AGRIVITA Journal of Agricultural Science. 2017. 39(2): 145-152

The Use of Electrophoretic Isozymes to Detect Tungro Infected Rice

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Received: August 28, 2015 /Accepted: March 30, 2016

ABSTRACT

Tungro is one of the most common diseases of rice plants which caused by double infection of RTBV (Rice Tungro Bacilliform Virus) and RTSV (Rice Tungro Spherical Virus), and it gives a significant economic loss. These viruses are transmitted by green leaf hopper (*Nephottetix virescens.* Distant), and the interaction between host plant and the viruses were still quite difficult to be fully understood. In order to look at whether there are any differences between the healthy and tungro infected rice, this study was set to examine the character differences between the infected and the healthy plants based on isozyme banding pattern. The infected plants were collected from three districts around Surakarta (Sragen, Sukoharjo, Klaten) followed by Polyacrilamide Gel Electrophoresis (PAGE) to evaluate the isozyme banding patterns. There were peroxidase, esterase and acid phosphotase isozymes used. The results showed that the real differences of isozyme banding patterns of both healthy and tungro infected plants were discovered. In all cases, the Tungro infected rice had thicker and more band numbers compared to the healthy one. This evident suggested that Tungro infected rice could be detected early using PAGE method.

Keywords: electrophoretic; isozymes; tungro; infected rice

INTRODUCTION

The use of electrophoretic approach especially plant isozymes have been widely conducted (Cahyarini, Yunus, & Purwanto, 2004; Medhabati, Nongalleima, Rajiv, & Sunitibala, 2013; Pushpa et al., 2014). This method was used not only to study plant species in general but also specifically dealing with staple food, such as rice. This food (rice) is one of the most crucial or rather vital for human consumption and mostly planted in the Asian tropical countries. However, the successful growth of rice plants has sometimes been disturbed by Tungro diseases. This disease was recorded as one of the most important problems in the agricultural activities, due to the impact of their damaging attack which could cause significant economic loss. This Tungro disease is caused by double infection of RTBV (Rice Tungro Bacilliform Virus) and RTSV (Rice Tungro Sphiracle Virus) which produce mosaic or necrosis symptoms. Further severe symptom could result stunting on the rice plants, and the reduction of internode and total number of plantlets could eventually result into criticism (Hibino, Saleh, & Roechan, 1979). Sama, Hasanuddin, Manwan, Cabunagan, & Hibino (1991) recorded that the infection of Tungro diseases could disturb the seed filling process and therefore, potentially could drop the total rice production drastically.

These two viruses had different capability in infecting the rice host plants. RTSV, for instance, can infect the rice independently and produce yellow to orange symptoms on the leaves. On the other hand, the RTBV does not have any capability to infect the rice plants without the existence of RTSV. The presence of RTSV is very useful in helping RTBV to attack the host rice plants. Accordingly, the presence of RTBV particles in the RTSV infection process would in some cases determine the severity produced symptoms (Hibino, Saleh, & Roechan, 1979). Actually, the two viruses are not serogically the same. The RTSV had ssRNA with 12 Kb in length and included in the *Leguviridae* family. Conversely, the RTBV was noted as ssDNA genome with approximately 8 Kb in length within the family of Caulimoviridae (van Regenmortel et al., 2000). These two different viruses were commonly known as Tungro diseases, and they attacked a number of rice varieties at the tropical countries which caused a significant economic loss.

Cite this as: Suranto, Arief, A., & Supyani. (2017). The use of electrophoretic isozymes to detect tungro infected rice. *AGRIVITA Journal of Agricultural Science, 39*(2), 145–152. http://doi.org/10.17503/agrivita.v39i2.643
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In 1999 the Tungro diseases attacked a number of rice fields around Surakarta (Central-Java) residencies causing major problems in the national rice stock production. Statistically, the research data showed that it was 90% of rice plants at that area which did not produce optimal yield (Center for monitoring pests and plant diseases of Surakarta, 2013). This was caused by Tungro attack and its impact remains until now, although the frequency has not been seen or recorded as often as before. Many efforts had been conducted to prevent Tungro diseases explosion; however, the results have not succeeded or yet. One of the most promising approaches to prevent the failure of harvesting is using the early detection system. The capability of detecting Tungro as early as possible during seedling period will be very useful in preventing further failure of farmers in the agricultural activities. This study, therefore tried to find out whether the use of electrophoretic isozymes would be useful in detecting the Tungro diseases in rice plants by comparing the banding patterns of isozymes of peroxidase, esterase and acid phosphatase, on both healthy and infected rice.

MATERIALS AND METHODS

The research activity from collecting sample in the field,followed by propagation of the plants infected virus in our glass house and electrophoretic isozymes conducted from june until September, 2014.

Collection of Plants Samples

Tungro infected rice leaves of *Oryza sativa L.* were collected from three different regencies around Surakarta, namely Sragen, Sukoharjo and Klaten. The infected plant samples were picked up directly from the natural fields (habitats) and then put in the pot containing compost soil. Each pot consists of three to five individual plants, and in every district 25 infected rice plants were picked and grown (planted) within 5 pots. These plant samples were kept at the glass house in order to make the plants available for electrophoretic procedures, whenever they were needed.

Confirmation of Tungro Infecting Rice

In order to make sure that all plant samples in this study were infected by Tungro disease, a field observation on the target of plant symptom was conducted, followed by evident of discovering vector (*N. virescens*) in the surrounding environment of infected rice. And to confirm this, iodine test was done to both infected samples and healthy plant as control.

Sample Extraction Procedures

The methods used for extracting the leaf samples and electrophoretic isozymes was according to Suranto (2001) which was slightly modified especially the treatment of samples used. About 350 milligrams of infected rice leaf samples were picked up from the third leaves of the top plant during anthesis. The lamina leaves were cut into very small sizes (ca. two mm in length) using scissors and grounded on the mortar using 500 -1000 µl buffer extraction, containing 0.018 grams cysteine, 0.021 grams of ascorbic acid and five-grams of sucrose which were diluted on 20 ml of tank buffer. The samples were then transferred into Eppendorf tubes and centrifuged at 3500 rpm for six minutes at room temperatures (28^{o}C). The resulted supernatant was then used for electrophoretic purposes. In this experiment, the researchers did not use liquid Nitrogen in crushing the sample instead of cutting the leaf samples smaller.

Electrophoresis

Mini Protean Tetra Cell of Vertical electrophoretic type, serial number 552BRO60043 produced by BIORAD USA was used in this experiment. This electrophoresis was run at the laboratory of molecular biology, in the Department of Biology-Faculty of Natural Sciences and Mathematics of Sebelas Maret University, Surakarta, Central Java - Indonesia. The total volume of samples loaded in each slot of resulted supernatants for peroxidase, esterase and acid phosphatase were 5 µL, 10 µL and 15 µL respectively, using constant voltage (100 volts) for one hour or until the bromophenol blue reached 5 millimeters above the bottom line of running gel. Each running sample was then stained separately for peroxidase, esterase and acid phosphatase.

Procedures of Gel Staining

The procedures of Peroxidase and Esterase staining were conducted according to Mills & Crowden (1968), while for Acid Phosphatase stain, the researchers used the method of Adam and Jolly (1980).

Data Analysis

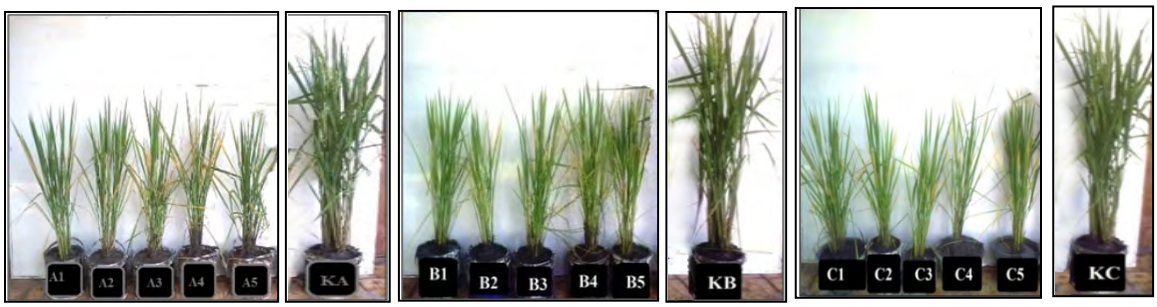
The emerging isozyme band pattern was then analyzed qualitatively by observing the presence and the absence of the band patterns, while the migration of the band was analyzed quantitatively using the Rf value as Ro used in 1994.

RESULTS AND DISCUSSION

As shown in Fig.1, the comparison between Tungro infected rice and plant control were clearly different. Beside the reduction of the high plant, the total number of plantlets also became apparent. In all cases, the Tungro infected rice was shorter than the healthy ones. Accordingly, the yellow mosaic symptom on the infected leaves was always seen in all single plants. These individual plants were than used to set up electrophoretic isozymes. The resulting isozymes of esterase, peroxidase and acid phosphatase were presented in Fig. 2.

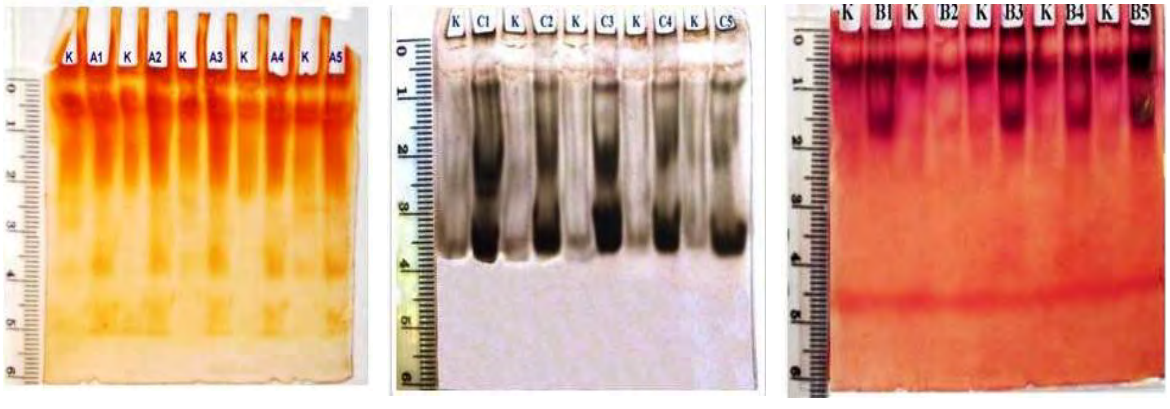
Peroxidase Isozymes

The resulted zymogram of peroxidase isozymes between the healthy and Tungro infected plants were presented in Fig. 3. Both of the healthy and infected rice Plants had the same number of Bands. The only significant difference between them was the thickness. The bands of the Tungro infected viruses always appeared thicker, and they were characterized by bands of Rf 0.092, 0.296, 0.537 and 0.719. The unique band at Rf 0.421 was detected particularly only from Tungro infected rice of Sukoharjo district. While the other two district of Klaten and Sragen were absent. This profile of peroxidase isozymes particularly the number and the pattern were of great interested to be studied. Clear difference between the two samples tested indicated that the infected rice showed thicker bands on certain positions compared to the healthy one. Although the main cause of thicker bands was not fully understood, the additional virus protein could have possibly been answered.



A B C

Fig. 1. Comparation between tungro infected rice (A, B, C) and the control (KA, KB, KC) plants collected from three districts and grown in our glass house to make available for Electrophoretic samples whenever used (A) Sragen; (B) Sukoharjo and (C) Klaten



A **B** **C**

Fig. 2. Examples of enzyme activities of (A) Peroxidase (B) Esterase and (C) Acid Phosphatase

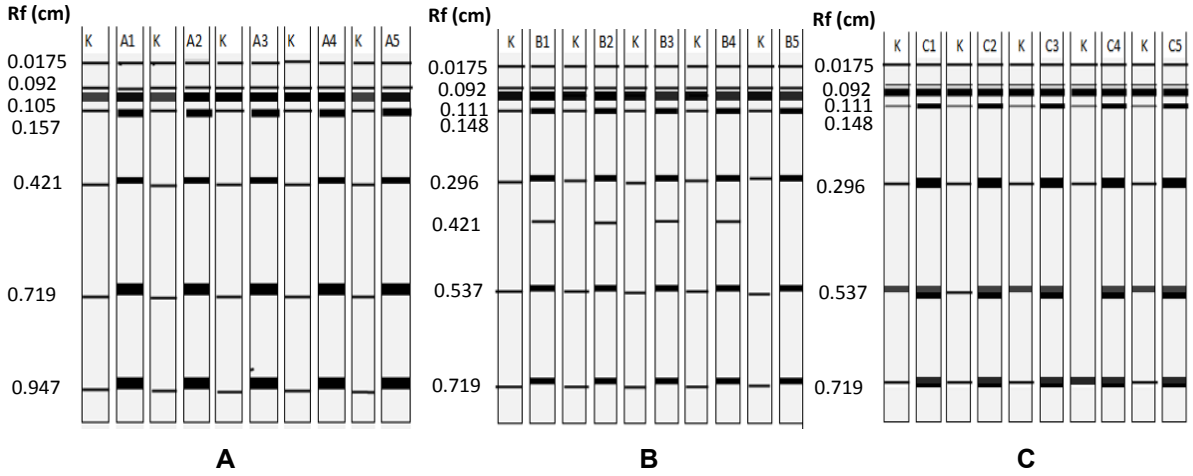


Fig. 3. Zymogram of peroxidase isozymes of tungro infected rice (A1; A2; A3; A4; A5; B1; B2;B3; B4; B5; C1; C2; C3; C4; C5) and (K) the control plants. Samples collected from districts: (A) Sragen; (B) Sukoharjo and (C) Klaten

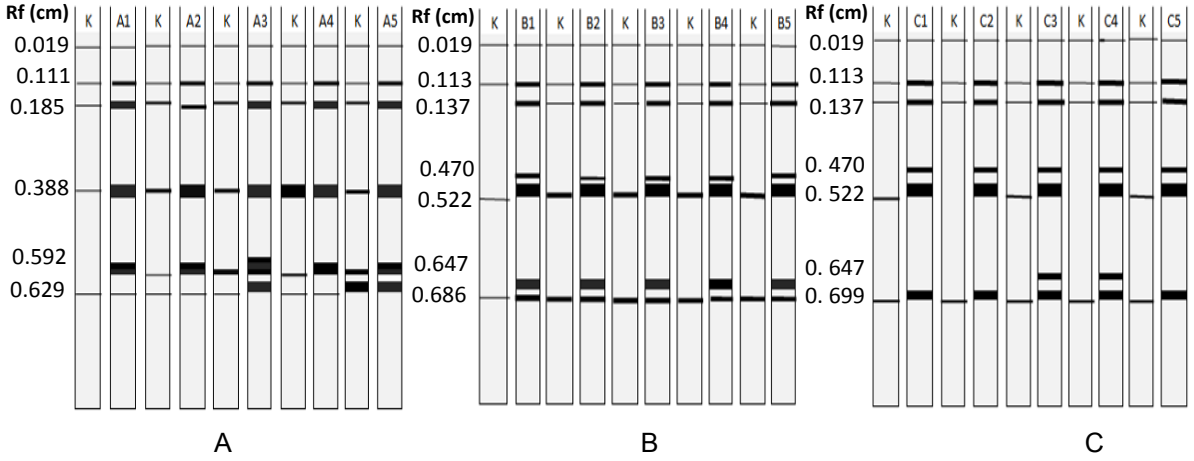


Fig. 4. Zymogram of eterase isozymes of tungro infected rice (A1; A2; A3; A4; A5; B1; B2;B3; B4; B5; C1; C2; C3; C4; C5) and (K) the control plants (K). Samples collected from districs: (A) Sragen; (B) Sukoharjo and (C) Klaten

Esterase Isozymes

Although the total number of esterase isozymes bands were not the same as peroxidase isozymes, this phenomenon of thicker bands on the Tungro infected plants were also exhibited by esterase activities within all the three districts. All samples tested were collected from these three different locations but at those areas they showed similar band patterns. Typical bands at the Rf values 0f 0.522 and 0.699 (see Fig. 4), they were detected at all sample locations. Meanwhile, the additional two bands at the Rf 0.470 and 0.647 were present only in Sukoharjo and Klaten districts respectively, and none for Sragen district. Total bands produced by the esterase activity was six or seven. This number of bands had wide variations, since only four bands were detected in certain varieties of good local rice – Rojolele (Widiyanti, Suranto, & Sugiyarto, 2008).

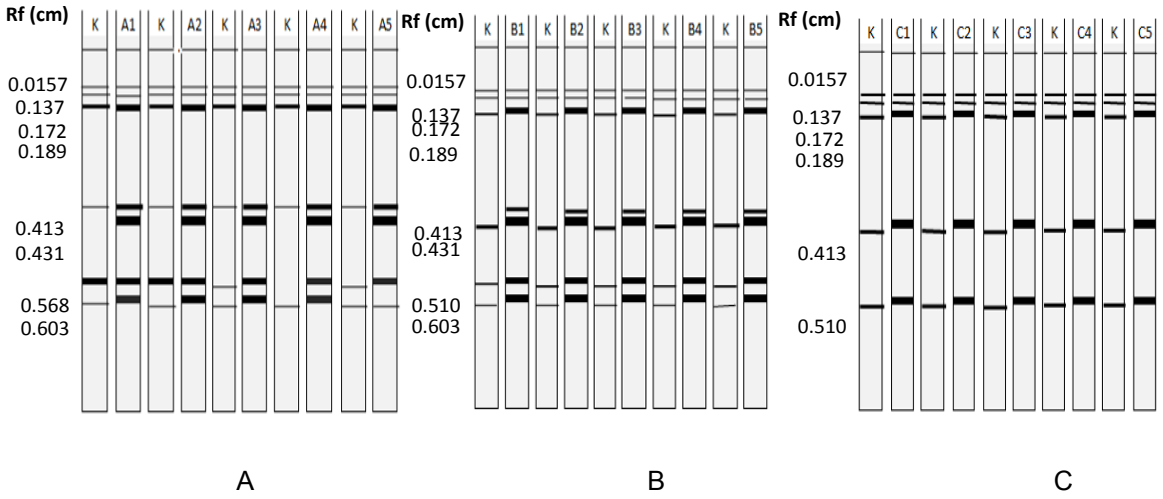


Fig. 5. Zymogram of acid phosphatase isozymes of tungro infected rice (A1; A2; A3; A4; A5; B1; B2;B3; B4; B5; C1; C2; C3; C4; C5), (K) the control plants. Samples collected from districs: (A) Sragen; (B) Sukoharjo and (C) Klaten

As seen in Fig. 5, the band patterns of Acid Phosphatase isozymes were clearly detected. In general, the color of acid phosphotase was quite close to peroxidase, but in reality the color would have been darker. It was interesting to note that, this enzyme was very slow in its activities. It only needed 1 – 3 minutes in order for the peroxidase bands to be detected. Conversely, the Acid Phosphatase needed more than six hours for the bands to appear. And sometimes the researchers needed to incubate overnight to observe the banding patterns.

Based on the first two enzymes before, this Acid Phosphatase was recorded to have similar behavior especially the thickness of bands, all samples tested from Tungro infected rice had thicker bands compared to the healthy ones. Three bands at the Rf values of 0.189, 0.430 and 0.560, were typical of peroxidase isozymes of the Tungro infected rice. Besides, the total number of six to eight, were the most common bands detected in Tungro infected rice of this enzyme activity. Meanwhile, the total band number of five was recorded for the rice sample which was not infected by plant viruses (Siva, Kumar, & Rajasekaran, 2013). Using other plant samples, Suranto (2001; 2002) also recorded a total of five bands in herbaceous plant of *Ranunculus amphitricus*.

The use of electrophoretic approaches especially plant isozymes for taxonomic purposes have been widely conducted (Degani & El-batsri, 1990; Degani, Beiles, El-Batsri, Goren, & Gazit, 1995). However, the application of this approach in clarifying the Tungro diseases was used for the first time. Similar work in confirming the isozyme banding patterns in cultivars of rice were done at Manipurj – North Eastern State of India as shown by Medhabati, Nongalleima, Rajiv, & Sunitibala (2013). They worked with esterase, alcohol dehydrogenase and glutamate dehydrogenase. Meanwhile Pushpa et al. (2014) using alcohol dehydrogenase (ADH) in distinguishing varieties, hybrid and its parental line of Indian rice.

Although the works conducted by Johnson, Janakiraman, & Irudayaraj (2012) were not really the same as what we have done, the results of clarifying the difference between the resistant and susceptible cultivars on Sugarcane were quite similar with this trial, in order to distinguish the infected rice by Tungro to the healthy one. They also looked for the difference in the profile of isozymes between different samples and this could be used to identify both varieties of sugarcane. The work at this preliminary results showed that using the electrophoretic isozymes would not only be useful in detecting the plants infecting Tungro, but also can be used to distinguished the varieties of rice resistant cultivars to certain diseases and the susceptible one. Similar work conducted by Sulistyarsi, Suranto, & Supriyadi (2012) using plant protein of rice. It showed that plants infected by Tungro had district bands compared to the uninfected samples.

In this point, it would be possible to consider that isozyme marker has been proven as reliable phylogenetical markers in plant breeding and crop improvement projects (Johnson, Janakiraman, & Irudayaraj, 2012). Furthermore, they argued, that at least isozyme forms of esterase and peroxidase have been the primary gene product and therefore can be used to deduce gene homology with high precision by comparing variation in their expression patterns in different varieties of sugarcane both of stress tolerance and disease resistance. There would be no doubt that the use of biochemical marker such as isozyme data in establishing distinctiveness of plant varieties due to the complexity of morphology or even clarifying symptoms of plants infecting virus would be very helpful (Patra & Chawla, 2010; Nurmiyati, Sugiyarto, & Sajidan, 2009)

The presence and the absence of bands in certain enzyme activities detected from certain plant organs may be related to the growth and development of the plants. Total number of bands detected from leaf samples of the seedling period can be differed from samples taken during anthesis. This phenomenon confirms that, for every single period of time, certain plants organs could express specific band patterns. This occurrence could be true because certain gene can only control certain plant organ within specific period of time. Thus the plants infected by certain viruses may express the metabolic activities differently compared to the normal plant. These differences could be detected with their enzyme activity within a particular enzyme staining. The three enzyme staining in this electrophoretic trial efforts have produced a very promising results of their isozymes banding patterns, indicating a clear cut different between the healthy and infected samples. This electrophoretic method particularly the peroxidase isozymes showed very thick bands especially at the last two from the based line of loading dye. Meanwhile, the other two enzyme activities produced similar pictures but, not as good as the peroxidase isozymes. It was interesting to note that thinner bands were sometimes still occurred at esterase and acid phosphatase activities, although in small percentage. This preliminary evident will be useful since the electrophoretic isozymes can employ many samples in a very small amount of time, and therefore it was not time consuming as well as cheap and easy to run.

CONCLUSION

The using of electrophoretic isozymes in order to detect Tungro infecting rice was quite promising to be employed. Although not all of the three enzyme systems gave the best results as expected, peroxidase activities at least showed a good quality of isozyme banding patterns indicating clear difference between the healthy and infected rice plants. This electrophoretic isozymes approach could be considered as one of the very simple and easy method in detecting plant infecting virus. Thus, in the near future this electrophoretic method could hopefully be used not only in detecting virus infected plants but also for other plant diseases

ACKNOWLEDGEMENTS

This research was partly of Suranto's Professorship Project and conducted with the financial support of State University operation Budget of Sebelas Maret University, Under Contract Number 165a/UN27.11/PN/2013. Therefore, we would like to thank the Rector of UNS for the financial help. We also thank to Mrs. Atik and Nita for their technical assistance during electrophoretic experiment in the Department of Biological Science, Faculty of Natural Sciences and Mathematics UNS. I do appreciate my student (Arief A) for his hard working in compliting my research project successfully. In addition, we thank to Mr. Douglas Obura for the English correction.

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