Evaluation of pollen viability, seed viability and vigor of two Bt rice lines Y7 and P8 carrying cry1B::cry1Aa fusion genes

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Abstract. Pollen viability, seed viability and seed vigor of two BT lines Y7 and P8 expressing Cry1B-Cry1Aa fusion proteins were assessed to determine if these two lines equivalence to its wild type rice cv Rojolele. Pollen viability was estimated using in vitro pollen germination methods. Pollen were collected at 0, 1, and 2h after shedding. Both seed viability and vigor were observed using rolled paper test methods at incubation temperature of 30, 33, 37, and 42 °C. The results showed that there was no significant difference between pollen viability of BT lines and Rojolele wild type. The highest pollen viability was obtained when the pollen grown shortly after anthesis (0h). Seed viability and vigor were significantly affected by the incubation temperature. The highest seed viability and vigor were obtained at incubation temperature of 33 °C. Both seed viability and vigor were decreased significantly when the seeds grown at higher temperature (37 °C). However, seed viability and vigor of BT lines were not significantly different from its wild type, except for the fresh weight parameters. Thus, based on these observations, BT lines were agronomically equivalence to wild type rice cv Rojolele. This data is important for the environmental risk assessment.

1. Introduction
Rice is staple food of Indonesian people and almost half of the world's population, therefore the availability and accessibility of this staple food are very important. Rice productivity and production should be increased to meet the domestic demand in accordance with the high rate of population growth (1.3%) [1], and land conversion (2.7% or 96,512 ha per year) [2]. On the other hand, yield losses should be reduced to a minimum level.

Rice production greatly affected by both biotic and abiotic stresses. Biotic stresses include insect pest, bacteria, and viruses. The yellow rice stem borer (Schirpophaga incertulas Walker) is a major pest in the rice fields. The yellow rice stem borer (YSB) attacks rice plants both in the vegetative and generative stages, which causes deadhearts and whiteheads symptoms, respectively. Based on 28 years observations, 1% of rice plants showing deadhearts, whiteheads, or both symptoms caused a yield loss of 2.5%, 4%, or 6.4%, respectively [3]. Without insecticide applications yield losses could reached up to 87.66% [4].

Up to present time, routine spraying with insecticides has been a common practice to control YSB in the rice fields. The use of insecticides is, however, ineffective since YSB larvae are hidden inside the rice stems. Furthermore, the uncontrolled application of insecticides could harm the health of farmers, consumers and the environments. Since insecticides are toxic, not only to the target insects, but also to the non-target insects, and mammals, the development of a new rice variety resistance to
YSB is necessary. However, efforts to develop a new rice variety with conventional breeding have not successful yet. No genes with high level of resistance to YSB has been found or mapped in rice or its wild relatives [5]. Therefore, the use of genetic engineering technology is an alternative way for rice breeding resistant to YSB by utilizing genes from other unrelated species.

BT toxin has long been used as biopesticide to control rice stem borers. BT toxin is a crystal protein that is naturally produced by the soil bacteria *Bacillus thuringiensis*. Cry toxins are encoded by the *cry* genes. Today, many *cry* genes have been found and characterized [6]. Some of these *cry* genes have been used singly or by stacking two or more *cry* genes together for the development of rice plants resistant to YSB using plant genetic transformation techniques. The *cry1Ab* and *cry1Ac* genes are the most popular *cry* genes and have been transformed into many important crops, including rice [7-9]. This genetic transformation approach had been used to produce transgenic Iranian aromatic rice Taroom Molai, the first genetically modified (GM) rice grown commercially, that expressed *Cry1Ab* ([http://www.sciencedev.net/Docs/ Iran%20Releases%20World.htm](http://www.sciencedev.net/Docs/ Iran%20Releases%20World.htm)). Then, Huahui1 and Shanyou63 carrying *cry1Ab*/*1Ac* fusion genes have received environmental safety certificates from the Chinese Government in 2009 for planting in Hubei Province [10,11]. Huahui1 had also received food and environmental safety approval from the FDA and EPA [12]. However, up to the present time the Huahui1 not planted commercially yet.

We have developed transgenic *BT* rice cv Rojolele carrying the *cry1B* and *cry1Aa* genes through *Agrobacterium* transformation. These *cry1B* and *cry1Aa* genes encodes Cry1B and Cry1Aa proteins, respectively, and have different binding sites in the midgut of YSB larvae that targeted to prolong rice resistance to YSB. Six of transgenic events were found to be highly resistant to YSB based on previous *in vitro* and *in planta* bioassay experiments. Before released to the environment, these transgenics rice must go through various environmental risk assessments both in the biosafety containments or confined field trials to get approval and environmental safety certificate from the regulatory agencies. One of the important information to be collected for the environmental risk assessment is the data on agronomic characters of newly GM crop. A new GM crop should agronomically equivalence to its non-GM counterparts, except for the target character(s). Whereas to be released commercially, a new crop variety (GM or non-GM) should be similar or better than the existing varieties.

In this study, pollen viability, seed viability and seed vigor of *BT* lines, Y7 and P8, were assessed to determine if these two *BT* lines were agronomically equivalence to its wild type of rice Rojolele. These agronomic characters are important to be observed in addition to the agronomic characters that are routinely observed by the breeders. Pollen viability plays an important role in the pollination and fertilization process. Pollen can flow from crop to crop or to its wild relatives. Whereas seeds viability and vigor are important for the competition in the fields. A new GM crop is expected to have no risk of becoming invasive or weedy, which is among others characterized by the seeds ability to grow quickly under unfavorable conditions, such as heat stress. In this study, we observed the percentage of pollen germination, maximum growth potential, seed germination, vigor index, germination rate, root and plumule length, fresh and dry weight. This data is required for the environmental risk assessments.

2. Materials and methods

2.1. Plant materials

Two *BT* rice lines P8 and Y7 were used in this experiment. Lines P8 and Y7 were derived from rice cv Rojolele event RFz 4.2.4-21-8-16-7 and RFz 4.2.2-1-27-13-6, respectively, transformed with *Agrobacterium tumefaciens*. The *A. tumefaciens* carrying plasmid pCAMBIA 1300 ubi *cry1B:*::cry1Aa (kindly provided by Dr. Guiderdoni, CIRAD France) was used to transformed rice cv Rojolele. The *A. tumefaciens* carrying *cry1B:*::cry1Aa fusion genes that joined by a linker (;) under the control of a constitutive maize ubiquitin promoter. Our previous study showed that these *BT* lines contained a single copy of the transgenes and resistance to the neonate larvae of YSB [13]. Untransformed (wild
type or non-\textit{Bt}) rice cv Rojolele was used as control. All rice plants were grown in the biosafety containment facility of RC Biotechnology in Cibinong from January to November 2019, 10 plants per line and 1-2 plants per pot.

2.2. \textit{In vitro pollen germination}
Selected panicles were detached from the plants at 9.00-10.00 AM and the base were kept immersed in water while taking into the laboratory. The panicles were then transferred into bottles containing ethanol solution. The pollen grains were harvested by gently shaking the spikelets on white HVS paper and carefully collected using a soft brush. Pollen were then cultured on a concave slide containing liquid pollen germination (PG) media [20\% (w/v) Sucrose, 10\% (v/v) polyethylene glycol 4000, 3 mM Ca(NO$_3$)$_2$.4H$_2$O, 40 mg/L H$_3$BO$_3$, and 3 mg/L vitamin B1] [14-15] at 0h (fresh, right after harvested), 1h, and 2h after collected and air dried at ambient temperature. Water (H$_2$O) was used as control media. The cultured pollen grains were then incubated at ambient temperature (±30°C) for about 30 minutes to allow them to germinate. During incubation all samples were kept on wet tissues in petri dishes to keep the sample in a humid condition. The number of germinated pollens were observed on a light microscope with 100x magnification (Will Wetzlar, Germany). A pollen grain was considered as germinated when the tube length was greater than the diameter of the grain [16]. Germinated and non-germinated pollen grains were counted in each field of view for a total count of no less than 100 pollen grains for each line with three replications. The percentage of pollen germination (PG) was determined by dividing the number of germinated pollen grains by the total number of pollens observed [17].

2.2.1. \textit{Seed viability and vigor}
As many as twenty five seeds were germinated in the rolled paper test method using wet stencil paper [18-20], with four replications. Seeds were incubated at temperature of 30, 33, 37, and 42°C. Observations were made on the fifth (5th) and seventh day (7th). Seed viability were measured with these following variables:

2.2.2. \textit{Maximum Growth Potential (\%).}
The maximum growth potential (MGP) was calculated on day-7 based on the number of normal and abnormal seedlings compared to the total seeds planted and expressed in percentage [21].

\[
MGP = \frac{\text{total number of normal seedlings} + \text{total number of abnormal seedlings}}{\text{total number of seeds tested}} \times 100\% \tag{1}
\]

2.2.3. \textit{Seed Germination (\%).}
Seed germination (SG) was calculated based on the percentage of normal seedling on day 5 and day 7 [21]. Normal seedling criteria were referred to ISTA (2014). The seed germination was calculated with following formulation:

\[
SG = \frac{\text{total number of normal seedlings}}{\text{total number of seeds tested}} \times 100\% \tag{2}
\]

While seed vigor was measured with these following variables: vigor index, germination rate, root and plumule length, fresh and dry weight.

2.2.4. \textit{Vigor index.}
Vigor index (VI) was determined by counting the percentage of normal seedlings at first count (day 5) [21,22] using following equation:

\[
VI = \frac{\text{total number of normal seedlings at day 5}}{\text{total number of seeds tested}} \times 100\% \tag{3}
\]
2.2.5. **Germination Rate.**

Germination rate (GR) was observed based on the percentage of normal seedling on day 5 and [23,24]. The formula used was as follows:

\[
\text{GR} = \frac{\text{SG at day } 5 + \text{SG at day } 7}{5}
\]  

(4)

2.2.6. **Shoot and root length, fresh and dry weight.**

Root and shoot length and fresh and dry weights of normal seedlings were recorded at last day of observation. Five seedlings were selected randomly per treatments with four replications. The seedlings dry weight was observed by using oven method at 60°C for 72 hours.

2.3. **Statistical analysis**

All experimental data were subjected to two-way analysis of variance (ANOVA), while differences among means were compared with the LSD test at the \(P=0.05\).

3. **Results and Discussions**

3.1. **Pollen viability**

In vitro pollen germination showed that rice cv Rojolele pollen grew very well in the liquid pollen germination (PG) medium [15]. Germinated pollens were observed when the pollen cultured in the PG medium right after harvested (0h) (Tabel 1). No viable pollen was observed after pollen air dried at ambient atmospheric temperature for 1 or 2 h (Figure 1). The highest percentage of pollen germination was obtained from BT line P8, with the average of 83.06%. Then followed by its wild type Rojolele and BT line Y7 with the pollen germination of 81.04% and 68.39%, respectively. These results indicating that pollen of both Bt rice line P8 and Y7 and its wild type were very sensitive to dehydration and lost its viability shortly after released from its anther sac. Bt and non-Bt rice pollen had high viability but short longevity. Similar results on the pollen of rice cv Nakdongbyeo and two transgenic rice, ABC and Ubi lines were observed [25]. Pollen viability of both Bt rice lines, ABC and Ubi, and its wild type of rice cv Nakdongbyeo were declined drastically 40 minutes after shedding. The sensitivity of rice pollen to desiccation that resulted in short longevity indicated that BT rice posed no harm to the environments were also reported [26].

| Time (h after shedding) | Pollen germination (%) |
|------------------------|------------------------|
|                        | Lines                  |
|                        | R          | Y7         | P8          |
| 0                      | 81.04 ± 2.61ab         | 68.39 ± 6.53b | 83.06 ± 10.06a |
| 1                      | 0          | 0          | 0           |
| 2                      | 0          | 0          | 0           |

Tabel 1. Comparison of pollen viability and longevity of BT lines and its wild type

Similar letters in a line indicating do not differ significantly at \(\alpha=0.05\) (ANOVA, LSD, n=3).
Figure 1. Example of germinated and non-germinated pollen cultured right after harvested (left) and air dried at ambient temperature for 1 hour (right). Pollen were observed under a light microscope with 100 x magnifications. Pt; pollen tube, Pg; pollen grain.

3.2. Seed viability and vigor
3.2.1. Effect of germination temperature

The observation on the maximum growth potential indicated that almost all (99-100%, or 99.9% in average) of the seeds tested were viable (Table 2). Two-way ANOVA showed that incubation temperature significantly affected the percentage of seed germination (Table 2). Rice seeds, both Bt and non-Bt, germinated very well at 30–33°C. The highest percentage of seed germination with normal seedlings (98.67%) was obtained at incubation temperature of 33°C, then at 30°C with 98% of normal seedlings. Number of normal seedlings were, however, dropped drastically to 55.67% when the germination temperature was increased up to 37°C. Higher incubation temperature (42°C) had completely inhibited rice seeds germination process since no viable or germinated seeds was observed (data not shown).

| Agronomic characters | Germination temperatures (°C) | P<0.05 | F<sub>6.94</sub> | Remarks |
|---------------------|------------------------------|--------|------------------|---------|
| Seed Viability:     |                              |        |                  |         |
| MGP<sup>2</sup> (%) | 100a                         | 99.67a | 100a             | 0.44    | 1       | ns<sup>1</sup> |
| SG<sup>3</sup> (%)  | 98a                          | 98.67a | 55.67b           | 0.002   | 42.56   | *       |
| Seed Vigor:         |                              |        |                  |         |
| Vigor Index         | 93.33a                       | 93.67a | 0b               | 2.82E-05| 374.67  | *       |
| Germination rate    | 19.25a                       | 19.6a  | 8.68b            | 8E-05   | 221.63  | *       |
| Root length (cm)    | 9.20b                        | 11.13a | 2.28c            | 7.32E-06| 737.14  | *       |
| Plumule length (cm) | 5.99a                        | 6.15a  | 4.37b            | 0.002   | 44.56   | *       |
| Fresh weight (g)    | 0.09a                        | 0.09a  | 0.07b            | 0.0004  | 95.78   | *       |
| Dry weight (g)      | 0.024a                       | 0.025a | 0.026a           | 0.20    | 2.43    | ns      |

<sup>1</sup> ns, not significant; * differ significantly; Similar letters in a line indicating do not differ significantly at P=0.05 (ANOVA, LSD, n=4).
<sup>2</sup> MGP, maximum growth potential.
<sup>3</sup> SG, seed germination.

Incubation temperatures also negatively influenced all vigor parameters observed, except the dry weight (Table 2). From Table 2 it can be seen that the highest vigor index, germination rate, root length, plumule length and fresh weight were obtained from seeds germinated at incubation temperature of 33°C, but not differ significantly to that of 30°C, except for the root length parameter. At higher incubation temperature (37°C), all vigor parameters decreased significantly, except for the...
dry weight parameter. This decrease might be caused by the reduction or inhibition of enzymes activities that important in the germination process [27].

3.2.2. Effect of rice lines
Seed viability of the BT lines were statistically not difference to its wild type rice cv Rojolele (R), both based on the percentage of maximum growth potential and seed germination (Tabel 3). Observation on the seed vigor parameters also showed that the BT lines (Y7 & P8) were not differ significantly from its wild type, except for the fresh weight parameter (Tabel 3). These indicating that based on these agronomic characters, the BT lines are similar to its wild type rice cv Rojolele.

Tabel 3. Comparison of BT and non-BT rice based on seed viability and vigor characters

| Agronomic characters | Lines | R       | Y7     | P8     | P<0.05 | F<0.94 | Remarks |
|----------------------|-------|---------|--------|--------|--------|--------|---------|
| Seed Viability:      |       |         |        |        |        |        |         |
| MGP (%)              |       | 99.67a  | 100a   | 100a   | 0.44   | 1      | ns      |
| SG (%)               |       | 85a     | 87.33a | 80a    | 0.45   | 0.98   | ns      |
| Seed Vigor:          |       |         |        |        |        |        |         |
| Vigor Index          |       | 57.67a  | 65.67a | 63.67a | 0.22   | 2.23   | ns      |
| Germination Rate     |       | 16.23a  | 16.13a | 15.16a | 0.25   | 2.01   | ns      |
| Root Length (cm)     |       | 7.08b   | 7.95a  | 7.58ab | 0.057  | 6.39   | ns      |
| Plumule Length (cm)  |       | 5.17b   | 5.93a  | 5.41ab | 0.05   | 6.92   | ns      |
| Fresh Weight (g)     |       | 0.081b  | 0.089a | 0.084ab| 0.029  | 9.69   | *       |
| Dry Weight (g)       |       | 0.025a  | 0.026a | 0.025a | 0.59   | 0.61   | ns      |

1 ns, not significant; *differ significantly; Similar letter in a line indicating do not differ significantly at P=0.05 (ANOVA, LSD, n=4).
2 MGP, maximum growth potential.
3 SG, seed germination.

4. Conclusions
In general, pollen viability, seed viability and seed vigor of the BT line Y7 and P8 were not differ significantly from its wild type of rice cv Rojolele, except for the fresh weight variable of the seedling vigor. On the other hands, incubation temperature significantly affected seed viability and vigor parameters, except for the dry weight variable. Based on these assessments, it can be concluded that BT line Y7 and P8 are agronomically similar to that of non-transgenic rice cv Rojolele.

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