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Utilizing In-Silico Techniques for Synthesizing bDNA for Crop Disease Diagnosis

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Abstract

The different DNA building block topologies include linear, circular, and branched DNA (bDNA). Complex molecular design has led to the production of various functional nanomaterials incorporating the significant properties of bDNA. In our study, we demonstrate two different methods for topology sampling of bDNA in modeling. The first method consists of sampling *Ralstonia solanacearum* cells and culturing them in a nutrient medium. The propagated cells are then stored from which their DNA is collected, and sequenced to form Y and X-shaped bDNA. The second method only took an *in-silico* approach where we formed branches Y and X of DNA in 3D mode using bioinformatics retainer databases. A set of sequences designed by software comprising Maya, bio-blender, and discovery studio (DS) software that can produce 3D models. These models will help us in the laboratory to carry out a precise laboratory simulation to detect bDNA design which can be useful in diagnostics. Although the first trial was unsuccessful, the use of a software simulation in a second trial was the most successful and yielded several 3D models of bDNA, which could be used in the laboratory simulation of molecular diagnostic strains that express genes. These models were used in molecular diagnostic assays which are sensitive, specific, and reliable tools in the detection of virulence genes of some diseases such as fusarium oxysporum, phytphthora fragariae, and botrytis cinerea. This platform not only offers a means for screening the potential activity of molecular diagnostics but also presents opportunities for time and cost savings.

Keywords: BDNA, in-silico, nanotechnology, Y and X-shape, 3D modeling

Introduction

The rapid and accurate detection of plant diseases is essential for sustainable agriculture as it reduces crop losses and improves global food security (Al-Hiary et al,.2011). Recent advancements in biotechnology have led to the development of innovative diagnostic methods that are fast and reliable (Falzone et al., 2021). One such method is bDNA (bDNA) technology, which is highly specific and sensitive in detecting plant pathogens (Tsongalis et al., 2006). The bDNA is a structure that consists of multiple DNA strands emanating from the point of branching, which can be ingeniously designed to possess isotropic or anisotropic properties, as well as symmetric or asymmetric attributes following specific requirements depending on the demand (Guo et al.,2019; Zhao et al.,2020).

The characteristics of bDNA include the following: (I) In a controlled manner, bDNA can act as an initiator core or as a growing building block for the creation of hyperbDNA nanostructures; (ii) the bDNA sequences can be logically constructed to meet desired specifications; and (iii) The bDNA has unique sticky ends that provide numerous modified sites for binding to specific functional units (Hu et al., 2022). These properties make bDNA molecules a versatile and ideal building block that can be used to produce highly complex, multifunctionally integrated materials. The Holliday junction, characterized by its four branched strands engaged in various recombination systems, stands as the most recognized biological illustration of bDNA. Since linear DNA is energetically favored over branches, branched structures are unstable. Due to its homologous sequence symmetry, the holiday compound can spontaneously resolve into linear duplexes through branch migration isomerization reactions. The creation of branched junctions in immobile DNA by Seeman, Kallenbach, and co-workers (Déclais et al.,2008; Dong et al., 2020).

Due to the intrinsic thermal instability of base pairing, bDNA building blocks are unstable at high temperatures. However, recent research has shown that psoralen can combine with the cross-linking to create a thermostable bDNA nanostructure and create

DNA origami structures with enhanced thermal stability (Vogel, H et al., 2006; Hartman et al.,2013; Kane et al., 2016; Li et al.,2021). The bDNA is always linked to nanotechnology, a new technology used in the diagnosis and treatment of diseases, and a lot of applications.

As known, the width of a DNA base pair is approximately 2 nm, and the base-to-base distance along one chain of DNA is 0.34 nm. Due to these dimensions, DNA can form structures ranging from nano to micro to macro. Also, the DNA serves as an excellent template for nanofabrication because it can be precisely controlled in terms of both location and orientation. In 1993, Tsu-Ju Fu and Nadrian Seeman successfully formed bDNA from single-stranded DNA. The amount of constituent single-stranded DNA determines the configuration of the bDNA or the number of branching arms (Li et al., 2004; Dong et al., 2020).

The concept of DNA nanotechnology was first introduced in 1982 by Nadrian Seeman, who developed the idea of constructing a mechanically strong tile structure using four single-stranded DNA strands. This structure, also known as a tile structure, consists of a four-way branched junction. The goal of this construction is to maximize the number of correct base pairs, and it is achieved by arranging complementary segments of the four individual ssDNA in a specific pattern (Wang et al., 2017)

we explore the application of *in-silico* techniques in synthesizing bDNA for diagnosing crop diseases. *In-silico* methods, involving computer-based simulations and analyses, offer a cost-effective and rapid approach to designing and optimizing bDNA structures (Vlachakis et al., 2014). These techniques predict the behavior and efficiency of bDNA in hybridization events, which are crucial for detecting specific nucleic acid sequences associated with pathogens and reinforcing the logic behind the development of innovative, safe drug candidates. By using computational modeling, researchers can simulate different scenarios and conditions to determine the most effective bDNA structures, without the need for extensive wet lab experiments (Pérez et al.,2012).

The software used to make DNA *in-silico* 2D and 3D forms, this software was used in the formation of bDNA as Blender (software), which is a free and open-source 3D computer graphics software toolset used for creating animated films, visual effects, art, and 3D-printed models, it was initially developed as an in-house application by the Dutch animation studio NeoGeo and was officially launched on January 2, 1994 (Doyle et al.,2021). Version 1.00 was released in January 1995 (Khoshnaw et al., 2019), and the bio-blender is part of the blender and is most often used in creating 3D models related to biology, genetics, chemistry, and so on. Maya was originally an animation product based on code from The Advanced Visualizer by Wavefront Technologies, Thomson Digital Image (TDI) Explore, Power Animator by Alias, and Alias Sketch. The IRIX-based projects (Kushwaha et al., 2015) were combined, and animation features were added; the project codename was Maya (Jinno, Y et al., 1990; Sharpe et al.,2008; Houston et al.,2016). We took advantage of these features and harnessed them to create 3D shapes of DNA and proteins. Discovery Studio (Wang et al., 2015; Jejurikar et al., 2021) is a suite of software for simulating small molecule and macromolecule systems (Singh et al., 2022). It is developed and distributed by Dassault Systems BIOVIA (Kemmish et al., 2017; Hameed et al., 2020) (formerly Accelrys). The product suite has a strong academic collaboration program, supports scientific research, and makes use of several software algorithms developed originally in the scientific community.

Herein we set the stage for discussing the principles behind bDNA technology, the role of *in-silico* methods in its development, and the potential these tools hold for revolutionizing plant disease diagnostics. Through a detailed examination of case studies and experimental data, this paper aims to demonstrate how synthetic bDNA, designed using computational tools, can effectively diagnose plant diseases *in silico*, rapidly and accurately. This will enhance the resilience of agricultural systems against pathogenic threats.

Materials and Methods

1. Molecular methods.

1.1. Collection of bacterial samples.

In the first trial, we used *ralstonia solanacearum* as a source of genomic DNA sequences for making bDNA. The isolates of bacteria were isolated from infected potato tubers and then transplanted on Petri dishes containing nutrient agar and Tween B medium as a selective medium triphenyl tetrazolium chloride (TTC) for 500 ml. Included, 500 ml of distilled water; 5 g of peptone; 2.5g of glucose; 0.5 g of casein hydrolysate; 0.025 g of triphenyl tetrazolium chloride, and 8.5 g of agar for *Ralstonia solanacearum* (kelman, A et al., 1954). For 48–72 hours, all infected plates were incubated at 28°C. Then bacteria were activated in liquid media called Luria Bertani broth (LB).

1.2. Genetic analysis of *R. solanacearum* isolates

1.2.1.DNA Extraction.

DNA was isolated by using the CTAB Bacterial DNA isolation protocol; Load a 2-milliliter centrifuge tube with bacterial culture. After centrifuging the material, place the pellets in the bottom. Take out the supernatant. For a 100 ml CTAB buffer, combine 1.5 M NaCl, 25 M EDTA, 100 M Tris HCl, and 2% CTAB. To get the desired final volume, add double-distilled water. Proceed to resuspend the pellets by vortexing them in about 400

µL of TE buffer and 100 µL of 5M NaCl. After thoroughly mixing the sample with 50 µL of CTAB buffer, incubate it for 60 minutes at 70°C in the dry bath. During the incubation period, stir the sample occasionally. After adding 500 µL of chloroform, vortex the mixture and let it sit on ice for half an hour. The sample should be centrifuged for 10 to 15 minutes at 4°C at a moderate speed. Gather the supernatant in an additional sterile tube, then thoroughly mix 500 µL of phenol: chloroform into the mixture. For mixing, use vortexing. After that, quickly centrifuge the sample to extract the top phase. Now use salt and alcohol to precipitate the DNA, then wash it with the alcohol. For a step-by-step tutorial, see the article "Role of alcohol in DNA extraction."

1.2.2. polymerase chain reaction (PCR) protocol

PCR amplification was carried out using a PerkinElmer/Gene Amp® PCR system 9700 (PE Applied Biosystems) for two samples one virulent bacteria and a virulant by using three primers, rated for forty cycles, following a five-minute initial denaturation cycle at 94°C. Each cycle consisted of a 45-second denaturation stage at 94°C, a 50-second annealing step (the annealing temperature varied according to the TM of each primer sequence), and a 1-minute extension step at 72°C. The primer extension step was increased to seven minutes at 72°C in the last cycle. was accomplished by using electrophoresis to separate PCR products on 1.5% agarose gels in 1X TBE buffer at 92 volts along with ethidium bromide (0.5 µg/ml). PCR results were photographed using a gel documentation system (BIO-RAD 2000) and seen under UV light.

Table 1. Primers were designed by using the NCBI database (primer blast/ Primer designing tool (nih.gov)

Primer name	Primer sequences	TM
RS- F	5'- GTCGCCGTCAACTCATTTCC – 3'	55
RS-R	5'- GTCGCCGTCAGCAATGCGGAATCG- 3'	
HrpB- F	5' – AGAGGTCGACGCGATACAGT- 3'	60
HrpB- R	5' – CATGAGCAAGGACGAAGTCA -3	

1.2.3 Cloning and Sequencing

The resulting DNA amplicons were eluted from agarose gel and purified using the QIAquick Gel Extraction Kit. The purified PCR fragments were ligated into pGEMR-T Easy Vector Systems according to its manufacturer. The competent cells of *E. coli* top 10 strain were prepared and transformed as described by (Inoue et al., 1990). From LB/Amp/Xgal plates, white colonies were selected and inoculated on LB/Amp broth media. Then it was incubated overnight at 33 °C with shaking to stabilize the plasmid inside the transformed cells. The alkaline method of Birnboim and Doly (Bimboim and Doly, 1979) was used to isolate the plasmid. To confirm the recombinant plasmids the purified plasmids were examined by electrophoresis on 1.5% agarose gel. Macrogen Company (South Korea) sequenced the obtained recombinant plasmids. The obtained sequence for Hrp and RS virulence and avirulence genes were examined for vector contamination using the VecScreen tool (http:// www.ncbi.nlm.nih.gov/tools/vecscreen). The obtained sequences were deposited at the NCBI database (http://www.ncbi.nlm.nih.gov) under accession numbers PP195597 and PP195598 virulence and avirulence Hrp, and PP214957 and PP214958 virulence and avirulence RS genes respectively.

1.2.4. multi-sequence alignment (MAS) and phylogenetic analysis

To identify sequence similarities with homologous DNA and proteins from other organismis, DELTA-BLAST tools were performed to Hrp and RS of *R. solanacearum.* Clustal omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo) were used to generate multiple sequence alignment for obtaind DNA sequencs as well as transcripts, RExML (https://github.com/ruby/rexml) and iTOL (https://itol.embl.de/) were used to form phylogentic tree.

1.3. The use of sequencing results to form bDNA.

We used PP214957, PP214958, PP195597, and PP195598 sequences of the two obtained isolates to form Y-X-shapes bDNA using *in-silico* software.

2 Genomics and network-based methods *in-silico.*

2.1 Genomics Methods and Databases

First, a search for the desired sequence for bDNA formation was conducted by scanning all the official databases. DNA can be taken from many sources of organisms. There are multiple shapes of bDNA, but we will focus on the Y and X shapes. The gene was chosen from genetic databases such as the National Centre for Biotechnology Information (NCBI), Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway (Kanehisa et al.,2010), European Nucleotide Archive (ENA), etc.

NCBI: Botrytis cinerea has been selected as a source of DNA. By using bioinformatics, we select a sequence (Botrytis cinerea strain Sl3 chromosome 01 (CP080979)) consisting of ≅4 million nucleotides) from botrytis.

Botrytis cinerea is a necrotrophic fungus that affects a wide variety of plant species, referred to as "*botrytis* bunch rot". This disease can cause significant financial losses to crops produced in greenhouses as well as fields. it can infect seedlings, plants that have not yet been harvested, or mature or senescent tissues. *B. cinerea* is a soft rot that causes soft fruit and leaves to seem crumpled and drenched in water. Grey Mold-infected twigs will eventually die back. Fruit drop and damage, such as ridging on growing and mature fruit, are caused by blossoms (SM et al.,2019).

Database Source Selection: The NCBI database was utilized as the primary source for acquiring genetic sequences. Sequences encoding the Y-shaped bDNA structures were extracted from these genes.

Sequence Retrieval and Selection: Sequences chosen for the Y-shaped bDNA structures were selected based on their complementarity in a predetermined manner. Specifically, three sequences, each comprising 38 nucleotides, were identified, and retrieved from the NCBI database.

Sequence Alignment and Verification: The identified Y-shaped bDNA sequences underwent further characterization. Every three thousand bases within the sequences were selected, followed by sequence alignment using Clustal Omega provided by EMBL-EBI. Subsequently, each set of aligned sequences was meticulously examined to ensure complementarity with other sets of aligned sequences, thereby establishing three templates essential for the formation of Y-shaped bDNA structures. X-shaped BDNA Sequence Identification: For the synthesis of X-shaped bDNA, four sequences possessing specific characteristics were required. Palindromic sequences, essential for X-shaped bDNA formation, were identified using the Palindromic Sequences Finder tool available at novoprolabs.com.

2.2. Software solutions for DNA-based modeling:

The first software to design DNA nanostructures was based on established molecular modeling software using standard structural files (e.g. pdb, mol, mae) (Williams S et al., 2008; Kekic et al.,2020). The tools discussed in the previous studies showed various changes in DNA configuration at the atomic level. In general, in molecular modeling software, the granularity of information is particularly sensitive to tasks involving small proteins and molecules. comparison of tools for the modeling of bDNA nanostructures (shown in Table2)

Table 2. Overall comparison of tools for the modeling of bDNA nanostructures. Tools can be accessed on the following websites: Blender (www.blender.org), bio-blender (www.bio-blender.org), Maya group (www.autodesk.com/products/maya/free-trial), biovia discovery studio (www.3ds.com/products/biovia/discovery-studio).

	Software	3D structure	Sequence Editing	Mesh input
1.	Blender & Bio-blender	✓	✓	✓
2.	Maya Group	✓	✓	✓
3.	BIOVIA Discovery studio	✓	✓	-

I. Blender and bio-blender:

Blender is a free and open-source 3D computer graphics software toolset used for creating animated films, visual effects, art, and 3D-printed models, it was initially developed as an in-house application by the Dutch animation studio NeoGeo and was officially launched on January 2, 1994 (Doyle et al.,2021). And bio-blender is used in the formation of structures related to biology, genetics, chemistry, etc

II. Maya group software.

Maya was originally an animation product based on code from The Advanced Visualizer by Wavefront Technologies, Thomson Digital Image (TDI) Explore, Power Animator by Alias, and Alias Sketch! The IRIX-based projects (Kushwaha et al., 2015) were combined, and animation features were added; the project codename was Maya (Jinno, Y et al., 1990; Sharpe et al.,2008; Houston et al.,2016). We took advantage of these features and harnessed them to create 3D shapes of DNA and proteins shown in.

III. Discovery Studio

Discovery Studio (Wang et al., 2015) (Jejurikar et al., 2021) is a suite of software for simulating small molecule and macromolecule systems (Singh et al., 2022). The product suite has a strong academic collaboration program, supporting scientific research, and makes use of several software algorithms developed originally in the scientific community, we used it to form bDNA.

3. Diagnosis.

3.1. Selection of crop.

We select strawberry crop due to its nutritional properties and suggested health advantages Strawberries are a highly sought-after fruit whose production has expanded over the past several decades. But because of its high perishability, erratic behavior in seasonal markets, and stringent quality requirements, it can produce large surpluses that are essentially highly polluting waste and negatively affect the social and economic conditions in production areas as well as the environment. As of right now, surplus strawberries are puréed and used as food additives and vitamins. Its chemical composition, however, makes strawberry purée (SP) ideal for a range of biotransformations that might

produce new products with greater added values and advantageous properties. (García -García et al., 2020)

3.2. common disease.

There are many common diseases, but we focused on these three diseases. These diseases are wilt diseases *(fusarium oxysporum*), In the 1960s, fusarium wilt in strawberries was first documented in Australia and Japan. Since then, instances of the pathogen*, Fusarium oxysporum* f. sp. fragariae (Fof), have been reported worldwide, with the majority of cases happening in the 1990s and 2000s. Numerous of these recent findings are connected to adjustments made to pre-plant soil fumigation protocolsThe disease has significant economic ramifications since infected plants may collapse and die. In the field, *Fusarium wilt* can be difficult to diagnose since its symptoms are similar to those of other soilborne infections.(Koike et al.,2015); grey mold (*botrytis cinerea*); red stele or red core (*phytphthora fragariae*), The oomycete *Phytophthora fragariae* is a highly destructive pathogen of cultivated strawberry (*Fragaria* × *ananassa*), causing the root rotting disease, "red core". The pathogen responsible for red core or red stele root rot in farmed strawberries (Fragaria × ananassa) is *Phytophthora fragariae*, which can cause complete plant collapse. According to Robinson Boyer et al. (2016), most commercial strawberries cultivated in the UK are grown in glasshouses, under polytunnels, or on tabletops using soilless substrate. Because the motile zoospores of Phytophthora spp. are so easy to spread via the irrigation system, they pose a special risk in these systems. Except for China and the Southern Mediterranean areas of Europe, this disease has spread to most strawberry-growing regions since it was first reported in Scotland in 1920 (van de Weg, 1997b; Efsa Panel on Plant Health [PLH], 2014). The European and Mediterranean Plant Protection Organization (EPPO) currently lists it as an A2 pest and treats it as a quarantine pest (van de Weg, 1997b; EPPO, 2018).

From each disease we select virulence gene, SIX8 (HQ260604.1) from fusarium oxysporum, BchX (XM024694372.1) from botrytis cinerea, and RxLR (MK530529.1) from phytphthora fragaria. (Home - Gene - NCBI (nih.gov)).

3.3. primer design.

Primers (forward and reverse) have been designed by using NCBI database (primer blast/ Primer designing tool (nih.gov)

Table 3. primers of the diseases.

Gene name	Primer sequences	
	Forward	Reveres
SIX8	5'-TATTTCGTCGTTCCCGCACA-3'	5'-CAATAATGGCGGCTTGGTCG-3'
BchX	5'-ATCACTCCCTTCGTGGAGGT-3'	5'-TACGGATAACCTGGCAGGGA-3'
RxLR	5'-CAACAAGTTCAAGGCGTGGG-3'	5'-AACTTGAGCTTGGGGTCGAG-3'

Table 4. Single-stranded overhang sequences for ligation (append to 5' end of core sequences)

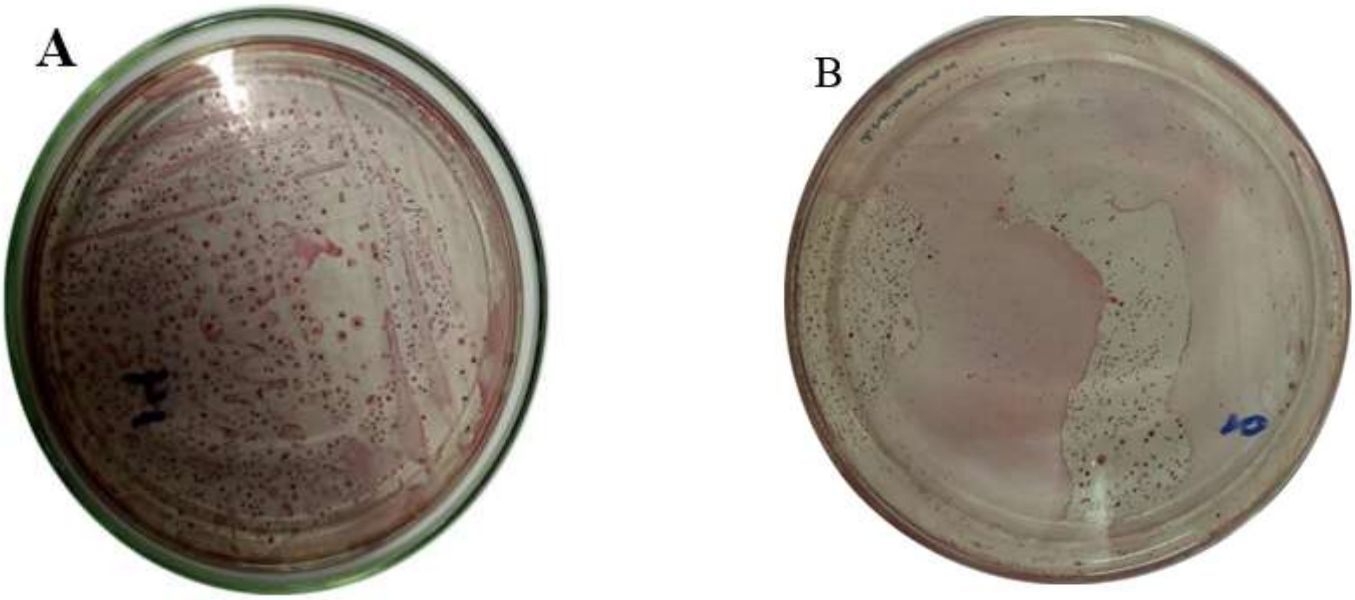
ssOverhang1F	**/5Phos/GCAA**
ssOverhang1R	/5Phos/TTCC
ssOverhang2F	/5Phos/GGAA
ssOverhang2R	/5Phos/GGAT
ssOverhang3F	/5Phos/ATCC
SsOverhang3R	/5Phos/TTGC

Before using primers in introduction, we checked them by using *in-silico* PCR (In silico PCR amplification (ehu.es)), or by using UCSC *in-silico* PCR (UCSC In-Silico PCR)

Results and Discussion

1. Collection of bacterial samples.

We isolated bacteria from infected potato tubers and then motivated them on Petri dishes containing nutrient agar and Tween B medium as a selective medium triphenyl tetrazolium chloride TTC. This medium was developed to differentiate between the two colony types: virulent colonies appear white with pink centers and non-virulent colonies appear dark red.



Virulent bacteria Avirulent bacteria

Figure 1. Solid specific medium (TTC) colonies of R. solanacearum usually are visible after 48-72 hours and incubated at 28ºC. This medium was developed to differentiate between the two colony types: Fig (1A) virulent colonies and Fig (1B) non-virulent colonies**.**

2. Molecular analysis of *R. solanacearum* isolates

2.1. Polymerase chain reaction (PCR) product.

PCR amplification was carried out using the designed primers from NCBI primer/blast. By using Invitrogen™ 50 bp DNA Ladder from Thermo Fisher Scientific. The results of PCR product of two isolates of R. solanacearum using two primers RS, and Hrp. RS in virulence and avirulance are 651 bp in length and in Hrp is 412 bp.

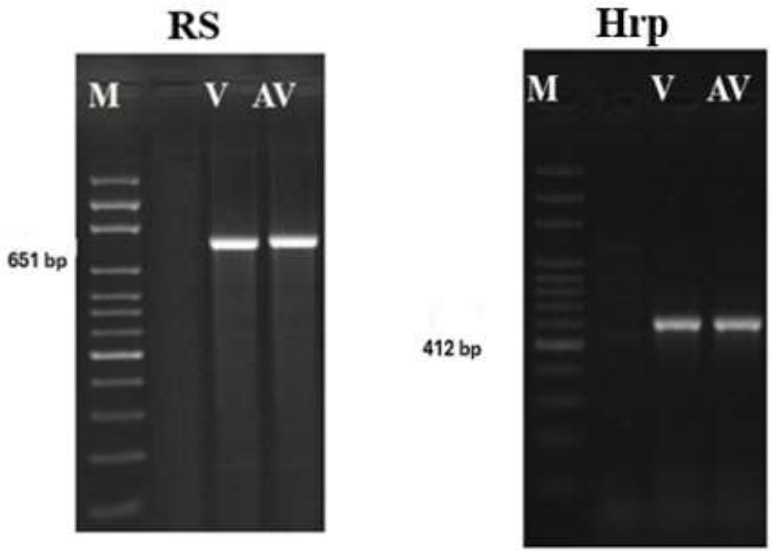


Figure 2. The PCR product of two isolates of R. solanacearum using two primers RS, and Hrp. M: 50 bp DNA ladder. RS is 651 bp in length and Hrp is 412 bp.

3.2.2. multi-sequence alignment (MAS) and phylogenetic analysis

To identify sequence similarities with homologous DNA and proteins from other organismis, DELTA-BLAST tools were performed to Hrp and RS of *R. solanacearum.* Clustal omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo) were used to generate multiple sequence alignment for obtaind DNA sequencs as well as transcripts, RExML (https://github.com/ruby/rexml) and iTOL (https://itol.embl.de/) were used to form phylogentic tree.

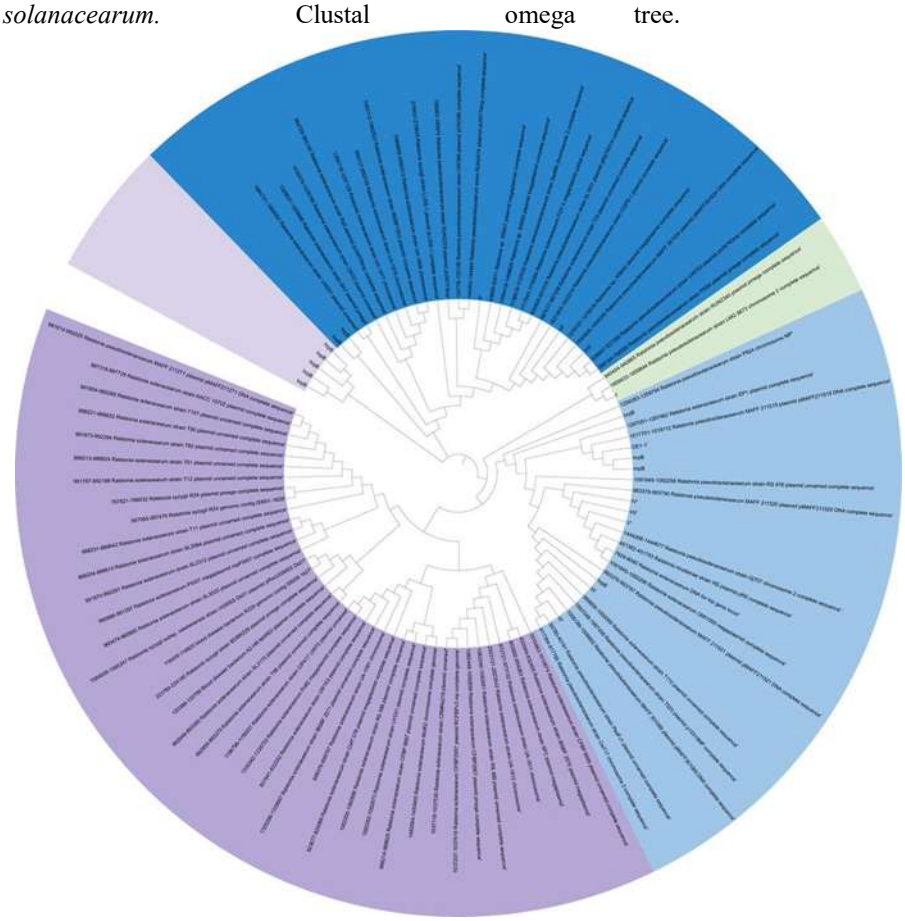


Figure 3. phylogentic tree showing the relation between the Hrp and RS with the all species of *Ralstonia*.

2.3. Using *R. solanacearum* sequencing results to form bDNA.

The used programs for generating bDNA from R. solanacearum obtained no results where the sequences did not contain the desired bases series, which the criteria needed to design bDNA Y-X-shapes which are 1) Y-shape consists of three arms and these arms consist of three sequences are complementary to each other, 2) X-shape consists of four arms from four palindromic sequences form also the palindromic sequences, then sequences were too small to create Y-bDNA, and they contain a large number of bases (GC), Guanin- Cytosine, only, which were not suitable for designing bDNA X-shapes four palindromic sequences (Hartman et al., 2013)

To differentiate strains within *R. solanacearum*, a race/biovar (biotype) system of classification based on host range and specific biochemical properties was used (Paudel et al., 2020) This system was informal and did not adhere to the Code of Nomenclature because no particular characteristics were found that allowed for a formal classification system. Despite its value, the race and biovar system was unable to provide light on *R. solanacearum's* origins and evolutionary history. Notable variety was also present within strains isolated from the same host, even if certain strains were restricted to affecting the host cultivated in a particular geographical location. This made it difficult to classify the strains according to their original host (Paudel et al., 2020). Because it is a bioype we have not succeeded in manufacturing bDNA.

So, that's why we shifted to *in-silico* methods (bioinformatics informatics and 3D modeling) to avoid repeating the same mistake and to save time, money, and effort. We used *in-silico* methods to ensure the validity of this experiment before conducting it in the laboratory.

3. *In-silico* method.

3.1. Genomics and Network-based methods *in-silico*.

As a result of searching for the required sequence in the botrytis cinerea genome registered on different databases, we reached the sequence strain Sl3 chromosome 01 (CP080979) sequence which consists of ≅ 4 million nucleotides. The NCBI database was utilized as the primary source for acquiring genetic sequences. Sequences encoding the Y-shaped bDNA structures were extracted from these genes. Sequences chosen for the Y-shaped bDNA structures were selected based on their complementarity in a predetermined manner. Specifically, three sequences, each comprising 38 nucleotides, were identified, and retrieved from the NCBI database. For the synthesis of X-shaped bDNA, four sequences possessing specific characteristics were required. Palindromic sequences, essential for X-shaped bDNA formation, were identified using the Palindromic Sequences Finder tool available at novoprolabs.com.

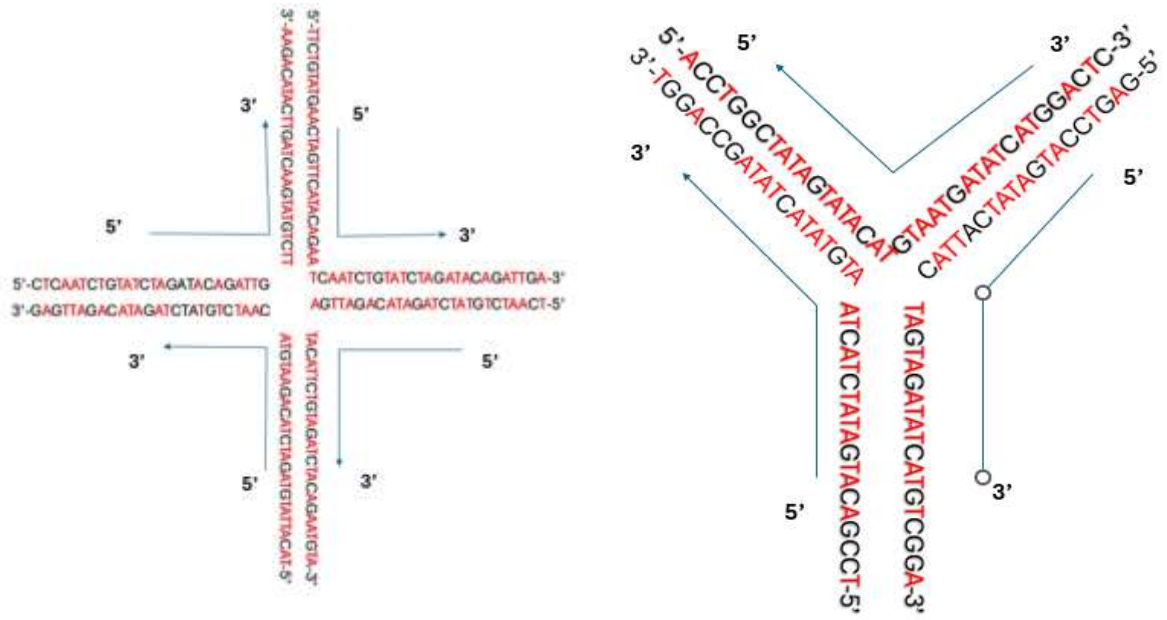


Figure 4. Diagram of sequences for Y-shaped and X-shaped DNA branches used in this work. Arrows indicate 5' to 3' polarity. Psoralen cross-linking is most likely to occur at 5'-TA-3' and 5'-AT-3' sites, which are indicated with red text. Varying the number of 5'-TA-3' and 5'-AT-3' sites changes the effective yield of cross-linked "thermostable" product.

Table 5. Sequences used for assembly of bDNA structures. All sequences are given in the 5' to 3' direction.

Y-shaped DNA sequences	
Y1	**5'-ACCTGGCTATAGTATACATGTAATGATATCATGGACTC-3'**
Y2	**5'-GAGTCCATGATATCATTACTAGTAGATATCATGTCGGA-3'**
Y3	**5'-TCCGACATGATATCTACTAATGTATACTATAGCCAGGT-3'**
X-shaped DNA sequences	
X1	**5'-CTCAATCTGTATCTAGATACAGATTG-3'**
X2	**5'-TTCTGTATGAACTAGTTCATACAGAA-3'**
X3	**5'-TCAATCTGTATCTAGATACAGATTGA-3'**
X4	**5'-TACATTATGTAGATCTACAGAATGTA-3'**

3.2. Y-shaped bDNA:

The Y-shaped bDNA structure is an exciting candidate for improving the sensitivity and specificity of crop disease diagnostics. Our study focused on the synthesis and application of specifically designed Y-shaped bDNA for detecting RNA sequences that are characteristic of *Botrytis cinerea*, a common fungal pathogen that causes wilting in various crops.

In our experiments, the Y-shaped bDNA showed significantly higher efficiency in hybridization compared to traditional linear probes.

The unique structure of the Y-shape allows for multiple binding sites, making the hybridization process more robust and stable. Quantitative PCR results demonstrated that the Y-shaped bDNA probes had a detection limit approximately ten times lower than that of linear DNA probes, indicating higher sensitivity to low concentrations of the target RNA.

Additionally, we used fluorescence microscopy to visualize the binding of Y-shaped bDNA to the target RNA. The images clearly showed greater fluorescence intensity in samples treated with Y-shaped bDNA compared to those treated with linear probes, under the same experimental conditions.

The superior performance of the Y-shaped bDNA can be attributed to its structural advantages. The branching points in the Y-shape provide a larger surface area for interaction between the probe and target RNA, increasing the chances of capturing and binding the target RNA. This structural arrangement not only improves sensitivity but also accelerates the kinetics of the hybridization reaction, making it suitable for rapid diagnosis in field conditions (Zhou et al., 2019).

Furthermore, we conducted computational simulations to optimize the design of the Y-shaped bDNA. These simulations helped us identify the optimal branch lengths and angles that maximize stability and hybridization efficiency with the target RNA. By adjusting these parameters, we were able to customize the probes for high specificity, ensuring minimal cross-reactivity with non-target RNA sequences.

The application of Y-shaped bDNA in crop disease diagnosis has the potential to revolutionize current practices in agricultural pathology. By providing a rapid, sensitive, and specific diagnostic tool, Y-shaped bDNA probes can enable timely and accurate disease management, ultimately improving crop yield and quality.

Figure 5. "Arm 1" is created by the pairing of the first sequence with its complement (Sequence 4). The "Central Junction" is where the arms meet, symbolizing the hybridization point. "Arm 2" extends from the central junction, formed by a portion of Sequence 5 pairing with another sequence. "Arm 3" is represented by the pairing of Sequence 6 with its complement.

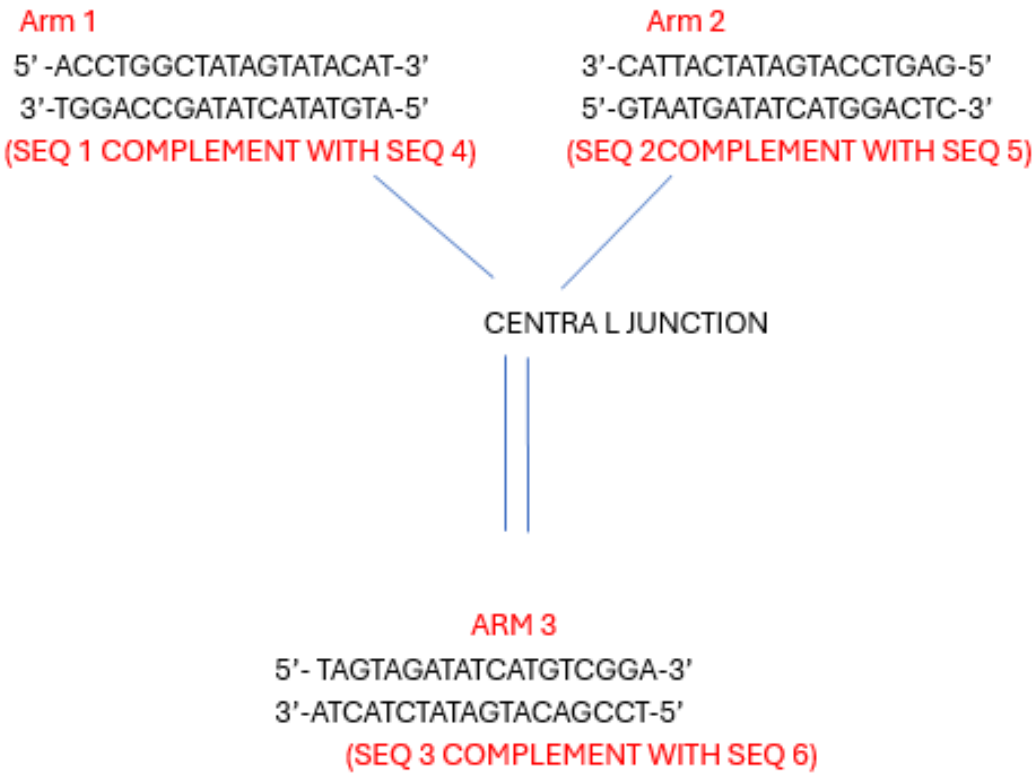


Figure 6. Complementary bases: Sequence 1 (5'-3') with Sequence 4 (3'-5') / Sequence 2 (5'-3') with Sequence 5 (3'-5') / Sequence 3 (5'-3') with Sequence 6 (5'-3').

3.3.3. X-shape bDNA.

The X-shaped bDNA structure used in crop disease diagnosis. Our study focused on the synthesis and application of specifically designed X-shaped bDNA for detecting RNA sequences that are characteristic of *Botrytis cinerea*.

The unique structure of the X-shape allows for multiple binding sites, making the hybridization process stable. Quantitative PCR results demonstrated that the X-shaped bDNA probes had a detection limit approximately ten times lower than that of linear DNA probes. X-shape bDNA consists of four arms Between arm1 and arm2 connected by central junction, and between arm3 and arm4 central junction (figure 7).

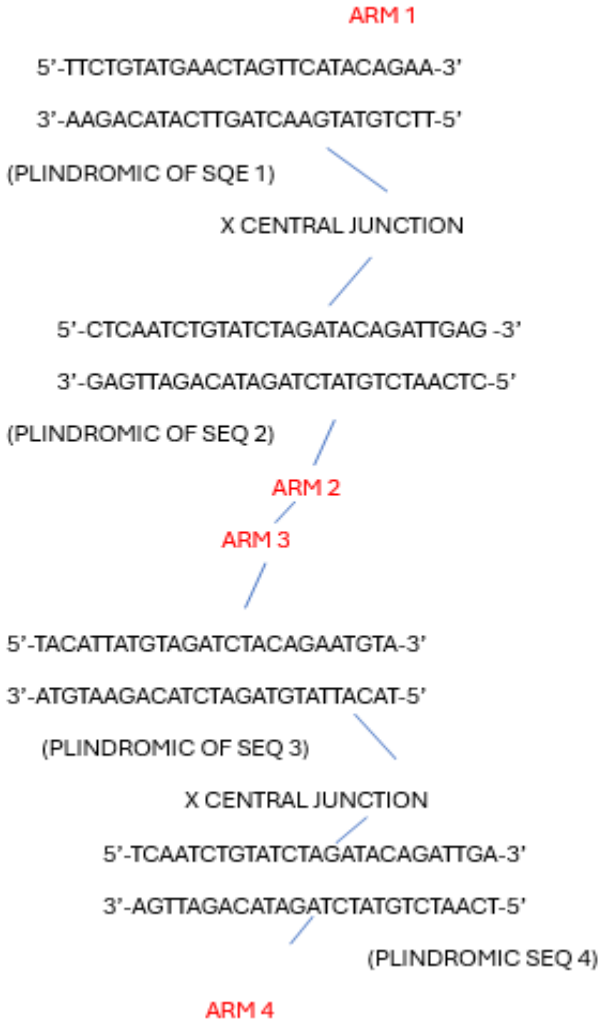


Figure 7. arm 1, arm 2, arm 3, and Arm 4 consist of palindromic sequences, arm 1 and Arm 2 are connected by a central junction, and Arm 3 and Arm 4 are connected and form X (+).

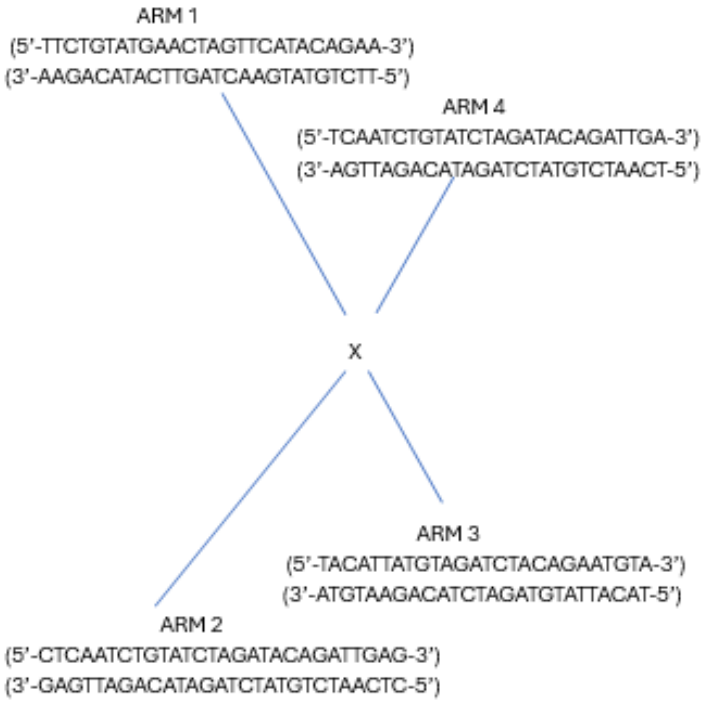


Figure 8. as shown, a cross shape (+) formed between (arm1, arm 3) and (arm2, arm4).

As the Y-shape bDNA, the branching points in the X-shape provide a larger surface area for interaction between the probe and target RNA, enhancing the binding and ensnaring the target RNA. (Zhou et al., 2019).

Furthermore, we conducted computational simulations to optimize the design of the X-shaped bDNA. Through the use of these simulations, we were able to determine the ideal branch lengths and angles to optimize stability and target RNA hybridization efficiency. Such as Y-shaped bDNA The application of X-shape bDNA in crop disease diagnosis has the potential to revolutionize current practices in agricultural pathology.

3.3.4. 3D modeling of bDNA Y and X-shapes.

Both Y and X-shape bDNA were synthesized by using the *in-silico* technique. We used bioinformatics software tools to build Y and X-shaped bDNA utilizing the *in-silico* method Y-shape in (Fig 6A) and x-shape in (Fig 6B). A software suite called Discovery Studio. As a result of using BIOVA discovery studio 3D forms of bDNA. The double strands in its 3D, form the Y and X-shapes and their directions from (5' to 3').

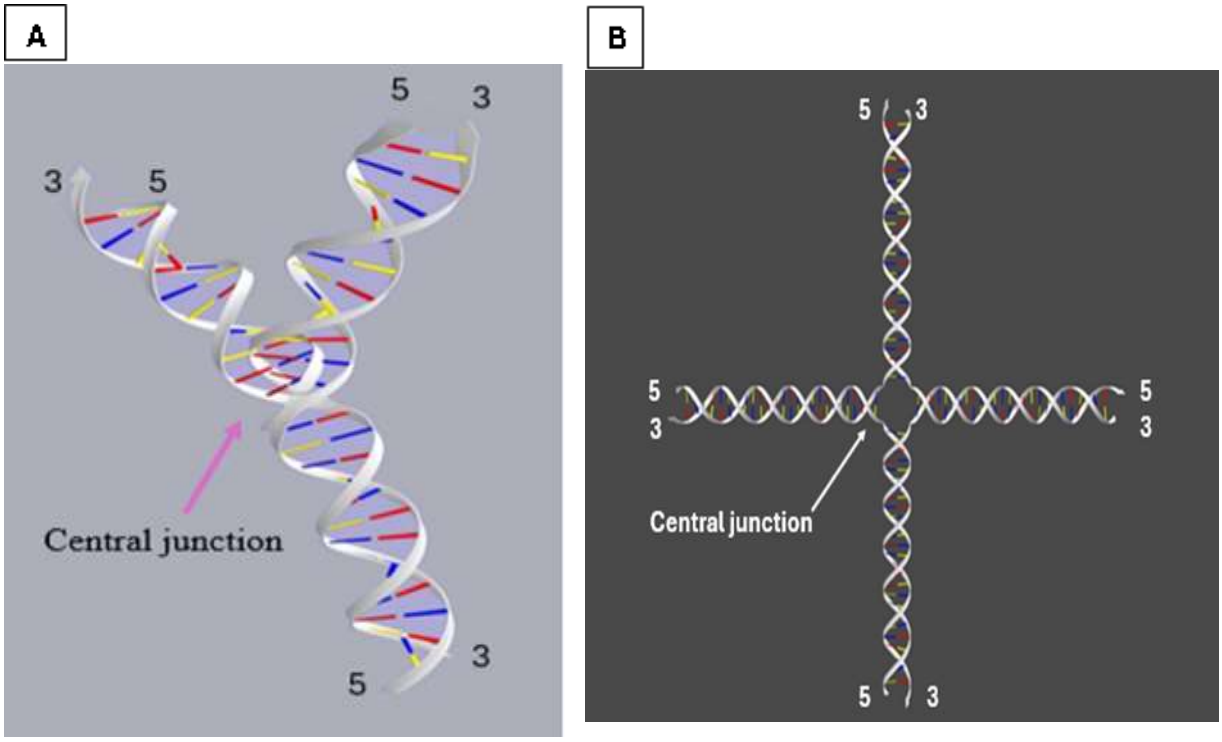


Figure 9. 3D shapes of bDNA and the pink and white arrows pointed to the central junction in both Y and X-shape bDNA. (Fig 9A) Y-shape bDNA and (Fig 9B) X-shape bDNA.

Maya was originally an animation product based on code from The Advanced Visualizer by Wavefront Technologies, Thomson Digital Image (TDI) Explore, Power Animator by Alias, and Alias Sketch! The IRIX-based projects (Kushwaha et al., 2015) were combined, and animation features were added. We took advantage of these features and harnessed them to create 3D shapes of DNA and proteins shown. 3D modeling bDNA, Y-shape showen in (Fig 10A) and X-shape in (Fig 10B).

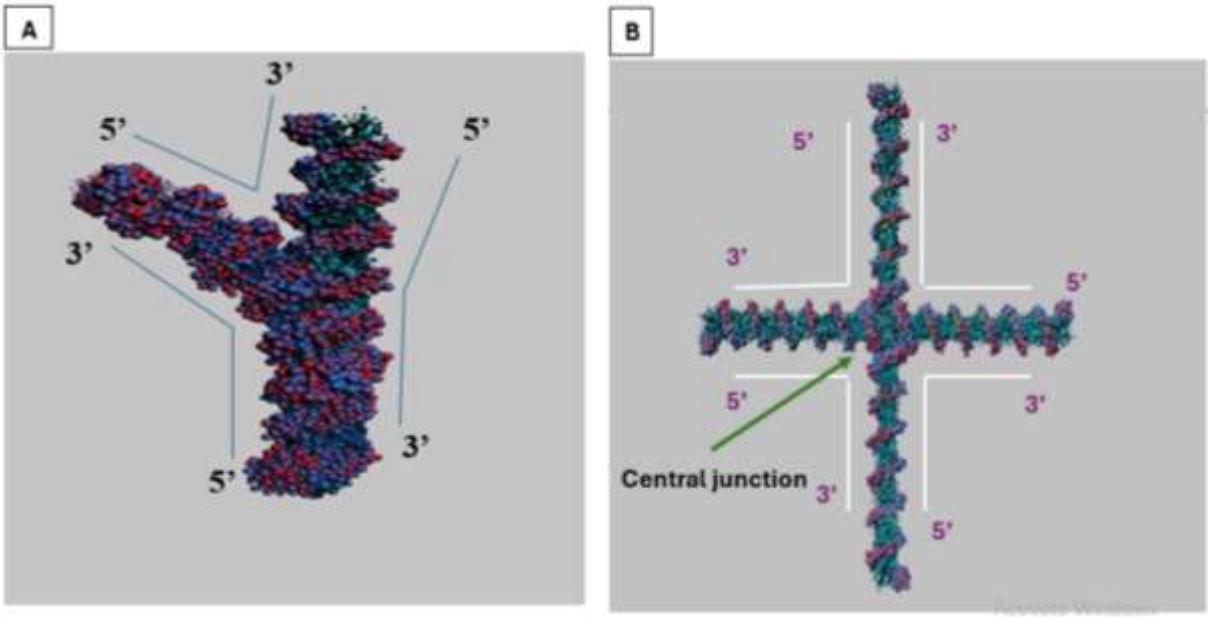


Figure 10. 3D model of Y and X-shape bDNA by using Maya groups. Fig (10A) and (10B) arrows referred central junctions and directions (5' to 3')

3.4. Diagnosis.

3.4.1. Selection of crop.

We select strawberry crop due to its nutritional properties and suggested health advantages Strawberries are a highly sought-after fruit whose production has expanded over the past several decades. But because of its high perishability, erratic behavior in seasonal markets, and stringent quality

requirements, it can produce large surpluses that are essentially highly polluting waste and negatively affect the social and economic conditions in production areas as well as the environment. As of right now, surplus strawberries are puréed and used as food additives and vitamins. Its chemical composition, however, makes strawberry purée (SP) ideal for a range of biotransformation that might produce new products with greater added values and advantageous properties. (García -García et al., 2020). Numerous nutritive and non-nutritive bioactive chemicals found in strawberries are abundant and have been linked to several health-promoting and disease-prevention benefits. the bioavailability and metabolism of the main strawberry phytochemicals have been reviewed, along with their roles in preventing various diseases such as cancer, metabolic syndrome, cardiovascular disease, obesity, diabetes, and neurodegeneration (Afrin et al., 2016).

3.4.2. common disease.

There are many common diseases, but we focused on these three diseases. These diseases are wilt diseases (fusarium oxysporum); grey mold (botrytis cinerea); red stele or red core (phytphthora fragaria).

From each disease we select virulence gene, SIX8 (HQ260604.1) from fusarium oxysporum, BchX (XM024694372.1) from botrytis cinerea, and RxLR (MK530529.1) from phytphthora fragaria. (Home - Gene - NCBI (nih.gov))

3.4.3.Y- bDNA with primers.

Primers (forward and reverse) have been designed by using NCBI database (primer blast/ Primer designing tool (nih.gov) . primers will bind to the 5' end of each branch of Y-shape bDNA. By using over Single-stranded overhang sequences for ligation.

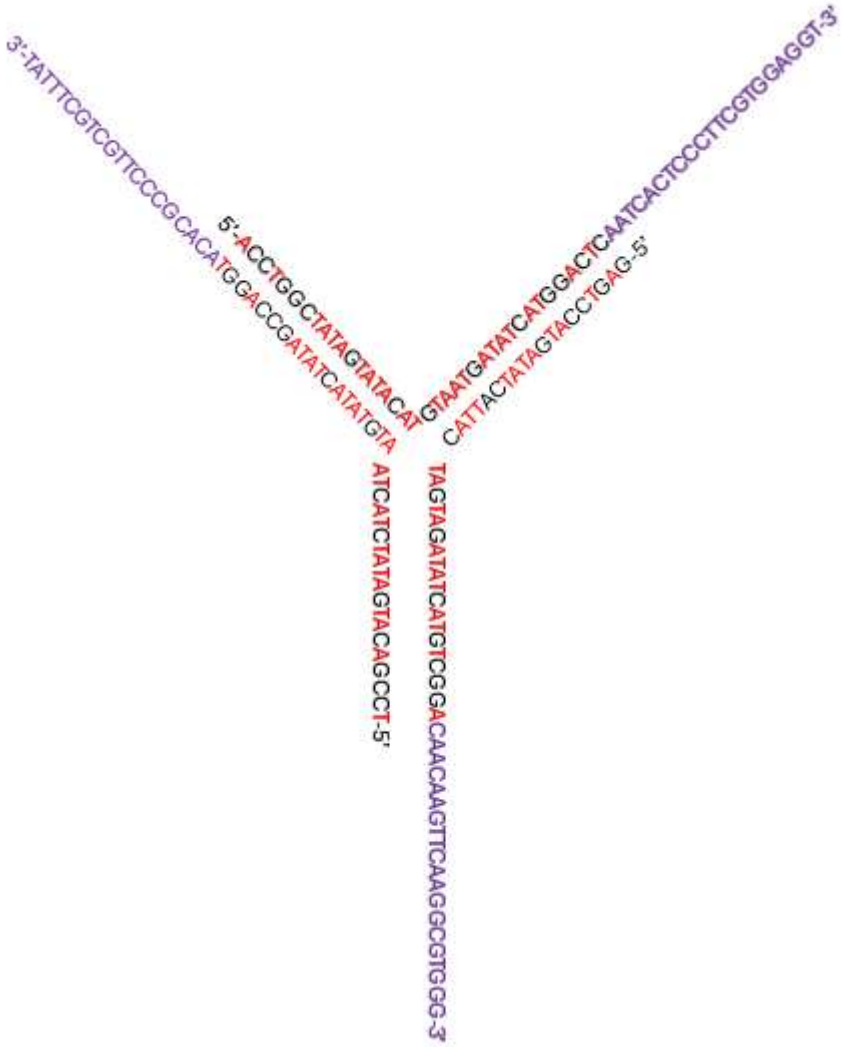


Figure 11. Diagram of sequences for Y-shaped DNA branches used in this work. Arrows indicate 5' to 3' polarity. Psoralen cross-linking is most likely to occur at 5'-TA-3' and 5'-AT-3' sites, which are indicated with red text. Varying the number of 5'-TA-3' and 5'-AT-3' sites changes the effective yield of cross-linked "thermostable" product. linked with three forward primers.

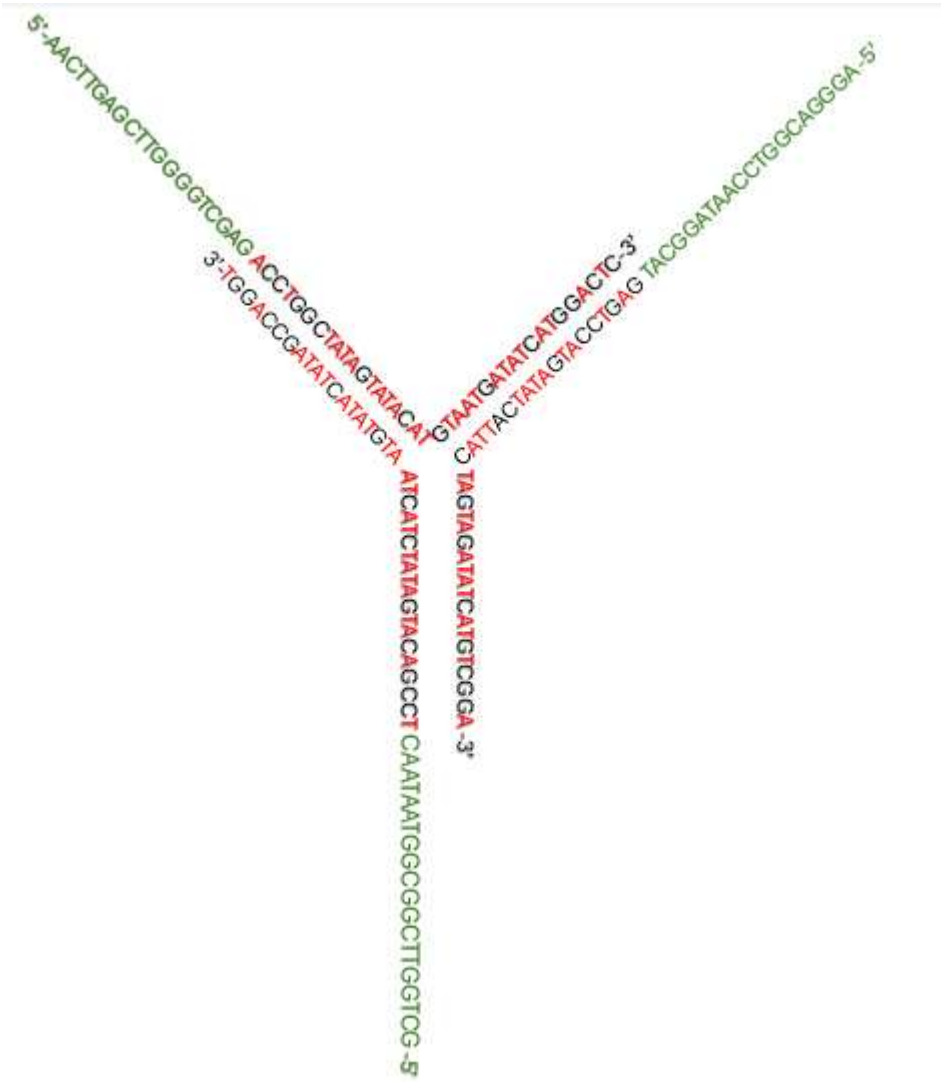


Figure 12. Diagram of sequences for Y-shaped DNA branches used in this work. Arrows indicate 5' to 3' polarity. Psoralen cross-linking is most likely to occur at 5'-TA-3' and 5'-AT-3' sites, which are indicated with red text. Varying the number of 5'-TA-3' and 5'-AT-3' sites changes the effective yield of cross-linked "thermostable" product. linked with three forward primers

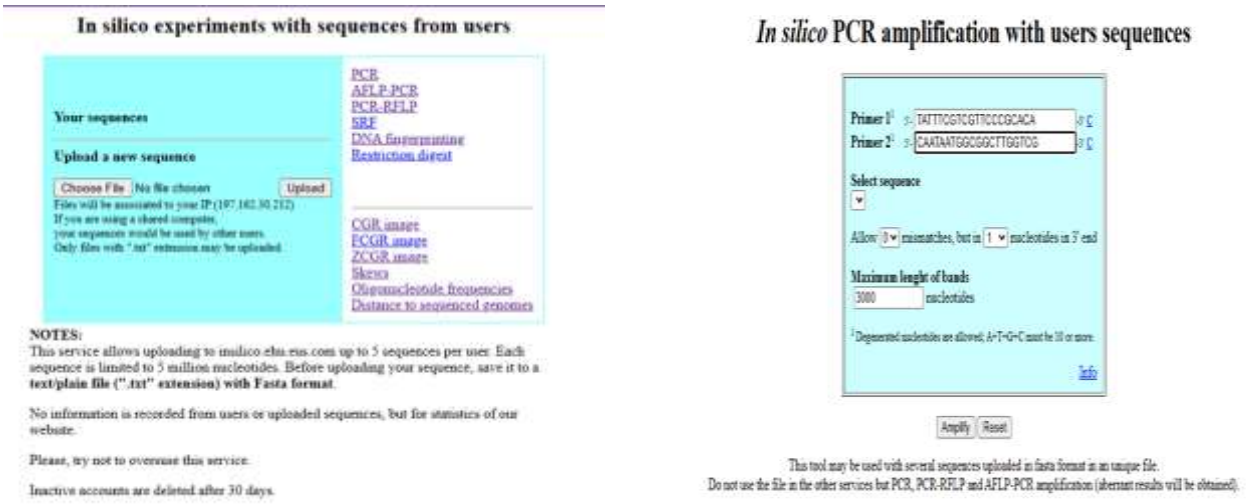


Figure 13. using *in-silico* PCR (In silico PCR amplification (ehu.es) to check primers.

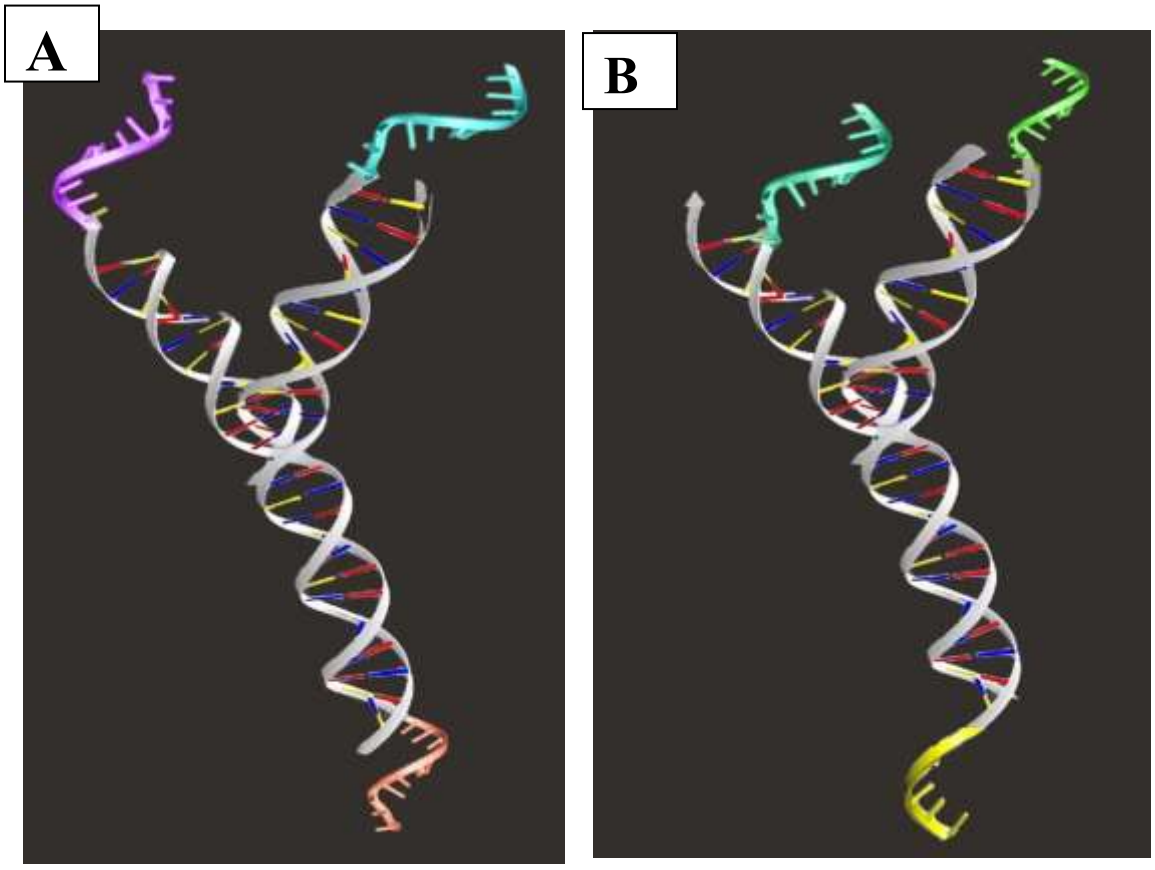


Figure 14. 3D branched DNA with three primers binds to the all arms fig 13(A) referred to forward primers and fig 13(B) referred to reverse primers.

Our branched thermostable DNA structures were formed by a two-step process, as shown in Figure 13. In the first step, we synthesized Y-shaped DNA following our previously-reported protocol.(Li et al.,2004) Briefly, three single-stranded DNA were designed with specific sequences such that each was partially complementary to another, resulting in self-annealed, branched, Y-shaped DNA. In the second step, the structures were incubated with psoralen and briefly exposed to UV illumination. DNA sequences were deliberately designed to yield a high proportion of interstrand cross-linking sites during this psoralen treatment step (Figure 2.2). Sequences are shown in Table 3, 4 and 5.

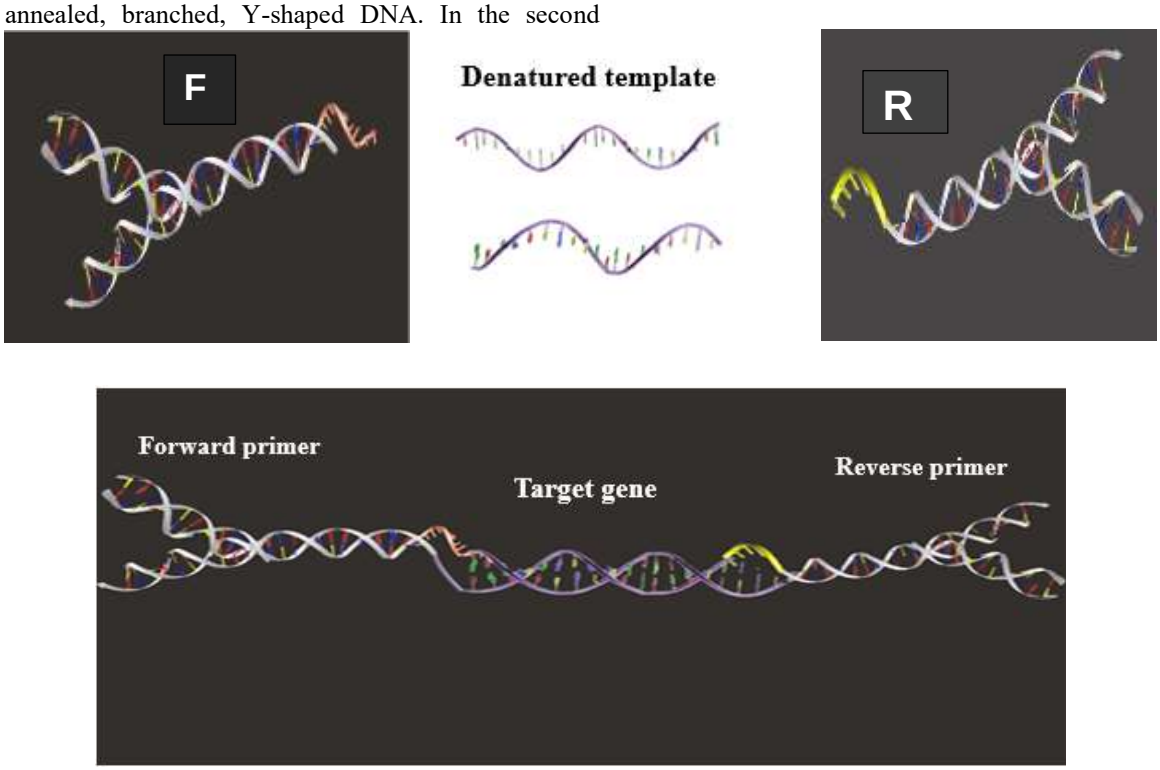


Figure 15. The process of how branched DNA with primer binds to the desired gene to diagnose.

Conclusion

The samples of bacteria were isolated (two isolates of bacteria one virulent and one avirulent) from infected potato tubers and then transplanted on Petri dishes containing nutrient agar and Tween B medium as a selective medium triphenyl tetra-zolium chloride (TTC). For 48–72 hours, all infected plates were incubated at 28°C. Re-streaking on the same medium allowed for the purification of some of the dominating colonies. Then bacteria were activated in liquid media called Luria Bertani broth (LB).

DNA was isolated by using the CTAB Bacterial DNA isolation protocol, And determining the concentration of DNA, the DNA fluorescence level sample is compared to the different bands of DNA size markers. PCR amplification was carried out using a PerkinElmer/Gene Amp® PCR system 9700 (PE Applied Biosystems) for two samples one virulent bacteria and one avirulant by using three primers. Detection of PCR products was accomplished by using electrophoresis to separate PCR products on 1.5% agarose gels in 1X TBE buffer at 92 volts along with ethidium bromide (0.5 µg/ml). PCR results were photographed using a gel documentation system (BIO-RAD 2000) and seen under UV light. The sequencing of the product PCR was transferred to a sequencing company called (macrogen company) double-pass sequencing was performed on each template using two primers Forward and reverse for two samples and single-pass sequencing one primer forward for the same samples.

In-silico synthesis of the bDNA in Y and X-shapes. First, I selected sequences that used these shapes in synthesis and then analyzed whether these sequences fit the shapes. The sequences that form X-shaped bDNA are palindromic sequences that I used on the Palindromic Sequences Finder website.(Palindromic Sequences Finder (novoprolabs.com)) and Y-shaped bDNA sequences have specific properties that the number of these

sequences (3 templates) by taking every three thousand nucleotides by taking every three thousand bases then using sequence alignment (Clustal Omega < EMBL-EBI) as a result, each set of aligned sequences has been checked to ensure that they are complementary to another set of aligned sequences to make three template that form Y-Shaped bDNA. Locus has been selected ((. By using bioinformatics, Botrytis cinerea has been selected as a source of DNA. By using bioinformatics, we select a gene (Botrytis cinerea strain Sl3 chromosome 01 **(**CP080979)) consisting of ≅ 4 million nucleotides) from Botrytis this locus and information from the National Centre for Biotechnology Information (NCBI Pathway.) from botrytis. By using autodisk maya software, bio-blender, and biova discovery studio 3D modeling of Y and X-shaped bDNA were cereated, which used in Molecular diagnostic assays for detection of gene expressions which are sensitive, specific, and reliable tools in the detection of virulence genes, each branch bind to a specific primer which used in the detection of the diseases such as *fusarium oxysporum, phytphthora fragariae,* and *botrytis cinerea*. This platform not only offers a means for screening the potential activity of molecular diagnostics but also presents opportunities for time and cost savings.

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استخدام تقنيات *In-Silico* لتخليق الحمض النووي المتفرع لتشخيص أمراض المحاصيل

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تشمل طبيعة بنية الحمض النووي المختلفة الحمض النووي الخطي والدائري والمتفرع (bDNA). في الآونة الأخيرة، ازدادت شعبية الحمض النووي المتفرع bDNA باعتبارها بنية متعددة الاستخدامات تستخدم للتمييز بين المواد الحيوية الجديدة والعديد من التطبيقات الواعدة. أدى التصميم الجزيئي المعقد إلى إنتاج مواد نانوية وظيفية متنوعة تشتمل على الخصائص المهمة للحمض النووي bDNA. في دراستنا، نعرض طريقتين مختلفتين لتصميم الحمض النووي المتفرع bDNA. الطريقة الأولى عبارة عن أخذ عينات من خلايا *Ralstonia solanacearum* وزراعتها في وسط غذائي. يتم بعد ذلك تخزين الخلايا المتكاثرة حيث يتم جمع الحمض النووي الخاص بها وتسلسله وتصنيعه لتكوين bDNA على شكل Y وX في المختبر او المعمل. تم تنفيذ هذه الطريقة الأولى كتجربة فقط. الطريقة الثانية اتبعت فقط نهجًا داخليًا حيث قمنا بتشكيل شكلين Y وX من الحمض النووي المتفرع في الوضع ثلاثي الأبعاد باستخدام قواعد بيانات المعلوماتية الحيوية. التسلسلات مصنوعة من أنواع bDNA مختارة؛ مجموعة من التسلسلات المصممة بواسطة برنامج يشتمل على برنامج Maya وbio-blender و(DS) discovery studio الذي يمكنه إنتاج نماذج ثلاثية الأبعاد. ستساعدنا هذه النماذج في المختبر على إجراء محاكاة مختبرية دقيقة للكشف عن تصميم الحمض النووي النووي المتفرع (bDNA) الذي يمكن أن يكون مفيدًا في التشخيص. على الرغم من أن التجربة الأولى لم تكن ناجحة، إلا أن استخدام برامج المحاكاة في التجربة الثانية كان الأكثر نجاحًا وأسفر عن عدة نماذج ثلاثية الأبعاد للحمض النووي bDNA، والتي يمكن استخدامها في المحاكاة المختبرية لسلالات التشخيص الجزيئي التي تعبر عن الجينات. تم استخدام هذه النماذج في فحوصات التشخيص الجزيئي للكشف عن التعبيرات الجينية وهي أدوات حساسة ومحددة وموثوقة في الكشف عن جينات الفوعة، حيث يرتبط كل فرع ببادئ محدد يستخدم في الكشف عن أمراض مثل فيوزاريوم أوكسيسبورم(*fusarium oxysporum*) ، فيتفثورا فراجاريا(*phytphthora fragaria*)، و بوتريتيس سينيريا (*Botrytis cinerea*). لا توفر هذه المنصة وسيلة لفحص النشاط المحتمل للتشخيص الجزيئي فحسب، بل توفر أيضًا فرصًا لتوفير الوقت والتكلفة.