Optimization of the Detection Method for Xanthomonas axonopodis on Chili Pepper Seeds Using Polymerase Chain Reaction with Two Difference Primers

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Abstract. The detection of Xanthomonas axonopodis pv vesicatoria (XAV) from chili seeds was carried out to avoid spreading XAV through seed. Detection of XAV in chili seeds through Polymerase Chain Reaction (PCR) using XAVF/XAVR and RST65/RST69 primers sometimes got unclear electrophoresis results. This research was conducted to determine the optimal time and temperature of primer annealing, and primer concentration to detect XAV on chili pepper seeds. Optimization was conducted in three phases, (1) optimization of primer annealing temperature, (2) optimization of annealing time, and (3) determinate primer concentration. The results show that XAVF/XAVR primer is optimal at 60°C for 30 seconds on the primer concentration of 10 pmol, and primer RST65/RST69 is optimal at 58°C for 20 seconds on the primer concentration of 10 pmol. The optimal time, temperature of primer annealing, and primer concentration were validated by three repetitions using positive and negative control. The validation test result shows that optimal time and temperature of primer annealing, and primer concentration consistently produce clear electrophoresis results without smear.

Keyword : Detection, Xanthomonas axonopodis, chili seeds, Polymerase Chain Reaction, primer optimization

1 Introduction

Xanthomonas campestris pv *vesicatoria* or *Xanthomonas axonopodis* pv *vesicatoria* (XAV) is an important pathogen in chili plants worldwide. Bacterial spot disease caused by XAV is difficult to control due to limited control methods. Chemical control has had limited success, making the disease difficult to control after an epidemic. So this disease needs to be controlled before planting through detecting XAV in seeds before planting.

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One method for detecting the presence of XAV in chili seeds with accurate results, in a short time, and without having to culture the samples used is the Polymerase Chain Reaction (PCR) method. The result of the PCR process is many nucleotide parts because they have been multiplied and visualized using electrophoresis in the form of DNA bands. DNA bands are not always clearly visible, but can also appear blurry, smear, or be invisible. Overcoming the occurrence of blurry, smear, or invisible products, one of which is by optimizing or varying the conditions during the PCR process. One way to optimize the PCR process is by optimizing temperature, annealing time, and primary concentration.

Primer XAVF/XAVR and RST65/RST69 can be used for XAV detection in chili seeds. XAV detection using PCR on chili seeds using primers XAVF (5' AGAAGCAGTCCTTGAAGGCA 3') and XAVR (5' AATGACCTCGC CAGTTGAGT 3') with an estimated amplification product length of 517 bp using annealing temperature of 58 ºC and 30 seconds [1]F. XAV can also be detected using the primers RST65 (5' GTCG TCGT TACGG CAAGG TGGTCG 3') and RST69 (5' TCGCCCAGCGT CATCAG GCCATC 3') with an estimated 420 bp amplification product using annealing temperature of 63 ºC and 30 seconds [2].

However, the PCR results from previous studies showed smeared DNA bands. In this study the aims were to determine temperature, annealing time, and primer concentration in the PCR reaction in XAV detection from chili seeds, to determine the consistency of the results of optimizing temperature, annealing time, and primary concentration during the validation test, and to determine the consistency of the results of optimizing temperature, annealing time, and primary concentration when used in the detection of XAV bacteria from chili seeds.

2 Material and Methods

2.1 Optimization of annealing temperature

The annealing temperature optimization process begins with mixing the reagents. Each treatment used 9.5 µL of ddH2O, 12.5 µL of Master Mix, 1 µL of forward and reverse primers, and 1 µL of XAV DNA. In order for uniform reagent composition each treatment, the reagents for 12 treatments were mixed in a 1.5 mL tube as a cocktail. 114 µL ddH2O, 150 µL Master Mix, 13 µL of forward and reverse primers were mixed in a 1.5 mL tube. 24 µL of cocktail was transferred to a PCR tube and added 1 µL of H_2O as a negative control. The remaining cocktail was transferred equally to 12 PCR tubes and add 12 µL DNA XAV of each tube. The amplification process for each pair of the same primer was carried out simultaneously with gradient PCR. The time and temperature settings were according to Table 1 and Table 2 but the annealing temperature followed Table 3.

Tabel 1. Amplification program for XAVF/XAVR primers [1].

Step	Temperature	Time	Cycle
Initial Denaturation	94 °C	5 minutes	1
Denaturation	94 °C	60 seconds	25
Annealing	*Following tabel 3*	30 seconds	
Extension	72 °C	60 seconds	
Final extension	72 °C	10 minutes	1

Tabel 2. Amplification program for RST65/ RST69 primers[2].

Step	Temperature	Cycle
Initial Denaturation	95 °C	1
Denaturation	95 °C	29
Annealing	*Following tabel 3*	
Extension	72 °C	
Final extension	72 °C	1

Tabel 3. Various temperature of annealing XAVF/R and RST65/69 primers used at optimization of annealing temperature.

Treatment code	Annealing temperature using primer XAVF/R	Annealing temperature using primer RST65/69
1	56,0 °C	
2	56,2 °C	
3	56,5 °C	
4	56,9 °C	[b]
5	57,3 °C	
7	57,8 °C	
8	58,7 °C [a]	
9	59,1 °C	
10	59,5 °C	
11	59,8 °C	
12	60,0 °C	
[a] Annealing temperature used at previous research [1].		
[b] Annealing temperature used at previous research [2].		

2.2 Optimization of annealing time

The process of optimizing the annealing time begins by mixing the reagents for each treatment mention in Table 4. Each treatment used 9.5 µL ddH2O, 12.5 µL Master Mix, 1 µL Primer forward and reverse, and 1 µL DNA XAV. In order for each treatment to get a uniform reagent composition, the reagents for all treatments were mixed as a cocktail. 24 µL of cocktail was transferred to PCR tubes and added 1 µL ddH2O as a negative control. The remaining cocktail was added with 3 µL of DNA XAV, then transferred to 3 PCR tubes. The amplification process is carried out alternately by setting the time and temperature according to Tables 2 and Table 3. The annealing temperature following the results of annealing temperature optimization.

Tabel 4. Various time of annealing XAVF/R and RST65/69 primers used at optimization of annealing time.

Treatment code	Annealing time using primer XAV/R	Annealing time using primer RST65/69
1	20 second	20 second
2	30 second [a]	30 second
3	40 second	40 second
[a] Annealing time used at previous research [1].		

2.3 Optimization of primer concentration

The process of optimizing the primer concentration begins with mixing the reagents for each treatment in Table 5. Each treatment used 9.5 µL ddH2O, 12.5 µL Master Mix, 1 µL primer forward, 1 µL primer reverse, and 1 µL DNA template. In order for each treatment to get a uniform reagent composition, the reagents except the primers for all treatments were mixed in a 1.5 mL tube as a cocktail. 76 µL ddH2O and 100 µL Master Mix were mixed in a 1.5 mL tube as a cocktail. The cocktails were transferred to 4 PCR tubes of 22 µL each and 1 µL of XAV DNA was added to each tube except for the negative control using ddH2O. Forward and reverse primers with concentrations of 5 pmol, 10 pmol, 15 pmol, and 20 pmol were added to the tube. The amplification process was carried out using the program according to Table 2 and Table 3. The annealing temperature and time used the optimal time and temperature from the previous stage. The primer concentration treatment of the same primer was amplified simultaneously.

Tabel 5. Various XAVF/R and RST65/69 primers concentration used at optimization of primer concentration.

Treatment code	Concentration of Primer XAV/R	Concentration of Primer RST65/69
1	5 pmol per µL	5 pmol per µL
2	10 pmol per µL[a]	10 pmol per µL[a]
3	15 pmol per µL	15 pmol per µL
4	20 pmol per µL	20 pmol per µL
[a] Primer concentration used at previous research by [1].		

2.4 Validation test for annealing temperature and time & primer concentration

Validation of the optimal annealing time, temperature, and primer concentration for primers XAVF/XAVR and RST65/RST69 was carried out with three repetitions on 3 different reagen mixing time and machine running time. This stage was conducted to make sure the optimal time, temperature, and primer concentration are consistently optimal on several replications of amplification. Each treatment used 9.5 µL ddH2O, 12.5 µL Master Mix, 1 µL primer forward, 1 µL primer reverse, and 1 µL DNA template. Every amplification used negative control and XAV DNA template as positive control.

3 Result and discussion

3.1 Optimization of annealing temperature

Figure 1 shows the result of optimization of annealing temperature using XAVR/XAVF (A) and RST65/RST69 (B). DNA band appear all samples except control sample. DNA band with annealing temperature 60 ^{o}C seems clearer than other annealing temperature for XAVR/XAVF. Smear on other DNA bands is caused by the temperature used being too low so that the primer does not stick to the target DNA piece. An annealing temperature that is too low causes the primer to stick to the target. DNA band with annealing temperature 58 ^{o}C seems clearer than other annealing temperature for primer RST65/RST69. The temperature is lower than XAVR/XAVF because the primer base length of RST65/RST69 is lower than XAVR/XAVF. If the temperature is too high for a short DNA size it can cause the primer to separate from the target DNA so that the DNA is not read perfectly [3].

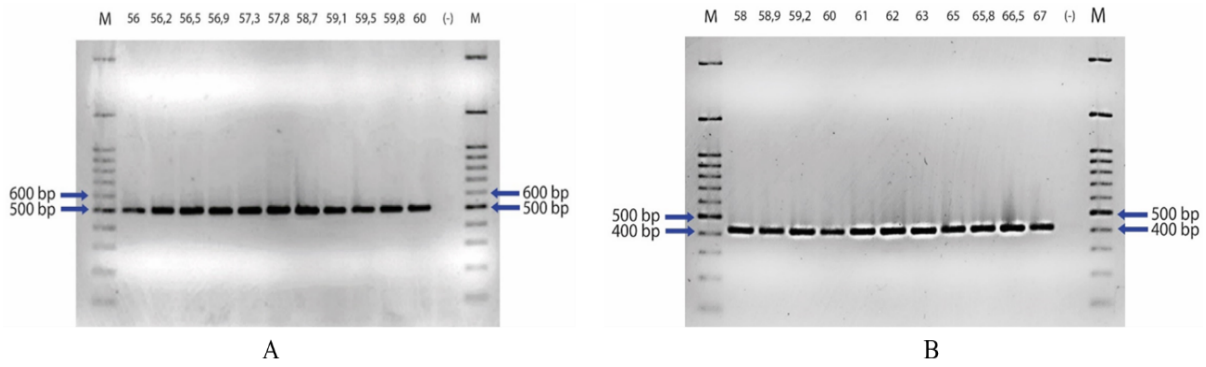

Fig 1. Electrogram of optimization of annealing temperature. (A) XAVR/XAVF primers; M: Marker 100bp; temperature 56-60 ºC. (B) RST65/RST69 primers; M: Marker 100bp; temperature range 58-67ºC; (-) control sample.

3.2 Optimization of annealing time

Figure 2 show the optimal annealing time using XAVF/XAVR (A) and RST65/RST69 (B) primers. All annealing time use negative control do not show any DNA band. It shows that the reaction running well. The one treatment that effect the result is only the annealing time. The best annealing time for XAVF/XAVR is 30 second. Annealing use 20 second show thin DNA band and 40 second has smear. Annealing RST65/RST69 use 20 and 30 second show clear DNA band. Annealing use 40 second show a thin smear. 20 second is chosen for the annealing time for RST65/RST69 because it shorter. The timing for the annealing process is related to the length of the primer. The longer the primer size, the longer annealing time it takes to be able to attach to the target DNA perfectly.

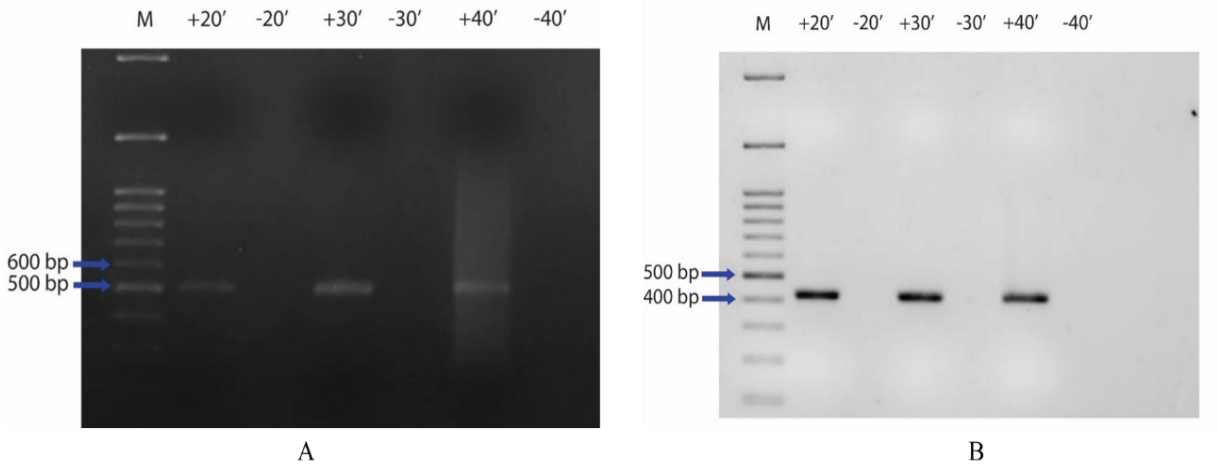

Fig 2. Electrogram of optimization of annealing time. (A): XAVF/XAVR primers. (B) RST65/RST69 primers. Annealing time tested: 20 second, 30 second, and 40 second. M: Marker 100bp. (+): using positive control. (-): using negative control.

3.3 Optimization of primer concentration

Figure 3 shows the optimal concentration of XAVF/XAVR (A) and RST65/RST69 (B) primers. All treatment uses negative control do not show any DNA band. It shows that the reaction running well. The one condition that affects the DNA band result is only the primer

concentration. The best annealing time for XAVF/XAVR is 30 second. Primer concentration 5 pmol and 20 pmol show a thin smear at XAVF/XAVR and RST65/RST69 primer. Primer concentration 15 pmol show smear at XAVF/XAVR and thin DNA band at RST65/RST69. The best primer concentration is 10 pmol for XAVF/XAVR and RST65/RST69 primer. Primer concentrations that are too low or too high can cause poor amplification [4] and a low ratio or comparison between primers and DNA causes the resulting product to be inconsistent [5].

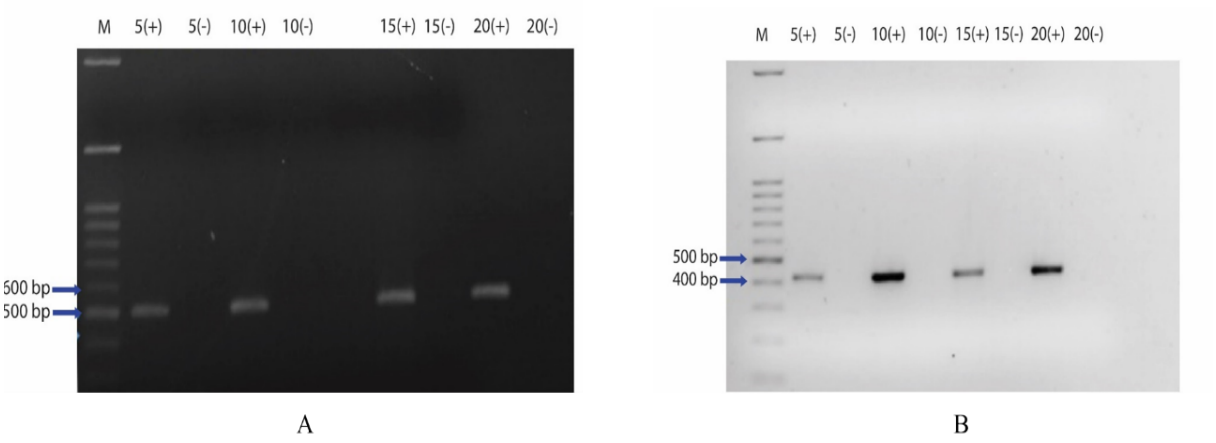

Fig 3. Electrogram of optimization of primer concentration. (A): XAVF/XAVR primers. (B) RST65/RST69 primers. Primer concentration tested: 5 pmol, 10 pmol, 15 pmol, and 20 pmol. M: Marker 100bp. (+): using positive control. (-): using negative control.

3.4 Validation test for annealing temperature and time and primer concentration

Validation of temperature, annealing time, and primer concentration for XAVF/XAVR is carried out using the best annealing temperature and time of 60°C, for 30 seconds, and a primer concentration of 10 pmol. [5] Validation of temperature, annealing time, and primer concentration for RST65/RST69 is carried out using the best temperature and annealing time of 58°C for 20 seconds and a primer concentration of 10 pmol. Figure 4 shows DNA bands that were clearly visible from the 3 replications showing that the optimal temperature, time, and primer concentration from the previous stage consistently gave good results. The results of PCR can be good or bad results, this is not only influenced by temperature, annealing time and primary concentration, but can also be caused by human factors and the tools used. Validation is carried out aiming to minimize false good results, or luck. From Figure 4, the DNA bands that appear clear at that temperature, time and concentration are not the result of mere luck.

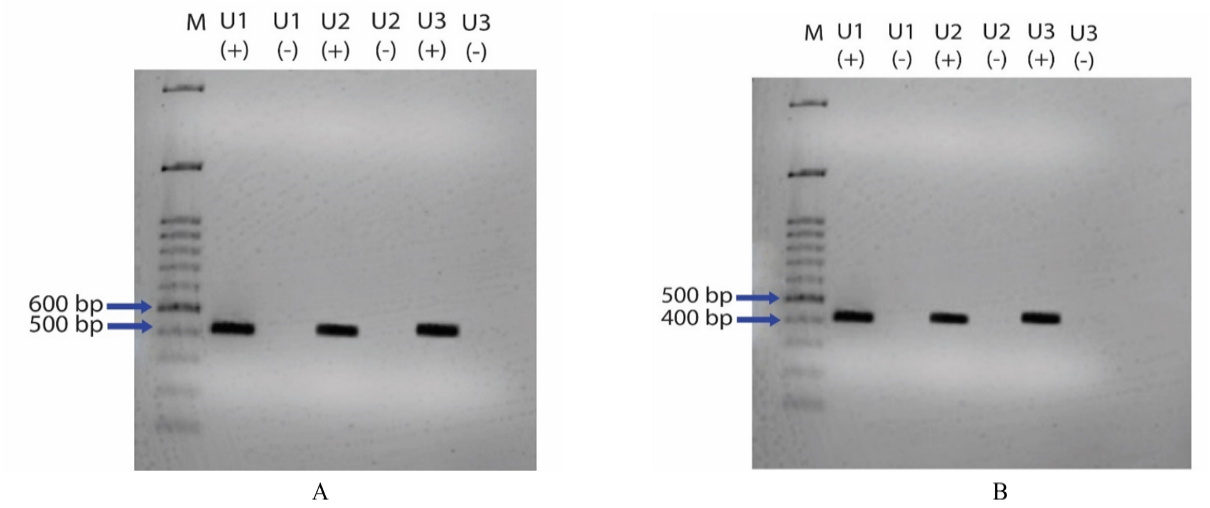

Fig 4. Electrogram of validation test for annealing temperature and time & primer concentration. (A): XAVF/XAVR primers. (B) RST65/RST69 primers. U1: repetition 1, U2: repetition 2, U3: repetition 3. M: Marker 100bp. (+): using positive control. (-): using negative control

4 Conclusion

Based on the results and discussion, it can be concluded that the optimum temperature, annealing time, and primer concentration for the detection of XAV bacteria on chili seed using XAVF/XAVR primers is 60 °C for 30 seconds and 10 pmol primer concentration, RST65/RST69 primer 58 °C for 20 seconds and 10 pmol primer concentration. The results of the temperature validation test, anneling time, and primary concentration obtained consistent results.

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