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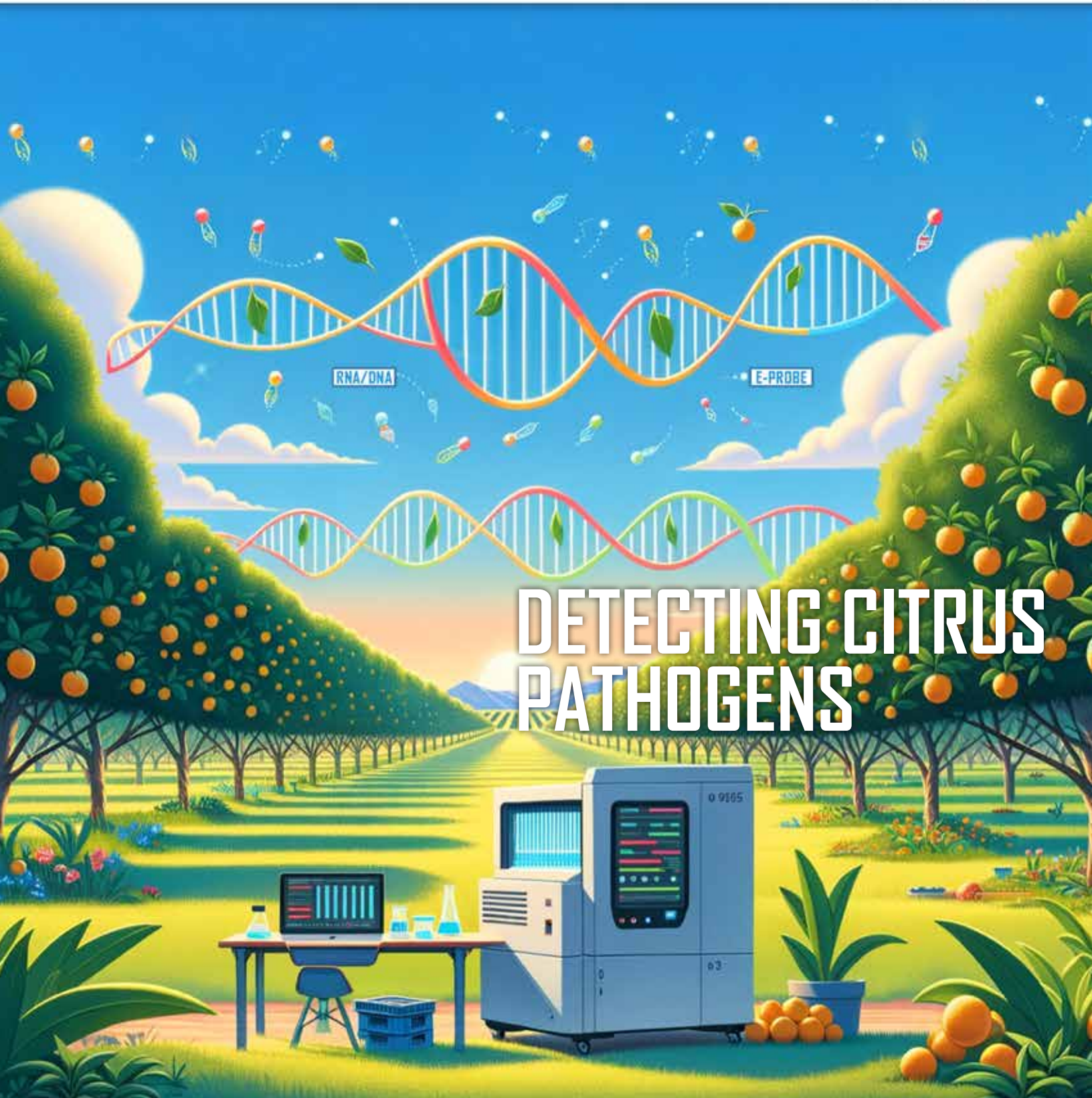

DETECTING CITRUS PATHOGENS

E-PROBES TARGETING CITRUS PATHOGENS AS A NEW DIAGNOSTIC STANDARD

Conceptual illustration showcasing the application of advanced molecular biology and computer programming to detect citrus pathogens.

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Project Summary

At the conclusion of the project, we demonstrated the use of high-throughput sequencing (HTS), a groundbreaking technology that merges molecular biology and computer science for citrus pathogen detection and identification. Our primary objective was to simplify HTS data analysis, making it accessible to individuals without a bioinformatics background. The Electronic (E)-probe Diagnostic Nucleic Acid Analysis (EDNA) technology, developed by the Oklahoma State University (OSU) Institute of Biosecurity and Microbial Forensics, proves to be a promising tool for achieving this objective. In the project's final stages, we conducted a comprehensive assessment of EDNA analysis alongside existing diagnostics within the Citrus Clonal Protection Program (CCPP) variety index (VI) process.

Our achievements include the successful calculation of the statistical limits of detection for E-probes targeting citrus pathogens, the design of E-probes for internal controls and the organization of workshops tailored for regulators and future EDNA users. Additionally, we published EDNA protocols and validation data, expected to serve as a foundational basis for regulatory acceptance of the technology. EDNA analysis has the potential to simplify the diagnostic tests required for the release of citrus varieties from quarantine. Furthermore, the user-friendly HTS/EDNA technology online platform holds considerable potential. This project marks a significant step toward the widespread adoption of EDNA technology in citrus pathogen detection.

Background

The California citrus industry, valued at approximately $3.6 billion with an economic impact of $7.6 billion in the state (Babcock 2022), faces threats from various pathogens spread through tree grafting with infected materials. In its ongoing efforts to protect California's citrus, the CCPP-associated scientists are working toward incorporating advanced pathogen detection methods like HTS/EDNA in the citrus variety introduction process. HTS, integrating molecular biology and computer science, allows simultaneous detection of multiple pathogens in plant samples. Due to significant reductions in HTS costs and the increased affordability and well-documented nature of EDNA (Dang et al. 2022a and b), these technologies are expected to become valuable for citrus germplasm trade partners worldwide, bolstering biosecurity measures for the citrus industry.

In the case of the CCPP, the variety introduction process includes diagnostics based on biological and laboratory assays complimenting each other for the detection of various pathogens and diseases. Biological indexing[1] is based on seven species of citrus indicators while the laboratory assays are based primarily on 30 individual polymerase chain reaction (PCR) tests. Combined, these diagnostic approaches comprise CCPP's variety index (VI), which requires approximately eight to ten months to be completed. New technologies such as HTS/EDNA provide an opportunity to consolidate the molecular tests of the VI process by consolidating the 30 PCR tests into a single multiplex assay, thus generating savings in effort, cost and time.

Table 1. High-throughput-Electronic (E)-probe Diagnostic Nucleic Acid Analysis list of targeted citrus pathogens.

A. TARGET PATHOGENS FOR WHICH VALIDATION IS COMPLETE	
1	Citrus tristeza virus
2	'*Candidatus* Liberibacter asiaticus'
3	Citrus exocortis viroid
4	*Spiroplasma citri*
5	Citrus vein enation virus
6	Citrus psorosis virus
7	Citrus leaf blotch virus
8	Citrus tatter leaf virus
9	Citrus variegation virus
10	Citrus concave gum associated virus and Citrus virus A
11	Hop stunt viroid (citrus isolates)
B. TARGET PATHOGENS FOR WHICH *IN PLANTA* VALIDATION IS IN PROGRESS	
12	*Xanthomonas citri* subsp. *citri*
13	*Xylella fastidiosa* subsp. *pauca*
14	'*Candidatus* Phytoplasma aurantifolia'

Ongoing testing of HTS/EDNA compared with currently established standards will continue for several years to ensure the sustained reliability of E-probes over time. Moreover, the cost-effectiveness for detecting multiple citrus pathogens in a single sample is likely to drive regulatory acceptance and laboratory adoption in the near future. In our previous studies, we have demonstrated that HTS/EDNA was sensitive and specific, eliminating the need for personnel with a bioinformatics background or high-cost servers conducting extended analyses to acquire diagnostic results (Dang et al., 2019, 2021, 2022 a, b, 2023). The publications resulting from our studies serve as crucial documentation for the ongoing citrus HTS/EDNA regulatory acceptance proceedings.

Summary of Results

We successfully designed, curated and statistically validated E-probes targeting the citrus pathogens outlined in **Table 1**. We have addressed the challenge of calculating the limit of detection (LOD) for E-probes targeting citrus pathogens by incorporating housekeeping genes from non-citrus woody hosts. This ensures unique reads for statistical analyses and helps prevent false positives (Dang et al. 2023). Our recent E-probe validation has emphasized specificity, computerized analytical sensitivity and diagnostic sensitivity, entailing testing against both confirmed positive and healthy specimens. These E-probes are now available for implementation in the CCPP's VI process (**Table 1, A**).

Since June 2021, the HTS/EDNA protocol has been incorporated successfully into the CCPP VI testing pipeline. HTS/EDNA results were reported to regulatory agencies in parallel with bioindexing and laboratory tests, for seven VIs and 67 citrus varieties released from state and federal quarantine. This reporting process is integral to facilitating the regulatory acceptance of the HTS/EDNA protocol for the introduction and quarantine release of citrus varieties into California by the CCPP. Moreover, an *in silico* validation study was initiated between OSU and CCPP, covering metrics such as robustness, reliability and transferability, with comparisons made across various operators to report to the regulatory agencies in the future. In the past four years, we also conducted five workshops and training sessions for regulators and future EDNA users. These events were essential for evaluating the user-friendliness of the HTS-EDNA online platform and fostering interest in its adoption for citrus diagnostics. These activities, alongside our existing publications, showcase the alignment of HTS/EDNA results with the CCPP's established and regulatorily accepted bioindexing and laboratory protocols and laid the foundation towards regulatory acceptance of the new diagnostic technology.

Final Steps

For the remaining three target pathogens, all E-probe design and validation steps have been completed, except for the *in planta*[2] validation tests (**Table 1, B**). Due to the exotic nature of these pathogens to California, *in planta* validation was delayed by the unavailability of infected samples. To overcome this challenge, we acquired the necessary federal and state permits and initiated collaboration with both domestic and international scientists to obtain infected plant tissue samples. The final *in planta* validation steps for these bacterial pathogens are anticipated to conclude

in the coming months. Based on recent data accumulated from this project, along with upcoming publications, we further anticipate adoption of the HTS/EDNA assays for all 14 targeted citrus pathogens of this project.

Conclusion

As we conclude our data collection and present our findings to regulatory agencies, it is evident that HTS and EDNA are potent diagnostic tools, that can streamline the CCPP's citrus variety introduction and quarantine release processes. The widespread acceptance of HTS within scientific communities, alongside EDNA, leads us to confidently anticipate that this technology will become a mainstream tool for citrus pathogen detection. Moreover, the transferability of HTS/EDNA technology to various diagnostic laboratories, facilitated by the MicrobeFinder (MiFi®) online platform, and ease of use, holds significant promise.

This technology establishes a structured pipeline for the inclusion of potential emerging citrus pathogens into the CCPP's existing diagnostic pathogen detection program. Moreover, this will help reduce the overall duration of the variety indexing pipeline for the CCPP. The CCPP and other citrus germplasm programs can leverage this technology to benefit the citrus industry by providing access to pathogen-tested, certified budwood for citrus grove establishment. These advancements collectively enable a more efficient pathway for the introduction and distribution of new citrus varieties in California.

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Glossary

[1]Biological indexing (or Bioindexing): Plant-based diagnostic technique employed to identify specific plant pathogens causing disease under controlled environmental conditions. In citrus, this involves grafting a blind bud/bark piece from a suspected diseased plant onto a young, greenhouse-grown indicator plant, followed by monitoring for the development of characteristic symptoms. Indicator plants chosen for bioindexing usually are susceptible to a particular pathogen, exhibiting noticeable disease symptoms shortly after being graft-inoculated with the infected plant tissue.

[2]*In planta* (Latin): Processes, experiments or analyses conducted within a living plant or using a sample from a living plant. In the context of this article, the term refers to analyses of high-throughput data generated from citrus samples infected with citrus pathogens.

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