to efficiently amplify target DNA while in the presence of large amounts of genomic DNA, which can be from a host organism or from the target organism. Vickers and Graham (1996) were able to use a High-fidelity PCR protocol utilizing a DNA polymerase mixture containing *Taq* polymerase and a heat stable DNA polymerase with proof reading ability *(Pwo)* to amplify a single copy gene (*Bar*), a marker for the selection of transgenic plants, while in the presence of Barley genomic DNA. The High-fidelity PCR consistently amplified the target gene, while the Standard PCR only occasionally produced results. The High-fidelity PCR has also been shown to detect bacterial infections and microbial associations in numerous arthropod species. Jeyaprakash and Hoy (2000) demonstrated that the High-fidelity PCR was more sensitive than the Standard PCR in detecting *Wolbachia* infections in arthropods. When plasmids containing the *wsp* gene were amplified while in the presence of arthropod DNA, the High-fidelity PCR consistently amplified 1 fg of plasmid DNA containing the *wsp* gene while the Standard PCR could detect 1 ng of plamid.

Hoy et al. (2001) also showed that the High-fidelity PCR was more sensitive than Standard PCR in detecting the citrus greening pathogen '*Candidatus* Liberibacter asiaticus' while in the presence of genomic DNA from citrus psyllids, citrus trees, or citrus psyllid parasitoids. Furthermore, the High-fidelity PCR has been used to detect and characterize a new Microsporidium species from the predatory mite *Metaseiulus occidentalis* (Nesbitt) (Becnel et al. 2002), to identify and distinguish two parasitoids of the brown citrus aphid (Persad et al. 2004), to examine the microbial

diversity of *Metaseiulus occidentalis* and its prey, *Tetranychus urticae* (Hoy and Jeyaprakash 2005), and to amplify 16S ribosomal sequences of endotoxin producing bacteria in varying amounts of dust mite DNA (Valerio et al. 2005).

Plant disease diagnosticians rely on rapid and sensitive pathogen detection methods as a tool in their diagnostic arsenal. Our current research seeks to improve existing *in planta* molecular detection methods by comparing successful detection of pathogen DNA using several commonly used DNA extraction methods coupled with standard or High-Fidelity (Hi-Fi) PCR protocols using species specific primers for select pathogens. Hi-Fi PCR utilizes a proof reading DNA polymerase with *Taq* polymerase to increase product sizes as well as the sensitivity of the PCR reaction. It has been used to successfully detect *Candidatus* Liberibacter asiaticus in the psyllid vector *Diaphorina citri* (Hoy et al. 2001) and several pathogens from orchid tissue (Cating 2010). In the current study we demonstrated that Hi-Fi PCR is more sensitive than standard PCR in detecting *Phytophthora nicotianae* in symptomatic tissue of *Spathiphyllum*

OBJECTIVES

Both the Florida Extension Plant Diagnostic Clinic at the Tropical Research & Education Center and the diagnostic clinic at the University of Puerto Rico, Juana Diaz receive diseased plant samples representing a multitude of pathogens. These clinics serve their function as a valuable resource for the agricultural community by providing pathogen identification and disease management recommendations where appropriate. At the same time they are a viable means for researchers to monitor plant pathogen populations in the surrounding areas. We used this opportunity to investigate the following research objectives and thus the following procedures relied on both clinics for plant samples analyzed.

1) Compare DNA extraction methods including DNeasy Plant Mini Kit (Qiagen), Extract-N-Amp Plant Kit (Sigma-Aldrich), CTAB buffer with chloroform extraction and ethanol precipitation and Shorty buffer with isopropanol precipitation.

2) Determine whether the High-Fidelity PCR is more sensitive than the Standard PCR in detecting fungi, bacteria, and viruses from naturally infected plant species.

MATERIALS AND METHODS

DNA was extracted by using DNA extraction protocol 3 (below) and amplified by using the Standard PCR and High-fidelity PCR protocols (explained below) and the results compared. To quantify the differences between Standard and High-fidelity PCR, serially diluted plasmid DNA (1000 ng to 1 fg) will be spiked with 10 ng of plant genomic DNA. Two negative controls will be used, one containing the plant genomic DNA alone, the other without DNA. All reactions will be replicated three times.

DNA extraction protocols

The following protocols (1 and 2) were used to extract genomic DNA from plant host tissue.

1) Plant DNA: 3.866 g of leaf tissue was frozen in liquid nitrogen and ground in 8 ml of CTAB buffer (2% cetyltrimethylammonium bromide, 100 mMTris pH 7.5, 20 mM EDTA, 1% polyvinyl pyrrolidone, and 1.4 M NaCl) for 10 min and aliquoted into eight tubes. The samples were then incubated at 60ºC for 16 h. After two chloroform extractions, the eight DNA aliquots were combined into four and precipitated in 2-propanol and resuspended in 100 µL sterile water. All samples were pooled to make a 400 µL sample of genomic orchid DNA. Plant DNA was quantified by using an Eppendorf BioPhotometer G131 V1.35 (Eppendorf, Hamburg, Germany).

2) The leaf spot fungus *Pseudocercospora odontoglossii* was isolated from a *Cattleya* hybrid and identified based on morphological characters (Ellis 1976; Crous and Braun 2003) and host. Single spores were grown on V-8 juice agar for two weeks at 25° C under artificial light at 12L:12D photoperiod. A section of mycelium approximately 2 X 2 cm was scraped from the surface of the plate with a sterile wooden applicator stick and placed in a 0.5-mL tube. Then 100 µL of Extraction Solution (Extract-n-Amp, Sigma-Aldrich, St. Louis, MO) was added and the sample ground for 5 min with a sterile plastic pestle and heated to 95° C for 10 min. We added 100 µL of dilution solution (Extract-n-Amp, Sigma) to the sample; then the sample was briefly vortexed. Finally, 30 µL of the extracted DNA was added to 270 µL of sterile water (Harmon et al. 2003).

In order to compare the use of the High-fidelity PCR and the Standard PCR in the diagnosis of plant diseases, several types of commonly cultivated tropical foliage plants were inoculated with bacterial and fungal pathogens by using standard procedures. The DNA from these plants was extracted by using protocol 3:

3) A cork borer (~6 mm in diameter) was used to cut a section of plant material. The plant section will be placed in a 0.5-mL tube and the tissue will be frozen in liquid nitrogen and ground with a sterile plastic pestle for 5 min, after which time 100 µL of extraction solution (Extract-n-Amp, Sigma-Aldrich) will be added and heated to 95° C for 10 min. Then 100µL of dilution solution (Extract-n-amp, Sigma-Aldrich) will be added, and the sample briefly vortexed. Finally, 30µL of the extracted DNA will be added to 270 µL of sterile water (Harmon et al. 2003). After DNA extraction, the Standard and High-fidelity PCR reactions will be performed using the following protocols with the appropriate primers.

Total Plant RNA Extraction

Total plant RNA was extracted using a modified RiboPure Kit protocol.

Homogenization:

1. Homogenize 0.1 g of tissue samples in Liquid nitrogen using sterile pestle and mortar.
2. Re-suspend in 500 µl of TRI Reagent.
3. Incubate the homogenate for 10 min at room temp.
4. Centrifuge at 13,000 rpm for 15 min at 4° C and transfer the supernatant to a new tube.

RNA Extraction:

1. Add 100 µL of BCP to 1 mL of homogenate and mix well.
2. Incubate the homogenate for 10 min at room temperature.
3. Centrifuge at 13,000 rpm for 15 min at 4° C
4. Transfer aqueous phase to a new 1.5-mL micro centrifuge tube.

Final RNA Purification:

1. Add 200 µL of 100% ethanol and mix immediately.
2. Pass the sample through a Filter Cartridge.
3. Wash the filter twice with 500 µL of Wash Solution. Centrifuge one minute at 8,000 rpm.
4. Transfer the filter to a new eppendorf tube.
5. Elute RNA with 50 µL Elution Buffer and freeze until concentration reading.

Standard PCR protocol

Standard PCR will be performed using DNA extracted from inoculated plants and plants with suspected diseases in a 25 µL reaction volume containing 2.5 µL of 10X PCR Buffer +Mg (Boehringer, Mannheim, Germany), 200 µM dATP, dGTP, dCTP, and dTTP, 400 pM of primers ITS4 and ITS5 (White et al. 1990) for fungi, and 16S primers (Weisburg et al. 1991) for bacteria and .2 units of *Taq* DNA polymerase (Bioline, Taunton, MA). Samples will be covered with 50µL of sterile mineral oil and amplified using the following temperature profile: (i) 94°C for 5 min; (ii) 35 cycles consisting of denaturing at 94° C for 30 s, annealing at 53° C for 30 s, and extension at 72° C for 1 min. (the described annealing temperature is for the fungal primers).

High-fidelity PCR protocol

High-fidelity PCR will be performed by using DNA extracted from inoculated plants and plants with suspected diseases in a 50 µL reaction volume containing 50 mM TRIS, pH 9.2, 16 mM ammonium sulfate, 1.75 mM$MgCl_2$, 350 µL dATP, dGTP, dCTP, and dTTP, 800 pmol of primers ITS4 and ITS5 (white et al. 1990) for fungi and 16S primers (Weisburg et al. 1991) for bacteria, 1 unit of Accuzume (Roche Molecular Biochemicals) and 5 units *Taq* DNA polymerase (Bioline) (Barnes, 1994). Samples will be covered with 100 µL of sterile mineral oil and amplified using three linked temperature profiles (i) 94° C for 2 min; (ii) 10 cycles consisting of denaturing at 94° C for 10 s, annealing at 53°C for 30 s, and extension at 68°C for 1 min; (iii) 25 cycles consisting of 94° C for 10 s, annealing at 53° C for 30 s, and extension at 68° C for 1 min plus an additional 20 s during each consecutive cycle (Hoy et al. 2001; Jeyaprakash and Hoy 2000) (the described annealing temperature is for the fungal primers).

Molecular cloning for DNA sequencing

In order to confirm the identity of the obtained PCR product, the target DNA sequences will be cloned using the TOPO T/A cloning kit (invitrogen, Carlsbad, CA) and sent for sequencing to the ICBR at the University of Florida, Gainesville, Florida. Before ligation into the cloning vector, PCR products will be cleaned using the QIAquick PCR purification kit (Qiagen, Valencia, CA) following the manufacturer's recommendations and eluted in 50 µL of sterile glass-distilled, glass-collected water. A 3' A-overhang will be added to the PCR product after purification to facilitate ligation into the cloning vector by mixing the 50 µL DNA sample with 5.75 µL 10X High-fidelity

buffer (50 mM TRIS, pH 9.2, 16 mM ammonium sulfate, 1.75 mM $MgCl_2$), 100 mMdATP, and 1 unit *Taq* polymerase (Bioline). The reaction will be placed in a thermocycler at 72˚C for 45min. The product will be immediately cloned into the TOPO T/A cloning vector following the manufacturer's recommendations (Invitrogen Corporation, Carlsbad, CA), and *Escherichia coli* cells will be transformed with the recombinant plasmid. *E. coli* colonies will be selected from plates containing X-GAL, IPTG, and ampicillin and grown overnight in LB broth containing ampicillin at 37˚C. Plasmids will be extracted using the Qiagen Plasmid Mini Prep Kit (Qiagen, Valencia, CA) and digested with *Eco*RI restriction enzyme followed by gel electrophoresis on a 2% agarose TAE gel stained with ethidium bromide to confirm the correct size of the insert.

RESULTS AND DISCUSSION

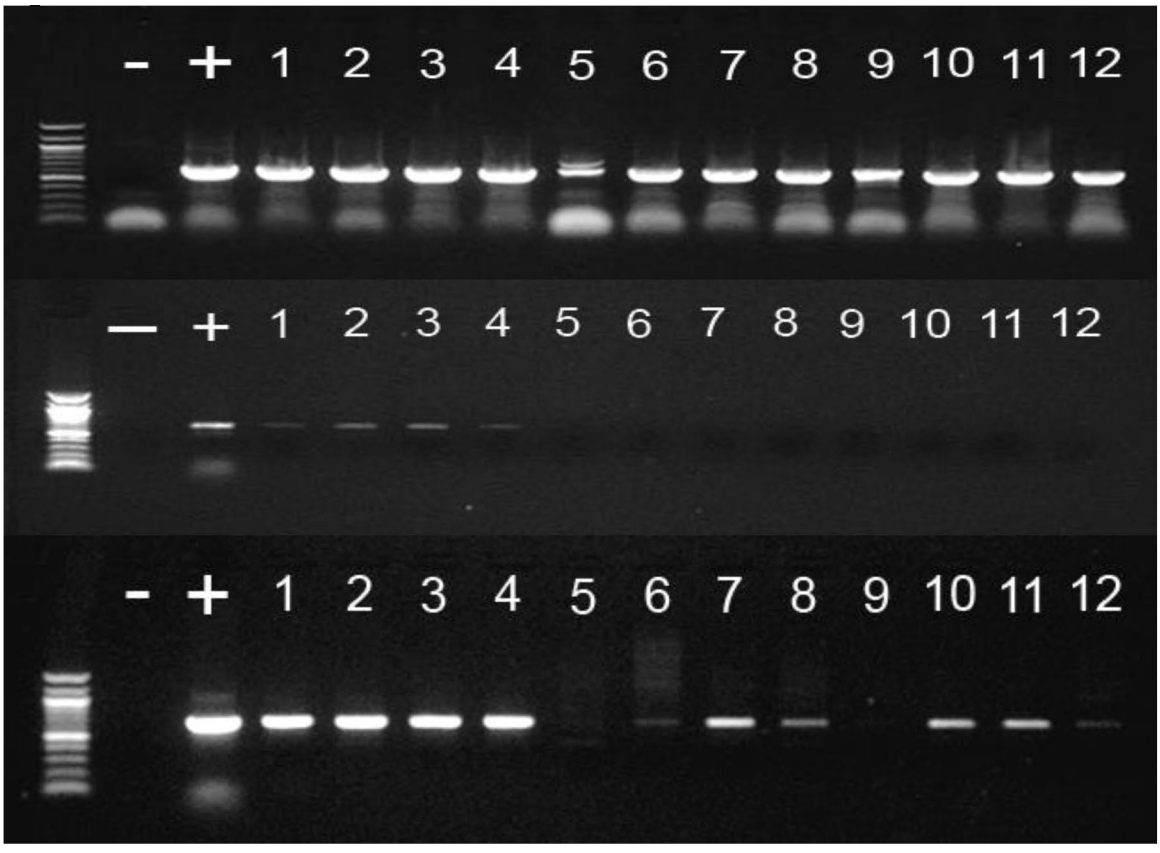
With the Extract-n-Amp and CTAB extractions only Hi-Fi PCR using Accuzyme coupled with DNAeasy and Extract-N-Amp extraction positively detected *P. nicotianae* DNA in all samples of all tissues (Figure 2). Accuzyme Hi-Fi PCR also produced positives for the majority of samples using CTAB extraction. LongAmp Hi-Fi PCR detected the pathogen for the majority of samples for all DNA extraction methods except the Shorty protocol (Figure 2). Standard PCR only detected the pathogen for leaf samples using Dneasy extraction. Extraction with Shorty buffer did not produce any positive detection of the pathogen from any sample save a single leaf sample using Accuzyme Hi-Fi PCR.

These results indicate that Hi-Fi PCR is a more sensitive in detecting pathogen DNA from symptomatic host tissue samples. Our study also indicates that a quick DNA extraction method like the Extract-N-Amp kit produces similar results as a longer method like the DNeasy Kit, even though DNA purity may be lower. The Extract-N-Amp protocol also requires minimal tissue processing.

The advantage of Hi-Fi PCR is that it is similarly easy to run as Standard PCR and does not require special equipment, making it less costly than other molecular detection methods that confer increased detection sensitivity, like real-time PCR. When combined with species-specific primers, this detection method enables diagnosis of specific pathogens directly from plant tissue in a matter of hours, which is especially useful for time sensitive disease problems.

In the future we plan to implement the Hi-Fi detection method for other important pathogens in south Florida, including citrus greening, lethal yellowing of palms, and laurel wilt of avocado.

Figure 1.

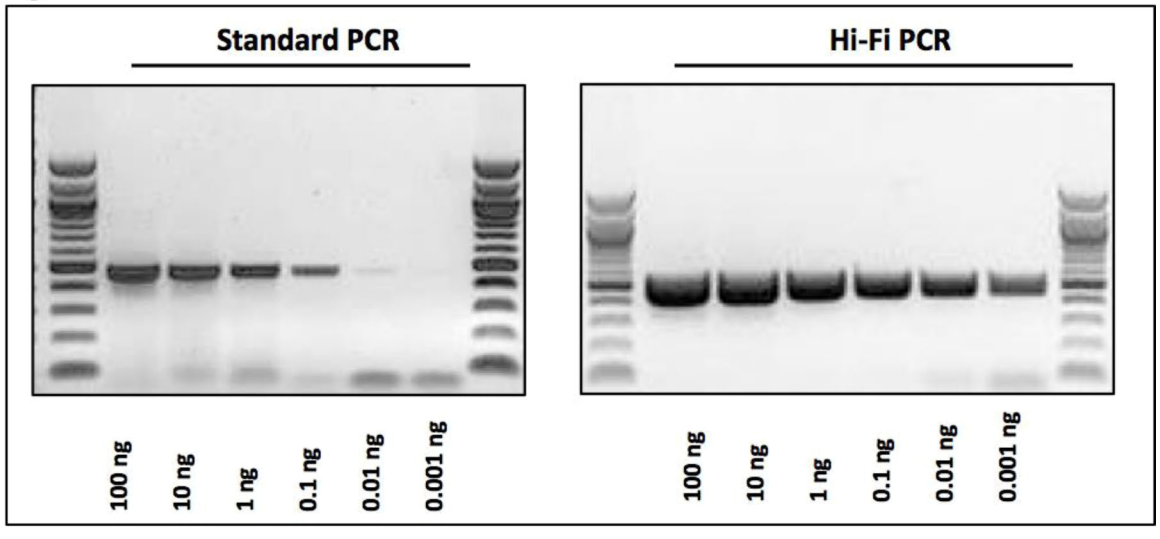


Only Hi-Fi PCR using Accuzyme coupled with DNAeasy and Extract-N-Amp extraction positively detected *P. nicotianae* DNA in all samples of all tissues. Accuzyme Hi-Fi PCR also produced positives for the majority of samples using CTAB extraction. LongAmp Hi-Fi PCR detected the pathogen for the majority of samples for all DNA extraction methods except the Shorty protocol. Standard PCR detected only the pathogen for leaf samples using Dneasy extraction. Extraction with Shorty buffer did not produce any positive detection of the pathogen from any sample save a single leaf sample, using Accuzyme Hi-Fi PCR.

Table 1. Average DNA quantity and purity ratio for four different DNA extraction methods on symptomatic leaves, petioles and roots of *Spathiphyllum* sp. inoculated with *P. nicotianae*

	Leaves		**Roots**		**Petioles**	
	DNA quantity (ug/ml)	Purity ratio	DNA quantity (ug/ml)	Purity ratio	DNA quantity (ug/ml)	Purity ratio
Qiagen	1.74	1.70	1.08	1.64	0.81	1.82
Extract-N-Amp	13.05	0.80	22.02	0.89	16.62	0.86
CTAB	16.71	1.86	8.26	1.72	2.05	1.87
Shorty buffer	7.66	0.99	4.29	1.22	1.56	1.25

Figure 2



ORSV Primers: (expected band of 474 bp) with Tm 48ºC.
CP-Forward 1: 5' ATGTCTTACACTATTACAGACCCG'3
CP-Reverse 1: 5' GGAAGAGGTCCAAGTAAGTCC '3

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