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MOLECULAR DETECTION OF FIVE MIXED RICE VIRUSES BY REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

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ABSTRACT

Rice plants are one of the important commodities for the livelihoods of people all over the world, including in South Sulawesi. However, rice plants often suffer from virus infections which can cause production losses and reduced quality. Mixed viral infections in rice plants in South Sulawesi have been detected through RT-PCR analysis. The results showed that the rice plant samples were infected by several types of viruses, including *Rice tungro spherical virus* (RTSV), *Rice tungro bacilliform virus* (RTBV), *Rice grassy stunt virus* (RGSV), *Rice ragged stunt virus* (RRSV), and *Rice black streaked dwarf virus* (RBSDV). The detected virus DNA molecule sizes varied between 195-1764 bp in different samples. This finding highlights the importance of considering mixed viral infections in rice plants in South Sulawesi as a significant issue. The determination of the type of viruses and the infection level is crucial in determining an effective control strategy and minimizing rice production losses. Preventive measures, such as maintaining the cleanliness of the planting location, destroying infected plants, and planting virus-resistant varieties, are necessary to prevent the spread of these viruses.

Keywords: Mixed viral infections, RT-PCR, rice viruses, South Sulawesi.

INTRODUCTION

Rice plants are a major source of food for a large portion of the world population (Elert, 2014; IRRI, 2010; Khush, 2005). Rice production is influenced by many factors, one of which is virus infection. Virus infection in rice plants can cause significant losses to the agricultural industry, including decreased productivity and quality of the harvest. Therefore, it is important to understand and effectively address this issue. Plant viruses are typically transmitted by insects that serve as vectors (Hogenhout *et al.,* 2008). Their effectiveness in spreading the virus depends on factors such as behavior, efficiency, and ability to travel from plant to plant and cross long distances (Fereres *et al.,* 2009; Blanc, *et al.,* 2016). Virus in rice plants can emerge and spread dynamically. Insect pathogens such as plant hoppers and leafhoppers cause double damage to rice yields due to their direct impact on the growth and development of rice, as well as their high efficiency in transmitting rice viruses (Wang *et al.,* 2022).

The Rice plant virus, such as *Rice tungro spherical virus* (RTSV), *Rice tungro bacilliform virus* (RTBV), *Rice grassy stunt virus* (RGSV), *Rice ragged stunt virus* (RRSV), and *Rice black*

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streaked dwarf virus (RBSDV), can be transmitted by planthoppers and leafhoppers, which have high efficacy in transmitting the virus. The infection of dwarf viruses in Rice plants has become a major problem for agriculture in South Sulawesi, making it imperative to address this issue as soon as possible. The diagnosis of virus infection is the first step in addressing this problem. RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) is a method commonly used in diagnosing virus infections in plants. RT-PCR is a fast and accurate diagnostic method that uses PCR technology to identify and evaluate the specificity and sensitivity of the virus (Syrmis *et al.,* 2004). This method is fast and accurate and is very useful in determining the type of virus using specific primers. Gel Analyzer, on the other hand, is a diagnostic method that uses electrophoresis gel to separate and identify DNA or RNA fragments taken from plant specimens. This method is easy to perform and useful for determining DNA/RNA/Protein Band molecular weight (Ahmed, 2021).

The current issue is the mixed infection of dwarf viruses in Rice plants in South Sulawesi that can spread quickly and affect many plants in a short time, causing great losses for the agricultural industry. In South Sulawesi, mixed infection of dwarf viruses in Rice plants is a major problem for agriculture. Dwarf viruses can spread quickly and affect many plants in a short time (Zhou, 2013), molecular detection is the first step in addressing this issue, however there are some challenges faced in this regard, such as: lack of information about the effectiveness of RT-PCR method in addressing mixed infection of dwarf viruses in Rice plants in South Sulawesi. The ability of RT-PCR method to address the problem of dwarf virus infections in Rice plants in South Sulawesi is still in question. The ability of RT-PCR method to distinguish between different virus types and ensure accurate results still needs to be investigated. The ability of RT-PCR method to address the problem of dwarf virus infections in Rice plants in South Sulawesi on a large scale also needs to be investigated. Thus, this research is necessary to address these issues and provide the appropriate solution to address mixed infection of dwarf viruses in Rice plants in South Sulawesi.

This research aims to evaluate the effectiveness of RT-PCR method in addressing mixed infection of dwarf viruses in Rice plants in South Sulawesi. The results of this research will be useful for agricultural experts and researchers to understand the level of effectiveness of this method and help in the development of better virus infection diagnostic methods in the future.

MATERIALS AND METHODS

Sample Collection: In this stage, rice plant samples were collected from various locations in South Sulawesi including Pinrang, Carawali, Sidrap, Wajo, Tanah Sitolo, and Maros (Indonesia). Samples were taken from plants showing symptoms of stunting virus such as changes in leaf color, leaf narrowing, and deformations. A visual examination was carried out to determine the symptoms appearing on the rice plants suspected to be infected with the stunting virus. After collection, the samples were stored in sterile containers at 4°C until further processing.

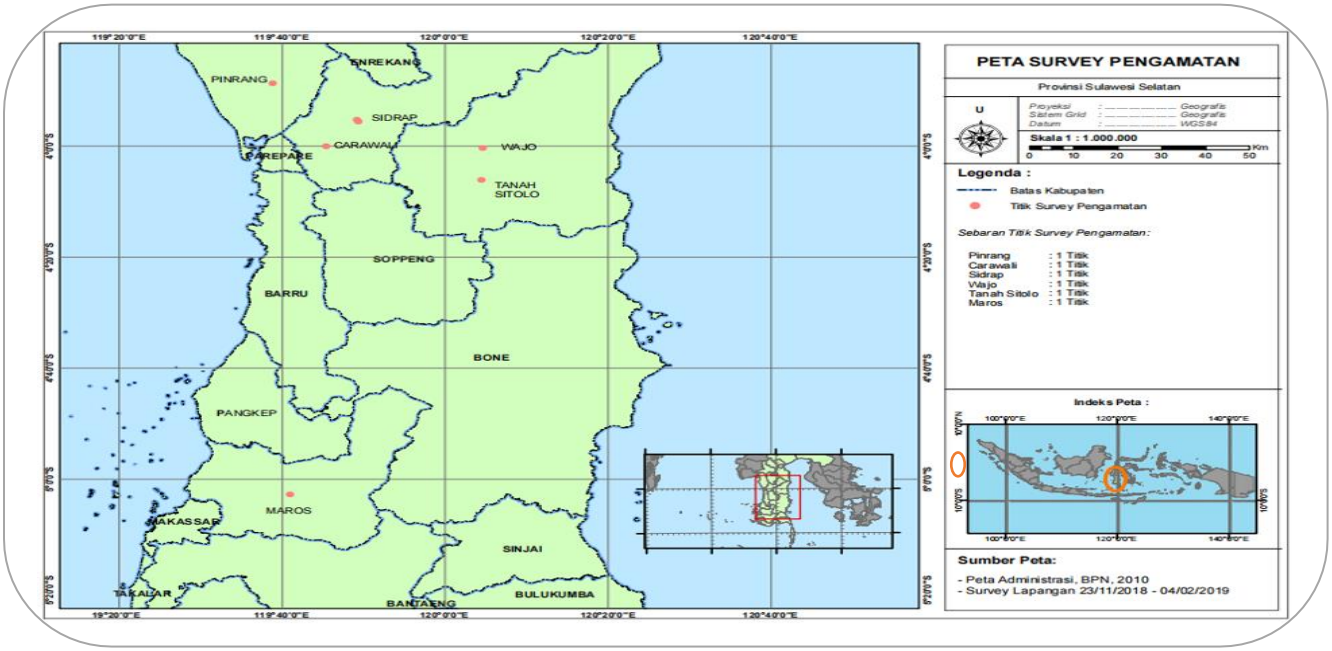


Figure 1. Map of Sample Collection Location of Rice Plants Symptoms of Rice Virus.

Total RNA/DNA Extraction: The Total RNA/DNA extraction was performed on plant samples using the commercial RNA/DNA extraction kit (GeneAid) by following the manufacturer's instructions. The kit contains the necessary reagents to isolate RNA/DNA from plant cells. The extraction was carried out to obtain the genetic material from the infected rice plant specimens. The infected specimens were cut and collected in a sterilized container, and the rice plant cells were lysed using a lysis buffer that contained cell-breaking enzymes such as pectinase and cellulase. RNA/DNA was separated from unwanted cellular components either by using the Total RNA/DNA extraction kit or manual methods. The purified RNA/DNA was further washed with a purification buffer to eliminate any unwanted components. Total RNA/DNA extraction is a crucial step in the research as it provides the genetic material required for RT-PCR and Gel Analyzer analysis. The method must be performed with care and in a sterilized environment to guarantee accurate and high-quality results.

RT-PCR (Reverse Transcriptase Polymerase Chain Reaction): cDNA synthesis was carried out through the RT-PCR process. In this stage, RT-PCR was performed using a commercially available kit (Toyobo Research Reagents) and following the manufacturer's instructions. The oligo primer (dt) in the RT-PCR reaction was used as the starting material to initiate the process of synthesizing cDNA from the RNA virus obtained from rice plant specimens. The function of the oligo primer (dt) was to facilitate the Reverse Transcription (RT) process by binding to the RNA virus and initiating the synthesis of new cDNA. The results of this process were then used as input for the PCR that was used to detect and verify the dwarf virus in rice plants in South Sulawesi. The following were the steps of RT-PCR and the reaction volume used to detect the dwarf virus with a slight modification.

Table 1. RT-PCR program stages and reaction volume used

Reaction Composition	Volume (µl)	RT-PCR Program	Temperature (º C)	Time	Cycle
RNase free H_2O 5x RT buffer	3,5				
dNTP mixture	2				
oligo primer (dt)	1	Incubation	42	20’	1
RNase inhibitor	0,5	Incubation	99	5’	1
Rever TraAce	0,5	Incubation	4	∞	-
RNA	0,5				
	2				

Note: The RT-PCR kit used in the study was the KOD Pluz Neo Kit Toyobo (Toyobo Research Reagents)

PCR (*Polymerase Chain Reaction*): Detection of the dwarf virus in rice plants in South Sulawesi was carried out using the molecular method of PCR with a Bio-Rad T100 TM Thermal Cycler machine. After the RT-PCR process was completed, the cDNA was then used as the input for the PCR. In this process, DNA polymerase was used to amplify the target virus through DNA amplification. In this method, a specific primer for the dwarf virus was used to initiate the amplification process. The following were the steps of the PCR and the reaction volume used to detect the dwarf virus, with a slight modification

Table 2. PCR program stages and reaction volume used

Reaction Composition	Volume (µL)	PCR Program	Temperature (°C)	Time	Cycle
PCR mix	5	Predenaturation	95	2’	1
Reverse primer	0,5	Denaturation	95	30”	35
Forward primer	0,5	Annealing	52	30”	35
DH_2O	3	Ekstension	72	30”	35
cDNA	1	Final extension	72	7’	1
		Stop	4	∞	-

Note: The RT-PCR reaction was carried out using MyTaq HS Red Mix (Bioline) as the PCR Mix. The primer concentration used was 10 pmol, and the total volume of each reaction was 10 µL.

The PCR method using specific dwarf virus primers for rice plants was used to detect the presence of the dwarf virus in rice plants. In this method, the cDNA sample obtained from RT-PCR was processed, and then molecular detection was performed using a PCR machine. The principle of PCR is to amplify a specific DNA sequence

originating from the rice plant dwarf virus using specific primers. Primers are short DNA sequences that encode specific DNA sequences of the rice plant dwarf virus and are used as targets in the PCR reaction. The amplification process is performed by adding reagents such as Taq polymerase, dNTP, and buffer to the PCR reaction. After several amplification cycles, the result was analyzed using a Gel Analyzer to determine the molecular weight of the rice plant dwarf virus DNA. The following are the specific primers used for detecting the virus. kerdil.

Table 3. Primers used for molecular detection of the presence of rice dwarf virus.

Viruses	Gene	Sekuence Primer (5'–3')	Expected Size (bp)	References
RTSV	Polypro tein	F: 5'-AAACGGTCATTGTGGGGAGGT -3' R: 5'-CAGGCCCAGCAACGACATAA-3'	1115	Shen *et al.,*(1993)
RTBV	P4	B2-F : 5'-GCAGAACAGAACTCTAAGGC-3' B2-R: 5'-GTCTAAGGCTCATGCTGGAT-3'	430	Cabauatan *et al.,* (1999)
RGSV	NCP	*NCP-F1: 5'-GGCTTATGATAGTCTGTGATTTG-3' NCP-R: 5'-GTGTAAGATGGGTAAAGTGCA-3'	450	Nam *et al.,* (2007),
RRSV	S8	F3:5'-GACTAGGGATGTGCGTTC-3' B3:5'-TGTAATCGACGTTCGCTC-3'	218	Le *et al.,*(2010)
		CPF: 5'-ACCGTCGTTGAGCTACCATCCATT-3' CPR: 5'-GGCGGGCCACTCAAACCAT-3'	494	Chen *et al.,*(2010)
RBSDV	CP	F3: 5'-CCCCAGAGACTTTCCGATAC-3' B3: 5'-GGTCTTTAAGTTGCCTGATGT-3'	228	Liu & Li (2012)

* Plant Virology laboratory collection at the Faculty of Agriculture, Universitas Gadjah Mada.

Electrophoresis: The electrophoresis method is a technique used to separate and analyze DNA fragments based on their size. In the electrophoresis method, a 4 µLsample of DNA obtained from the PCR result and 5 µl of 100 bp DNA ladder were placed on a 0.30 gram agarose gel diluted with 30 mL of 1x TBE electrophoresis buffer. Then, an electric current of 50 volts was applied for 50 minutes and the DNA fragments moved based on their size. Smaller fragments moved faster compared to larger fragments. After the electrophoresis process was completed, the DNA fragments were viewed on the gel by adding staining agents such as ethidium bromide or SYBR green and visualized with an ultraviolet (UV) transilluminator. The DNA bands formed in the electrophoresis result were then photographed using a digital camera.

The Gel Analyzer: The Gel Analyzer 19.1 software was used to analyze the results of the electrophoresis photo. The software, designed to analyze agarose or polyacrylamide electrophoresis gels, was found to be capable of accurately measuring the size and concentration of DNA fragments obtained from the electrophoresis results (Okasha *et al.,* 2021; Moradi *et al.,* 2022). The process of using the software involved importing the jpeg image of the gel into the program, cropping the gel, and measuring the size and concentration of the DNA fragments. The software facilitated the analysis, enabling the determination of the molecular weight of the DNA. The data obtained was then visualized in the form of a calibration curve and a table of the total molecular weight of the DNA.

RESULTS AND DISCUSSION

Sample Collection: Observations of rice plants in several locations in South Sulawesi were carried out to investigate the presence of mixed dwarf virus infections. The observations revealed the presence of mixed dwarf virus infections in rice plants, with symptoms such as leaf edges changing color, appearing spots, shoot bending, and stunted and dwarfed growth. The level of damage to the plants varied, some plants experienced light damage while others experienced severe damage. Samples of rice plants found had symptoms similar to those of tungro virus and *Rice grassy stunt virus* (RGSV), characterized by yellowing leaves and stunted growth. According to Srilatha *et al.,* (2019), the characteristic symptoms of tungro disease are yellowing leaves and stunted growth. Meanwhile, the characteristic symptoms of RGSV are dwarfed growth, yellowing leaves, and a large number of tillers (Satoh *et al.,* 2013).

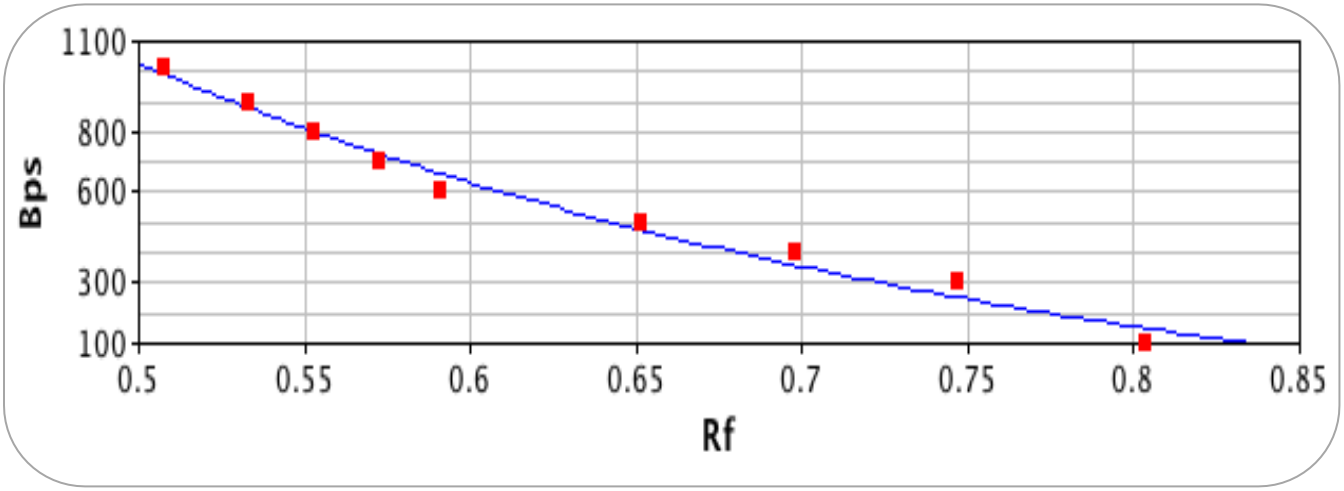
Figure 2. The symptoms of rice dwarf virus found at the sample collection location.

Molecular detections: Infection of 2 Types of Virus: The results of the study showed that mixed dwarf virus infections detected in rice plants in several locations were found to be high. Of the four samples taken, 50% were infected with the virus, of which half were infected by a combination of RGSV and RRSV in samples taken from the Sidrap and Wajo locations with DNA molecule sizes of around 284-794 bp using GelAnalizer 19.1 software. The other 50% were not detected for the presence of the virus, such as samples from the Maros and Pinrang locations. According to the research by Helina *et al.,* (2019), using similar specific primers (RGSV F1/R and RRSV F3/B3) with RT-PCR methods showed DNA bands of approximately 210-450 bp. This shows that the size of RGSV and RRSV viruses varies between species and types, even when using the same primer pair as previous research (Helina *et al.,* 2019). Some factors that can affect the base pair size of PCR results even when using the same primer include: variations in the quality and quantity of the DNA template (Wutke *et al.,* 2019), the presence of mutations and genetic variations in the DNA template (Silva *et al.,* 2017), the conditions of the PCR reaction such as optimal annealing temperature, extension duration, contaminant quality and concentration of primers, Taq polymerase, and other substances in the PCR reaction (Lorenz, 2012). All of these factors can affect the efficiency of amplification and the final PCR result, including the base pair size of the amplification product.

Figure 3. Detection of RGSV and RRSV using RGSV-NCPF1*/NCPR and RRSV-F3/B3 primers, 100 bp DNA Ladder marker.

Tabel 4. Detection of 2 types of rice viruses using RT-PCR and GelAnalizer 19.1 method.

Region	Code	RT-PCR		Molecular Weight (bp)	
		RGSV	RRSV	RGSV	RRSV
Maros	MRS	-	-	-	-
Sidrap	SDR	+	+	284	760
Wajo	WJO	+	+	318	794
Pinrang	PRG	-	-	-	
	M (Marker)			100 - 1013 bp	



y = 7543.457267 *exp (-3.441666 * x) - 322.936762 R^2 = 0.982

Figure 4. Calibration curve of Gel Analizer 19.1 software on the 4 detected samples.

The calibration curve graph generated by the Gel Analyzer software shows the relationship between the size of the electrophoresis product and the size of the standard used as a reference (Moradi *et al.,* 2022; Fernandes *et al.,* 2020). On the calibration curve graph, there are two lines: the y=x line (diagonal line) which showed the relationship between the size of the product and the size of the standard, and the regression line (straight line) which showed the relationship between the size of the product and the size of the standard used as a reference (Schneider *et al.,* 2010). A good calibration curve must have a high correlation coefficient (R2) and a regression line that approaches the y=x line (Stone *et al.,* 2003; Asuero *et al.,* 2006). This indicated that the size of the electrophoresis product could be well calibrated with the size of the standard used.

In this study, a 100 bp size marker was used, consisting of several DNA molecules with different sizes. The electrophoresis gel image used shows the result of the PCR carried out on several DNA samples. The Gel Analyzer calibration curve results given depict the relationship between an input (x) and an output (y). The mathematical equation y = 7543.457267 *exp (-3.441666 * x) - 322.936762 is the mathematical form of the calibration curve. The R^2 value (0.982) indicated the quality of the calibration curve, with a value closer to 1 indicating a better calibration curve (Angeli *et al.,* 2022).

Infection of 5 Types of Virus: The results of the PCR and Gel Analizer analysis showed that all six rice samples were infected with at least one of the five viruses (RTBV, RTSV, RGSV, RRSV, and RBSDV), except for the Carawali sample, which was only infected with RTBV and RRSV. The DNA molecular weight in each sample varied from 195-1764 bp. (2013) reported the amplification of the RBSDV virus using specific primers, and they Cho *et al.*(2013) found that the RBSDV virus amplification product had a size of 485-500 bp. The base pair size of the tungro virus varied depending on the primer used, ranging from 721-8110 bp (Sharma and Dasgupta, 2012), while the RGSV and RRSV had a size of 574-834 bp (Uehara-Ichiki *et al.,* 2013). Meanwhile, factors that influence the size of target DNA include: Size and complexity of the target genome (Green and Sambrook, 2019), quality and quantity of target DNA template (Faller *et al.,* 2019; Wijaya *et al.,* 2019), speed and efficiency of the target DNA cutting or amplification process (Tong *et al.,* 2011), and presence of inhibitors or contaminants during the target DNA cutting or amplification process (Borst *et al.,* 2004).

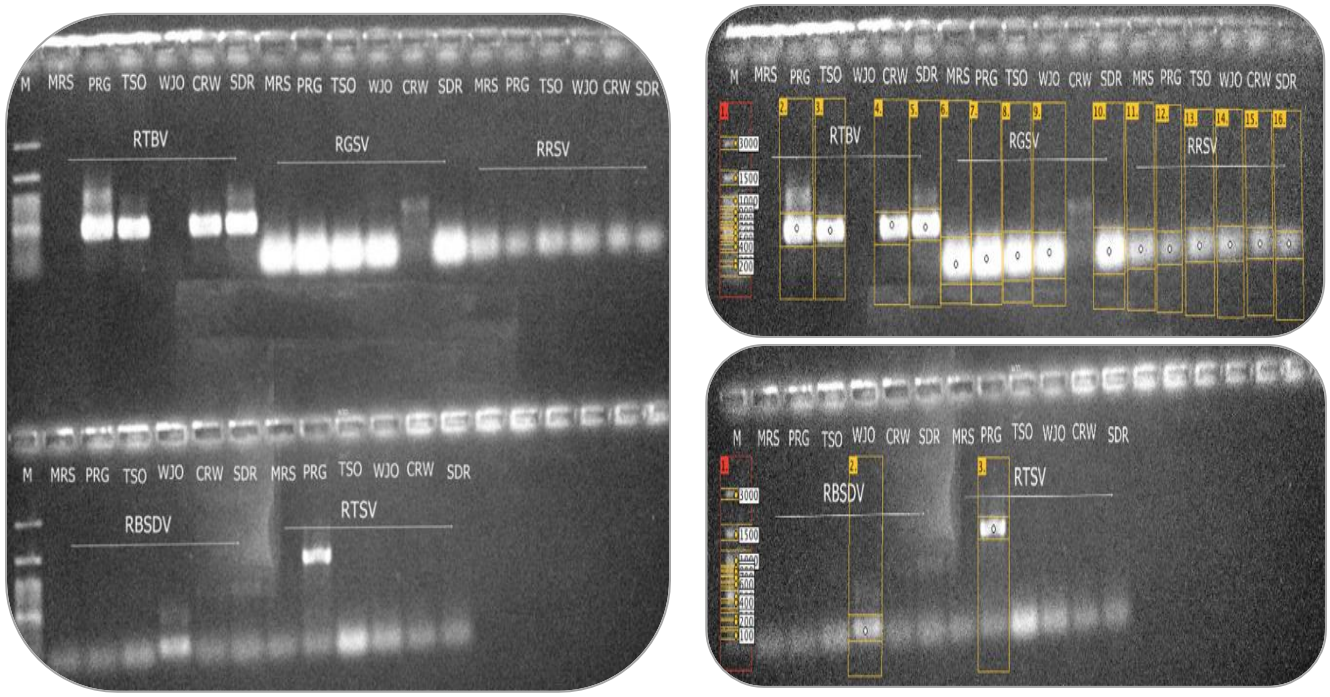
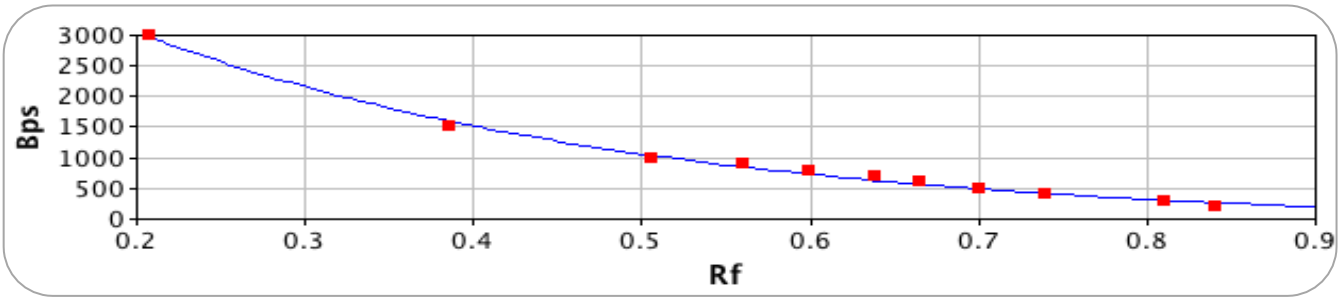


Figure 5. The result of PCR using the RRSV-CPF/CPR, RTBV, RTSV, RGSV, and RBSDV primers and a 100 bp DNA Ladder marker.

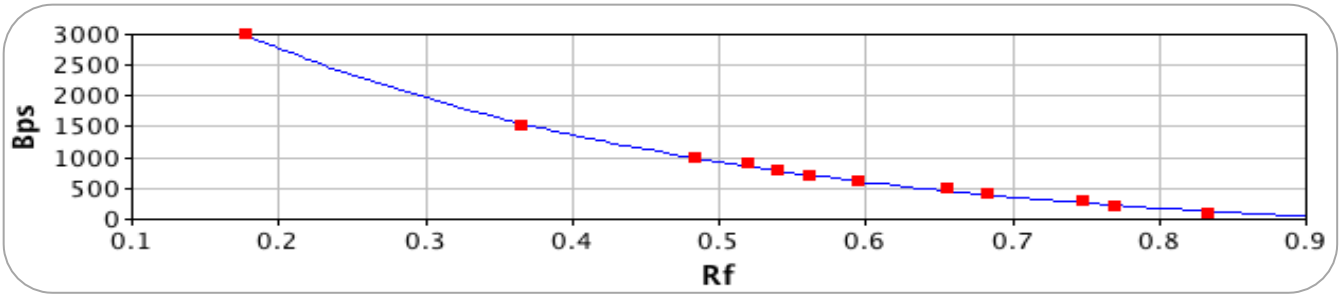
Table 5. The result of detecting 5 types of rice viruses using RT-PCR and Gel Analizer 19.1.

Region	Code	PCR	RT-PCR				Molecular Weight (bp)				
		RTBV	RGSV	RRSV	RBSDV	RTSV	RTBV	RGSV	RRSV	RBSDV	RTSV
Maros	MRS	-	+	+	-	-	-	315	489	-	-
Pinrang	PRG	+	+	+	-	+	611	367	510	-	1764
Tanah Sitolo	TSO	+	+	+	-	-	588	378	583	-	-
Wajo	WJO	-	+	+	+	-	-	430	605	195	-
Carawali	CRW	+	-	+	-	-	703	-	612	-	-
Sidrap	SDR	+	+	+	-	-	701	459	641	-	-
	M (Marker)						100 - 3000 bp				

The calibration produced by the Gel Analyzer software is shown in the figure below. In this example, a size marker consisting of DNA molecules of different sizes was used. The gel electrophoresis image shows the results of the PCR performed on several DNA samples. The calibration curve displays a y=x line (diagonal line) that illustrates the relationship between the product size and the standard size. The regression line (straight line) indicated by the calibration curve depicts the relationship between the product size and the standard size used as a reference. In this study, a high correlation coefficient (R^2) indicates that the size of the electrophoresis product can be accurately calibrated with the standard size used (Angeli *et al.,* 2022). This figure can also be utilized to assess the quality of PCR, for instance to verify if the PCR product produced has a size that matches the expected size.



y= 6120.319671 *exp (-3.303961 * x) - 110.573196 R^2= 0.996



y= 5666.567979 * exp (-3.000460 * x) - 331.831692 R^2= 0.999

Figure 6. Result of the calibration curve of the Gel Analizer 19.1 software on 6 detected samples.

The Gel Analyzer 19.1 calibration curve depicted shows the relationship between an input (x) and output (y) that was obtained in the study. The mathematical equation y = 6120.319671 * exp(-3.303961 * x) - 110.573196 represents the mathematical form of the calibration curve that was obtained. The R2 value (0.996) indicates the quality level of the calibration curve and a value closer to 1 indicates a better calibration curve. In this study, a high R2 value of 0.996 was found, indicating that the calibration curve had a good level of explanation, with about 99.6% of the variation in the output being able to be explained by the variation in the input. The calibration line between the equation y= 5666.567979 * exp(-3.000460 * x) - 331.831692, obtained in the study, showed that Y had a negative correlation with X and can be considered a good model of the relationship, as seen from the R2 value of 0.999 that was found, which indicates that 99.9% of Y's variation could be explained by X's variation.

CONCLUSION

There was a mixed virus infection in rice plants in South Sulawesi. The results of this research showed that the rice plant samples had a mixed virus infection, with viruses such as *Rice tungro spherical virus* (RTSV), *Rice tungro bacilliform virus* (RTBV), *Rice grassy stunt virus* (RGSV), *Rice ragged stunt virus* (RRSV), and *Rice black streaked dwarf virus* (RBSDV). The detected DNA molecular weight varied among samples, ranging from 195-1764 bp. These results emphasize that the mixed virus infection in rice plants in South Sulawesi is an issue that needs to be taken into consideration by farmers and researchers. Determining the type of virus and the level of infection is crucial for determining an effective control strategy and minimizing rice production losses. Preventive measures such as maintaining the cleanliness of the planting location, destroying infected plants, and planting disease-resistant varieties are necessary.

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