In vitro and in planta efficacy studies on T₆ generation of transgenic Rojolele rice lines against the rice yellow stem borer (Scirpophaga incertulas Walker)

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Abstract. One of the most destructive pests in cultivated rice (Oryza sativa L.) is the rice yellow stem borer (YSB; Scirpophaga incertulas Walker). YSB attacks rice plant throughout its life span, with the most devastating being during mature stage that causes a symptom called whiteheads. Every 1% of whiteheads symptoms resulted in 1% rice production losses. Planting YSB-resistant cultivars is a good strategy besides environmentally friendly. However, since the unavailability of YSB-resistant gene in rice germplasm, genetic engineering of rice to express Cry toxin from Bacillus thuringiensis (Bt) that is toxic to YSB is an alternative. Agrobacterium-mediated transformation has been conducted to introduce cry1B gene from Bt into a local javanica rice cultivar, Rojolele. The recombinant plasmid contains cry1B gene driven by wound-inducible promoter from maize proteinase inhibitor (mpI) gene and hygromycin phosphotransferase (hpt) selectable marker gene for transgenic plants selection. The presence of cry1B gene in transgenic rice plants was identified by PCR analysis. Four homozygous transgenic rice lines harboring cry1B gene have been obtained. The purpose of this experiment was to determine the efficacy of cry1B gene in homozygous transgenic rice lines against YSB at vegetative stage, in vitro and in planta at T₆ generation. In vitro assay was performed in aerated Petri dishes using leaf samples under growth room condition, meanwhile in planta assay was done by subjecting the four homozygous transgenic rice lines to YSB under greenhouse condition. The results showed that the four homozygous transgenic rice lines tested were highly resistant to YSB compared to the wild-type Rojolele rice.

1. Introduction
Rice (Oryza sativa L.) is one of the most important cereal crops in the world and become a staple food for people in Asia including Indonesia. However, rice production is always facing many problems that come from biotic and abiotic stresses. Yellow stem borer (YSB; Scirpophaga incertulas Walker) is one of the biotic stresses that contributes to the decline in rice production (Baehaki et al., 2013; Renuka et al., 2017). YSB attacks rice plant from seedling to maturity stage, and resulting in different symptoms (Ramesh et al., 2004). The deadhearts and whiteheads symptoms are produced when insects attack rice plant at vegetative and reproductive stages, respectively, with the most devastating being during mature stage (Cohen et al., 2008; Deka et al., 2010). The application of pesticides to control YSB is less effective since the larvae live inside the stem, thus inhibit pesticides to reach the larvae. Moreover, the excessive and indiscriminate use of pesticides resulted in severe adverse effect on environmental and human health (Ho et al., 2006; Chatterjee and Mondal., 2014; Tara et al., 2018). Thus, an alternative method to control YSB problems by minimizing the use of pesticides, should be necessary to be considered.
Planting YSB-resistant cultivars in the field is the best strategy not only to prevent yield loss but also to reduce pesticides application. Nevertheless, no resistant donor has been identified among rice germplasm collection, makes the option to use the conventional breeding method is difficult (Wu et al., 1997; Kumar et al., 2010).

*Bacillus thuringiensis (Bt)* is a gram-positive bacterium produces insecticidal crystal proteins, Cry proteins that are highly toxic to YSB (Kiani et al., 2009; Jisha et al., 2013). Genetic engineering technology is a method that can be used to overcome the barrier of sexual incompatibilities (Ramesh et al., 2004; Liu et al., 2016). By using genetic engineering technology, transformation of *cry1B* gene from *Bt* into a local javanica rice cultivar, Rojolele has been conducted in Genomics and Crop Improvement Laboratory-LIPI. The recombinant plasmid contains *cry1B* gene driven by wound-inducible promoter from maize proteinase inhibitor (mpi) gene and *hygromycin phosphotransferase (hpt)* selectable marker gene for transgenic plants selection was obtained from Dr. Emmanuel Guiderdoni, CIRAD, France. The *Agrobacterium*-mediated transformation method was carried out following method described by Hiei and Komari (2006) and homozygous transgenic rice lines have been obtained (Estiati et al., 2007; Estiati et al., 2012; Estiati et al., 2013). Among them, four homozygous transgenic rice lines were chosen for further research. Since stable inheritance and expression of *cry1B* gene in transgenic rice lines are important in determining the success of developing YSB-resistant cultivar, thus, the inheritance and expression of *cry1B* gene in successive generation have to be carried out. Studies on four homozygous transgenic rice lines at T6 generation demonstrated that the *cry1B* gene was stably inherited and the expression of *cry1B* gene confer resistance to plants against YSB compared to the wild-type Rojolele rice under *in vitro* and *in planta* conditions.

2. Materials and Methods

2.1. Plant materials

Four homozygous transgenic Rojolele rice lines at T6 generation (3R7.8.15.1, 3R25.7.27.1, 3R5.26.2.1 and 3R5.26.5.1) and control of susceptible cultivars to YSB (non-transgenic Rojolele and IR64) were used in this study.

2.2. The presence of cry1B gene and validation of homozygous transgenic Rojolele rice lines

The presence of *cry1B* gene in transgenic rice lines was tested at T6 generation (3R7.8.15.1, 3R25.7.27.1, 3R5.26.2.1 and 3R5.26.5.1) by PCR analysis. Fifteen individual plants from each transgenic rice line were subjected to PCR. The pCAMBIA1301 binary vector harboring *cry1B*, was used as a positive control. DNA was extracted from fresh leaf of transgenic rice plants following method described by Dellaporta et al (1993). PCR analysis to amplify *cry1B* fragment was performed using *cry1B* specific primers with the sequence as follow: *cry1B* forward: 5'-CCAGTTGACCGAGAGGTTTA-3' and *cry1B* reverse: 5'-CTGGGTTGATGGAGGTGTTAG-3'. The expected size of PCR product for *cry1B* fragment was 0.3 Kb.

Amplification of *cry1B* fragment were conducted by adding 1 µl of 100 ng DNA template into PCR mix reaction containing nuclease-free water, DreamTaq™ Green PCR Master Mix (2x) (Thermo Scientific), and 0.48 µM for each primer. PCR was conducted using Biometra Thermocycler (AnalytikJena) with cycle conditions as follow: 3 min pre-denaturation at 95°C, 1 min denaturation at 95°C, 1 min annealing at 60°C, 1 min extension at 72°C for 35 cycles, 7 min extension at 72°C. The *cry1B* amplicon were detected by loading the PCR’s product on a 1.2% agarose gel and visualized under UV light upon Ethidium Bromide staining.

2.3. *In vitro* bioassay of homozygous transgenic Rojolele rice lines against yellow stem borer in the laboratory

Five leaf sections were taken from each of four transgenic rice lines at T6 generation (3R7.8.15.1, 3R25.7.27.1, 3R5.26.2.1 and 3R5.26.5.1) and non-transgenic Rojolele as a control of susceptible cultivar. The leaf sections were placed on two sheets of filter paper in an aerated Petri dish under
growth room condition. Each leaf section was infested with ten first-instar larvae of YSB. To maintain moisture, the filter paper in Petri dish was moistened with sterilized distilled water. The observation on leaf damage and the number of live and dead larvae was conducted at 3 days after infestation.

2.4. In planta bioassay of homozygous transgenic Rojolele rice lines against yellow stem borer in the greenhouse

Bioassay was carried out under greenhouse condition. Five plants from each of four transgenic rice lines at T6 generation (3R7.8.15.1, 3R25.7.27.1, 3R5.26.2.1 and 3R5.26.5.1) and control of susceptible cultivars (non-transgenic Rojolele and IR64), were infested with ten first-instar larvae of YSB per tiller at 42 days after sowing. The resistance against YSB is evaluated based on the percentage of deadhearts. The incidence of deadhearts were recorded during the vegetative stage at 2 and 4 weeks after infestation (WAI) and the percentage of deadhearts were calculated as follow:

\[
\text{percentage of deadhearts} = \frac{\text{number of deadhearts counted}}{\text{total number of tillers observed}} \times 100\%
\]

Further, the percentage of deadhearts were converted to D value with the formula as below:

\[
D = \frac{\text{percentage of deadhearts in test entry}}{\text{percentage of deadhearts in susceptible check}} \times 100\%
\]

(Heinrichs et al., 1985).

And, transform the D value to a 0-9 scale, following Standard Evaluation System for rice: 0= no injury; 1=1-10%; 3=11-20%; 5=21-30%; 7=31-60% and 9= 61% and above (IRRI, 2013). Hereinafter, the rating scale of 0-9 was used to determine the category of plant resistance (Table 1) (Devasena et al., 2018).

### Table 1. Category of plant resistance based on the D value

| Damage (%) | Scale | Category of resistance            |
|------------|-------|-----------------------------------|
| 0          | 0     | Highly Resistant                  |
| 1-10       | 1     | Resistant                         |
| 11-20      | 3     | Moderately Resistant              |
| 21-30      | 5     | Moderately Susceptible            |
| 31-60      | 7     | Susceptible                       |
| 61% and above | 9     | Highly Susceptible                |

3. Result and Discussion

3.1. The presence of cry1B gene and validation of homozygous transgenic Rojolele rice lines

Homozygous transgenic rice lines was determined by the presence of cry1B gene in individual plant of a population. Eleven is the minimum number of plants to show at least one unexpected phenotype appeared under 95% probability for character controlled by a recessive allele. If one phenotype from unexpected recessive allele was revealed from 11 plants tested, it is indicated that the plant population was originated from heterozygous parent. On the contrary, if all of 11 individual plants tested showed the phenotype from expected dominant allele it is indicated that the plant population was originated from homozygous parent for the target gene (Satoto et al., 2008).
PCR analysis on four transgenic rice lines (3R7.8.15.1, 3R25.7.27.1, 3R5.26.2.1 and 3R5.26.5.1) in which each line consisted of 15 individual plants exhibited that all individual plants from each line were able to amplify the cry1B fragment with the same size which can be amplified by pCAMBIA 1301 as a positive control. These results prove that the four transgenic rice lines are homozygous for cry1B gene (Figure 1 and Table 2).

![Figure 1](image_url)

**Figure 1.** PCR analysis using cry1B specific primers in four transgenic rice lines at T₆ generation. (a) 3R7.8.15.1; (b) 3R25.7.27.1; (c) 3R5.26.2.1 and (d) 3R5.26.5.1. Each line consisted of 15 individual plants. M: λ HindIII; P: pCAMBIA 1301 harboring cry1B gene; W: dH₂O as a replacement for DNA; 1-15: transgenic plants.

**Table 2.** Inheritance of cry1B gene at T₆ generation of 3R7.8.15.1, 3R25.7.27.1, 3R5.26.2.1 and 3R5.26.5.1 transgenic rice lines

| No. | Transgenic rice lines | Total plants tested | + cry1B gene | - cry1B gene | Status          |
|-----|-----------------------|---------------------|--------------|--------------|----------------|
| 1   | 3R7.8.15.1            | 15                  | 15           | 0            | Homozygous     |
| 2   | 3R25.7.27.1           | 15                  | 15           | 0            | Homozygous     |
| 3   | 3R5.26.2.1            | 15                  | 15           | 0            | Homozygous     |
| 4   | 3R5.26.5.1            | 15                  | 15           | 0            | Homozygous     |

3.2. In vitro and in planta efficacy studies on transgenic Rojolele rice lines against rice yellow stem borer

The insect bioassays were conducted under laboratory by using Petri dish assays (in vitro) and whole plant bioassays (in planta). Non-transgenic Rojolele and IR64 were used as control of susceptible cultivars.

3.2.1. Insect bioassay in Petri dish

Transgenic rice lines at T₆ generation were evaluated to determine their stable resistance to YSB under Petri dish condition. Leaf sections from transgenic plants of four transgenic rice lines (3R7.8.15.1,
3R25.7.27.1, 3R5.26.2.1 and 3R5.26.5.1) and control of susceptible cultivar (non-trangenic Rojolele) were placed on a moistened filter paper in Petri dish and infested with first-instar larvae of YSB. Three days after infestation, the observation was conducted on leaf damage and the number of live and dead larvae for each Petri. The results showed that leaf damage of four transgenic rice lines exhibited little detectable damages caused by insect bites. Meanwhile, significant leaf damages were seen in non-trangenic Rojolele plant as a result of YSB feeding (Figure 2).

![Figure 2](image1.png)

**Figure 2.** Petri dish assay on transgenic rice lines harboring cry1B gene and control of susceptible cultivar (non-transgenic Rojolele). Leaf sections were infested with YSB larvae and each leaf section was infested with 10 larvae of YSB. a-d: transgenic rice lines (a) 3R7.8.15.1, (b) 3R25.7.27.1, (c) 3R5.26.2.1 and (d) 3R5.26.5.1; e: non-transgenic Rojolele cultivar

The observation on the number of live and dead larvae of YSB showed that the larvae mortality of 100% was observed in 3R5.26.2.1 line. In the remaining three transgenic rice lines i.e. 3R7.8.15.1, 3R25.7.27.1 and 3R5.26.5.1, percentage of dead larvae were 93.62%, 97.96% and 93.62%, respectively, whereas in non-trangenic Rojolele plant, the larvae mortality was only 11.11% (Table 2). Due to the very few surviving larvae on transgenic tissues, it is suggested that the toxin levels of Cry1B are sufficient to kill YSB larvae.
Table 3. Percentage of dead larvae of YSB on T6 transgenic Rojolele rice lines and non-transgenic Rojolele cultivar

| No. | Transgenic rice lines | Dead larvae of YSB at 3 days after infestation (DAI) | Percentage of dead larvae (%) |
|-----|-----------------------|-----------------------------------------------------|--------------------------------|
|     |                       | Total larvae | Alive larvae | Dead larvae |                               |
| 1   | 3R7.8.15.1            | 50           | 3            | 47          | 93.62                          |
| 2   | 3R25.7.27.1           | 50           | 1            | 49          | 97.96                          |
| 3   | 3R5.26.2.1            | 50           | 0            | 50          | 100.00                         |
| 4   | 3R5.26.5.1            | 50           | 3            | 47          | 93.62                          |
| 5   | Non-transgenic Rojolele | 50           | 45           | 5           | 11.11                          |

3.2.2. *Insect bioassay in greenhouse*

The efficacy of *cry1B* gene in four transgenic rice lines against YSB has been conducted in the greenhouse. Non-transgenic Rojolele and IR64 cultivars were also included in this experiment as susceptible checks. Five selected PCR-positive T6 plants were performed for each transgenic rice line and the same amount for each of susceptible checks. Ten first-instar larvae of YSB were infested into each tiller of individual plant. The category of four transgenic rice lines and control cultivars was determined based on the scoring system. Following the formula described by Heinrichs et al (1985), the percentage of deadhearts of 3R5.26.2.1 and 3R5.26.5.1 transgenic rice lines at 2 WAI were 0%, whereas the remaining two transgenic rice lines i.e. 3R7.8.15.1 and 3R25.7.27.1 were 16.97% and 13.96%, respectively. At 4 WAI, the percentage of deadhearts of 3R5.26.5.1 line were 0%, meanwhile the other three were 9.82%, 9.83% and 3.33, respectively. Very contrasting data were obtained from non-transgenic Rojolele and IR64 cultivars as susceptible checks, in which the percentage of deadhearts at 2WAI and 4WAI reached 93.5% and 98.18%, respectively. These percentage of deadhearts of four transgenic rice lines were significantly different from susceptible checks (Table 3).

Further, the percentage of deadhearts were converted to the D value following the formula described by Heinrichs et al (1985), with non-transgenic IR64 was used as a susceptible check. The D value of each transgenic rice line and control cultivars were then transformed to a 0-9 scale following Standard Evaluation System for rice (IRRI, 2013). Classification of transgenic rice lines and control cultivars based on scale of resistance at 2WAI and 4WAI, showed that four transgenic rice lines are categorized as resistant to YSB, even one transgenic rice line i.e. 3R5.26.5.1 is highly consistently categorized as highly resistant with score of 0. On the contrary, non-transgenic Rojolele and IR64 as susceptible checks are categorized as highly susceptible to YSB with score of 9 (Table 4). The data obtained from bioassays obviously indicated that the transgenic rice lines harboring *cry1B* gene can protect themselves from YSB attacks by using their own insecticide.
Table 4. Classification of transgenic rice lines and control cultivars against yellow stem borer at 2WAI and 4WAI

| Transgenic rice lines and susceptible control plants | Per cent deadhearts  | D value (%) | Score | Plant category |
|-----------------------------------------------------|-----------------------|-------------|-------|----------------|
|                                                     | 2WAI (D)               | 4WAI (D)    | 2WAI  | 4WAI  | 2WAI | 4WAI | 2WAI | 4WAI |
| 3R7.8.15.1                                          | 16.97 b                | 9.82 bc     | 17.28 | 10    | 3    | 1    |       |       |
| 3R5.26.2.1                                          | 0 c                    | 3.33 bc     | 0     | 3.39  | 0    | 1    |       |       |
| 3R5.26.5.1                                          | 0 c                    | 0 c         | 0     | 0     | 0    | 0    |       |       |
| Non-transgenic Rojolele IR64                        | 93.5 a                 | 93.5 a      | 95    | 95    | 9    | 9    |       |       |
| Non-transgenic                                      | 98.18 a                | 98.18 a     | 100   | 100   | 9    | 9    |       |       |

The mean values followed by the same letter on the same column are not significantly different at the 5% level in Duncan comparison test.

The appearance of transgenic homozygous rice lines harboring cry1B gene and two susceptible checks following infestation with YSB larvae, can be seen in Figure 3.

Figure 3. The appearance of transgenic homozygous rice lines and susceptible checks following infestation with the larvae of YSB at 14 days after inoculation. a: non-transgenic IR64 cultivar; b: non-transgenic Rojolele cultivar; c-f: transgenic homozygous rice lines i.e. 3R5.26.2.1, 3R5.26.2.5, 3R7.8.15.1 and 3R25.7.27, respectively.
4. Conclusion

The development of YSB-resistant transgenic rice lines have been conducted by introducing cry1B gene from Bt into a local javanica rice cultivar, Rojojale. Four transgenic rice lines at T6 generation were validated as homozygous for cry1B gene. Insect bioassay by using Petri dish assay (in vitro) and whole plant bioassay (in planta) showed that the expression of cry1B gene in transgenic rice lines confer highly resistance against YSB comparing with susceptible checks. At 2 WAI, the percentage of deadhearts of two transgenic rice lines i.e. 3R7.8.15.1 and 3R25.7.27.1 were higher than at 4 WAI (Table 2). This finding suggests that at 4WAI, the rice plants are able to produce new tillers, whereas the number of deadhearts (affected tillers) are not increased. Thus, by following the formula described by Heinrichs et al (1985), the percentage of deadhearts at 4 WAI are lower than 2WAI. Taking together, the inheritance and expression of cry1B gene in transgenic rice lines have been proven stable until T6 generation. However, the efficacy of cry1B gene in four transgenic rice lines should be tested in the field under real condition.

5. Acknowledgements

The author would thanks to all laboratory member for supporting this project.

6. References

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