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Gene flow from Clearfield® rice to weedy rice under field conditions

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ABSTRACT

Imidazolinone-herbicide-resistant Clearfield® (CL) rice permits the selective chemical control of weedy rice (*Oryza sativa*), a major weed problem in South-East Asian rice growing countries. However, there is major concern involving resistant individuals resulting from gene flow as the cultivated and weedy rice live side by side in the fields. An experiment was conducted in the rice fields of Kuala Rompin, Pahang, Malaysia to determine which Clearfield® rice cultivars and weedy rice cultivars are more prone to hybridization, and the effect on distance between the pollen donor and receptor plants. The experiment was piloted in a split plot design with four replications. Encircled population technique was used to determine the distance between the Clearfield® rice and detection of hybrids (F1). Resistance of progeny was determined after spraying with OnDuty™ and the confirmation of hybrids was done using the SSR primer RM251. Higher survival rate was recorded with cv. CL2 which was significantly different from cv. CL1. Weedy rice cultivar V1 and V2 in CL1 plots differed significantly from the same cultivar from CL2 plots. However, no significant difference was observed between weedy rice cultivars of V3 and V4, either in CL1 or CL2 plots. No survivors were found after second spraying. Suspected hybrids were found up to 5 m however the rate was much lower compared to only 1 m from the CL plots.

Keywords: herbicide resistance; hybridization; SSR primer; progeny; Malaysian rice

Weedy rice (red rice) originates from the rice genus *Oryza sativa* and has been known as a serious pest in rice growing countries. It has been speculated to be progeny of wild rice with cultivated rice or degradation of cultivated rice (Chin et al. 2007). Weedy rice is taller, has fewer tillers and competes for sunlight, water and nutrients with cultivated rice, which can cause significant reduction in rice yields and grain quality (Chin et al. 2007). Shivrain et al. (2008) reported that most of the nitrogen (N) fertilizers applied to the rice field were up taken by weedy rice which reached up to 60%, while the yield loss could be as high as 80%. This was further supported by Saito (2010), who found that the yield loss can extend up to 100% if the weedy rice is not managed properly. There are three possibilities from where weedy rice comes from. Firstly, weedy rice might have evolved from wild rice. Second hypothesis stated that weedy rice came from escaped commercial rice and successfully adapted to its natural surroundings. The third hypothesis presumes that weedy

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rice is the hybrid of wild rice and commercial rice and their progenies are highly competitive (Kane and Baack 2007). Most of wild rice and commercial rice have the same AA genotype as it is commonly found in the Asian region (Kumar et al. 2008). Due to the genetic similarities between weedy rice and cultivated rice, there has been no effective technique to control the former. This is due to the seeds that shatter easily and long dormancy of weedy rice's seed in the soil. Some practices in controlling weedy rice are eliminating of the panicles, manual weeding and spraying with herbicides (Rathore et al. 2013). The introduction of genetically modified herbicide-resistant rice in recent year has overcome the problem of weedy rice in rice field. One of the introduced herbicide-resistant rice is Clearfield® rice. Clearfield® is tolerant to imidazolinone-based herbicides. This herbicide works by inhibiting certain branch-chain based amino acids such as leucine, isoleucine and valine (Croughan 2003). These amino acids are important in cell growth. The Clearfield® rice was first developed in the Louisiana State University Agriculture Centre, USA (Burgos et al. 2011). It is not considered as transgenic rice because it was chemically induced for the seeds to be mutated. In Malaysia, the development of hybridization of Clearfield® rice with locally commercialized rice's started in 2003 at MARDI (Malaysian Agricultural Research and Development Institute) station in Penang (Azmi et al. 2008). Two popular cultivars MR219 and MR220 were hybridized with Clearfield® rice Line 1770 introduced from the USA. DNA analysis shows that cvs. MR220-CL1 and MR220-CL2 have genetic similarity of about 98.5% and 92.5%, respectively (Sudianto et al. 2013). The cvs. MR220-CL1 and MR220-CL2 were introduced to Malaysian farmers in 2010 (Azmi et al. 2012). The Clearfield® rice production in Malaysia can be up to 7 metric tons per hectare (Sudianto et al. 2013). However, there is still risk in planting the Clearfield® rice. The weedy rice was found to be a perfect plant for gene introgression because of its sexual compatibility (Lu and Snow 2005). Factors that influenced the gene flow are variation in flowering times, out-crossing rates, population sizes, distances between populations, wind speed and humidity (Lu and Snow 2005). Gene flow from herbicide-resistant rice to wild and weedy relatives has been widely studied in the United States, Europe and China with the natural hybridization rate ranging from 1–52% (Lang and Buu 2007). If the Clearfield® rice gene incidentally transfers to the weedy rice, this will cause the production of herbicide-resistant weedy rice and thus creating a super weed. Therefore, the objective of this study is to determine the variability of weedy rice seeds in terms of width and length of the seeds, height and number of tillers produced using Clearfield® rice as comparisons. This is because weedy rice and Clearfield® rice are known to have different morphology. The difference in the morphology can help in researches and ways to control weedy rice in the fields and to reduce chances of crossbreeding between Clearfield® rice and weedy rice.

MATERIAL AND METHODS

Experimental conditions and design in the field. The distance factor experiment was conducted at the Lembaga Kemajuan Perusahaan Pertanian (LKPP) Padi Sdn Bhd from May 2013 til November 2013. The experiment was arranged in a split plot design with four replications using so called encircled population technique. In this technique the Clearfield rice (CL1 and CL2) was in the central plot surrounded by cultivars of weedy rice (V1, V2, V3 and V4) planted randomly according to the distance from the central plot. This experimental plot had no previous history of planting Clearfield® rice. The weedy rice cultivars were planted 8, 10, 12, and 14 days before the Clearfield® rice planting to ensure there is overlapping of flowering period. All rice seedlings were grown in trays and 2-week old seedlings were transplanted in the main plot. Seedling of weedy rice was transplanted at a planting distance of 25 cm within the subplot. Each main plot was separated into 60 m by 60 m to avoid contamination from other plots. Urea fertilizer was applied 10 days after transplanting (DAT) with a rate of 100 kg per hectare. Mix fertilizers (17.5:15.5:10) were applied at 4^{th} and 10^{th} week after transplanting with a rate of 200 kg per hectare. Herbicides Bispyribac sodium (Nominee®) at the rate of 1 L per hectare and Pyrazosulfuron-ethyl (Basmin®) at the rate of 140 g per hectare were sprayed 1 week before transplanting to control weeds. Insecticides Imidacloprid (Confidor®) at the rate of 250 mL per hectare and Fipronil (Regent®) at the rate of 250 mL per hectare were applied at 2^{nd}, 5^{th} and

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9th week after transplanting. The field was flooded two days after transplanting to avoid fluctuation of weeds.

At the end of the season, mature seeds were collected by hand according to the cultivars and distance of Clearfield® rice to weedy rice. The seeds were dried under the sun for three days and stored before planting in trays accordingly. The stored fresh seeds were then sown in trays for further testing of survival rate after herbicide spraying.

After fourteen days, while the seedlings were grown-up with two to three leaves, OnDuty™ (imidazolinone) was sprayed at the rate of 220 g/ha. Survivors were counted after one week of spraying. The outcrossing rate was determined by the number of survivors divided by total number of seedlings.

Statistical analysis. All the recorded data were analysed with SAS 9.1 software. The means for survival rate were compared with ANOVA and the significant differences among means were compared with the least significant difference (*LSD*) and the significant difference was considered at $P \leq 0.05$.

DNA extraction. For confirmation of any gene flow from Clearfield® rice to weedy rice molecular analysis were done on the survivors. The leaves of survivors were collected one week after spraying with OnDuty™ (imidazolinone) and wrapped in aluminum foils. The leaf samples were stored in –80°C freezer to avoid degradation of DNA samples. Plant DNA was isolated using QIAGEN DNEasy® Plant Mini Kit. About 0.1 g of leaf tissue for each sample was disrupted with liquid nitrogen using mortar and pestle. The sample was placed in 1.5 mL microcentrifuge tube. Then 400 μL of Buffer AP1 and 4 μL of RNase were added into the tube and incubated in a water bath at 65°C for 10 min. The tube was inverted 2 to 3 times during the incubation period. After incubation, 130 μL of Buffer P3 was added and incubated on ice for 5 min. After that the lysate was centrifuge at 13 500 rpm for 5 min. Then the lysate was pipetted into a QIA shredder spin column that was placed in a 2 mL collection tube. The column was centrifuged for 2 min at 13 500 rpm. The flow-through was transferred into a new tube without disturbing the pellet. Then Buffer AW1 was added at 1.5 of the volume of the flow-through and mixed by pipetting. After that 650 μL of the mixture was transferred into DNeasy Mini spin column placed in a 2 mL collection tube. The column was centrifuged for 1 min at 8000 rpm and then the flow through was discarded. This step was repeated with remaining samples. The spin column was then placed into a new 2 mL collection tube. After that 500 μL of Buffer AW2 was added and centrifuged for 1 min at 8000 rpm. The flow through was discarded and another 500 μL was added and centrifuged for 2 min at 13 500 rpm. The spin column was then transferred into a 1.5 mL micro centrifuge tube. Later 100 μL of Buffer AE was added into the column for elution and incubated in room temperature for 5 min. The spin column was centrifuged for 5 min at 8000 rpm. The samples were then placed in a concentrator for about 15 min before keeping in –20°C freezer.

Confirmation with SSR markers. Initial study has shown that SSR primer RM251 is suitable to detect hybrids from CL1 and CL2 rice cultivars with V1 and V2 weedy cultivars as the alleles from Clearfield® rice and these two cultivars of weedy rice are of different sizes. The PCR reaction mixture contained 12.5 μL of MyTaq®, 0.5 μL of 10 nm of RM251 forward primer, 0.5 μL of 10 nm of RM251 reverse primer, 200 ng of genomic DNA and the rest was top up with ddH_2O to reach the final volume of 25 μL. The PCR amplification consisted of one cycle of 4 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 57°C, 1 min at 72°C and a final extension of 10 min at 72°C. The PCR products were run in 2% agarose gel using electrophoresis at 80 V for 80 min. Since the markers used are codominant, alleles from both parents should be found in any hybrid progenies.

RESULTS

Outcrossing rate from suspected hybrids. The overlapping period for the Clearfield® rice and weedy rice was twenty days from 14th August until 2nd September. The average temperature ranged from 24.87–33.17°C. The wind speed was from 5.2–5.4 km/h. The average humidity was high at 8 a.m. with an average of 83.87% but decreases to 66.6% at 2 p.m. No survivors were recorded after second spraying.

The figure below (Figure 1) shows the survival rate of weedy rice seedlings based on the Clearfield® rice cultivars. A significant difference was observed among CL1 and CL2 rice cultivars

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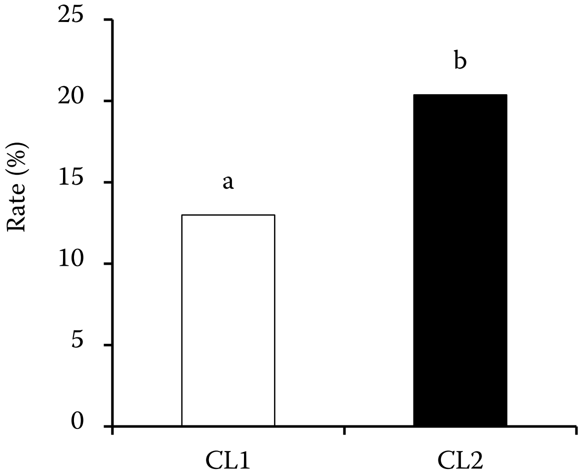

Figure 1. Survival rate of weedy rice one week after spraying based on Clearfield® rice cultivars

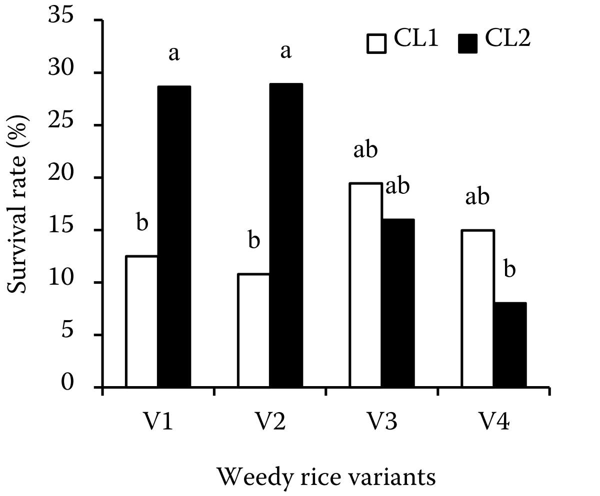

Figure 2. Survival rate of weedy rice progenies one week after spraying based on weedy rice cultivars

with the highest survival rate in CL2 (20.38%) compared to CL1 (13.00%) which is about 7% more over CL1.

Weedy rice cultivars. The survival rate of weedy rice progenies from different cultivars planted in CL1 and CL2 plots were compared. The mean survival rates of the weedy rice progenies at one week after spraying (WAS) are shown in Figure 2. The result revealed that, weedy rice CL2V1 and CL2V2 are the only progenies that shown significantly higher survival rate compared to CL1V1 and CL1V2 at $P \leq 0.05$ probability level. The weedy rice progenies from CL1 plots showed significantly lower survival rates compared to weedy rice progenies from CL2 plots.

Distance of Clearfield® rice and weedy rice. Figure 3 shows the survival rate of rice seedlings according to the distance of Clearfield® rice and weedy rice. From the figure, it is shown that there was a significant difference for survival rate based on different planting distances. Among all the distances only 1 m distance differed significantly from the distances of 2, 3, 4, and 5 m. Though minor differences were recorded among individual distances of 2, 3, 4, and 5 m but they were statistically non-significant (Figure 3). The mean survival rate of seedlings with distance of 1, 2, 3, 4 and 5 m were 37.51, 15.07, 10.72, 8.33, and 6.65%, respectively (Figure 3).

Treatment combination between Clearfield® rice and weedy rice. Figure 4 represents the number of survived seedlings at one week after spraying with OnDuty™. The weedy rice in CL2 plots showed the highest number of survival compared to CL1 plots. The seedlings planted at 1 m distance showed to have significantly higher survival rate compared to those planted at further distance. The highest survival rate (80.4%) was recorded in the treatment combination of CL2V1M1 (Figure 4). At least 8 combination treatments did not survive at the first week after spraying which were CL1V4M2, CL1V3M1, CL1V3M2, CL1V3M4, CL1V3M5, CL1V1M5, CL2V2M5 and CL1V4M5. Most of the combination treatments from CL1 plots thus did not survive after the first spraying.

Confirmation of hybrids. After the optimization, Clearfield® rice showed consistent band size. However, overall weedy rice showed two variations,

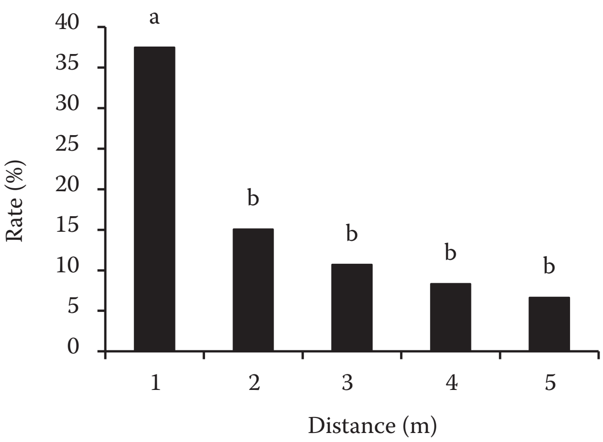

Figure 3. Survival rate one week after spraying based on planting distance

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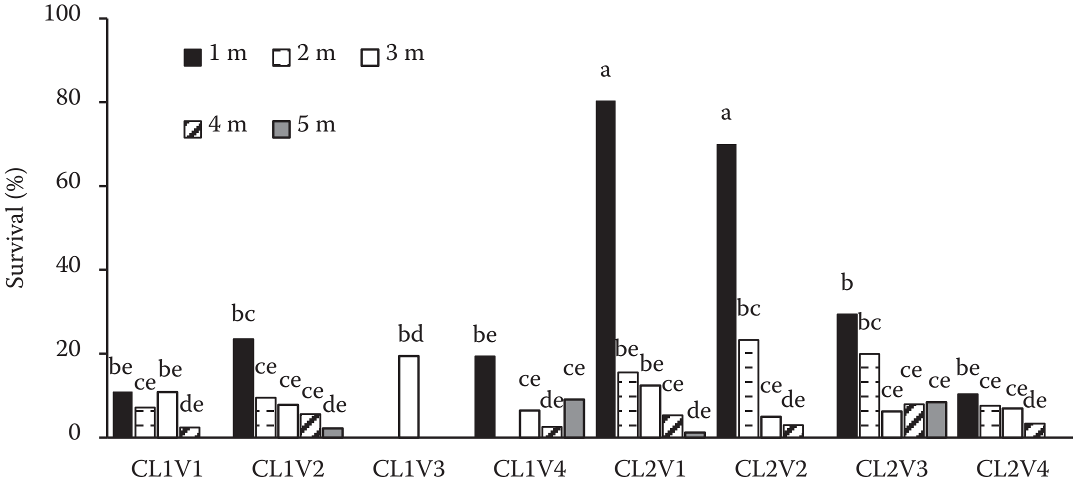

Figure 4. Survival rate of weedy rice after one week of spraying

regardless of cultivars. The hybrids produced three polymorphic bands using SSR Primer RM251 as shown by Li et al. (2006). From the result shown in the gel picture, Clearfield® rice produced two bands with the size of around 170–190 bp while weedy rice have one or two bands (Figure 5). In the second lane (L2) one variation was observed for weedy rice with one band at the size of around 120 bp. In the third lane (L3) for weedy rice had the second variation with two bands at 190 bp and 120 bp. The bands in the fourth to eighth lane were the hybrids with three bands roughly of the size 190, 170 and 120 bp. The bands with the size of 170 bp determined whether the rice seedlings were hybrids or not. The bands with sizes of 190 bp from weedy rice and Clearfield® rice proved that they are from common ancestry.

DISCUSSION

The conditions during overlapping were considered to be normal in the Malaysian climate. Personal observations showed that inflorescence of the flower started to open more as the temperature rises. On rainy days less inflorescence opened due to cloudy weather with high humidity and lower temperature. The wind speed was very erratic due to the annual monsoon season of the east coast of Malaysia. However the day by day averages do not show any major fluctuation during the overlapping period. Humidity, wind speed and other factors can affect the out crossing rate of rice (Lu and Snow 2005).

CL2 seems to have more tendencies to produce hybrids because more seedlings survived when

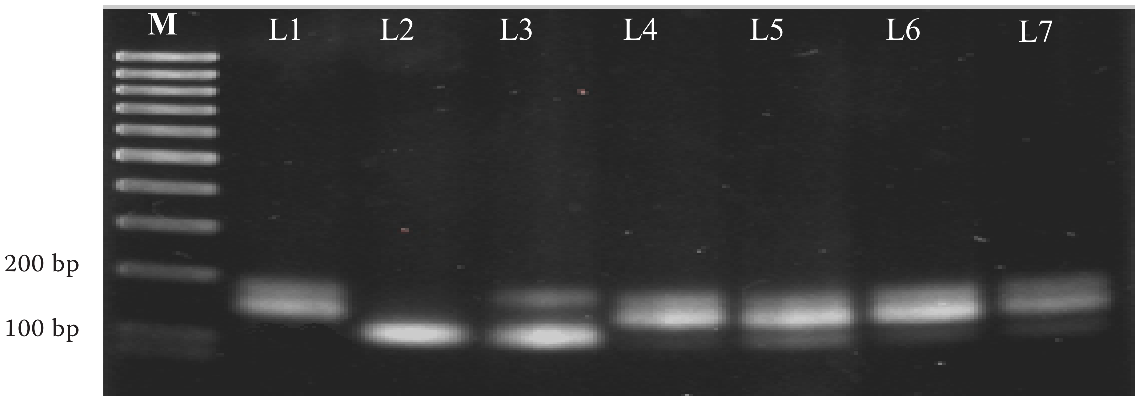

Figure 5. Confirmation of hybrids. L1 – Clearfield® rice; L2 to L3 – weedy rice; L4 to L7 – detected hybrids; M – DNA ladder

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planted in CL2 plots. From weedy rice parents, there was also a significant difference between the cultivars. Survival rates of V1 were significantly different with survival rates of V4 however there were no significant differences with the other two cultivars. The cv. V4 used in this study is considered an oddball out of the four. It has open panicle, which is different from the other three cultivars used in this study. However seedlings of V3 and V4 (seed-collected from CL1 plots) showed higher survival rates compared to seedling from CL2 plots. Certain cultivars of weedy rice also showed different significant survival rates with V4 which has the lowest rate (Figure 4). A significant difference was observed between the distance of 1 m and the other distances. The highest survival rate was recorded for the shortest distance (1 m) and the survival rate decreased with the increasing distances. The same result was also found by Shivrain et al. (2007) where the out-crossing rates of rice were decreased with the increasing distances. However, the result showed a significant difference in tolerance level of weedy rice towards imidazolinone herbicide when planted at different distances.

In a study by Li et al. (2006), it was reported that the SSR marker RM251 can produce three polymorphic bands. The important determinant of hybrids in using this primer is the second band. This band can only be found in Clearfield® rice cultivars and hybrids and absent in weedy rice cultivars. A study done by Shivrain et al. (2007) who showed that the bands of CL141 and CL161 have the same polymorphic band size. Their study also revealed that Clearfield® rice cultivars of CL1 and CL2 showed the same band size. It is important to note that the first band with the size of 190 bp can be found on certain weedy rice regardless on the cultivar and Clearfield® rice. Although it was not been proven, the band probably originated from cultivated rice. It can be assumed that some weedy rice hybridizes with cultivated rice and produce progenies. The progenies of cultivated rice with weedy rice are those with the two DNA fragments of the size 120 bp and 190 bp. From the result it can be concluded that gene flow from cultivated rice to weedy rice or vice versa is a natural phenomenon that occurs under field conditions.

Based on the findings of this study, the planting of Clearfield® rice and weedy rice side by side can have a negative impact on rice production in future due to the probability of hybridization. Insufficient control of escaped weedy rice during the early planting days before panicle initiation contributed to the gene flow. Continuation of ignorance can cause a major problem in future if this problem is not controlled. If the planting of Clearfield® rice is continued for years after years, undoubtedly it can be said that resistance to imidazolinone will increase in weedy rice. Suffice to say that Clearfield® rice is not recommended to be planted for long term but as a short term control for weedy rice before reverting back to commonly cultivated rice.

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