The effects of mixed direct seeding of parental lines with different hull colors on the seed production of hybrid rice

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ABSTRACT
Conventional production of hybrid rice seeds is based on sowing and transplanting parental lines separately (‘separate seeding and transplanting’, SST). To decrease the labour inputs of field management, a labour-saving model was implemented under a strategy of directly seeding the two parents as a mixture (‘mixed direct seeding’, MDS) to produce hybrid seeds. The strategy utilized a ‘yellow-hull/red-hull’ pair, ensuring that the female parent could be easily separated from the male parent after mixed harvesting. To assess the effects of MDS on seed production, two pairs, P88S/G-4 and Y58S/G-15, were tested and compared with those under SST at the same female/male seed ratio of 5:1 in 2015 and 2016. The parental lines for two pairs flowered synchronously, and the male sterile lines presented a high total stigma exertion rate in SST that ranged from 75.82% to 93.27%. At maturity, the hybrid seeds generated on seed parents were harvested together with those from pollen parents, and later, the hybrids were successfully colour sorted at a correctness rate of more than 99.90% on a machine. In MDS, the heading date of tested lines was advanced by 2–6 days. The outcrossing rate was improved by 8.59% and 7.61%, and the total panicle number increased by 4.78% and 3.80%, respectively. Ultimately, the actual yield in MDS significantly exceeded that in SST by 12.56% for P88S/G-4 and 8.95% for Y58S/G-15. This model succeeded in recycling hybrid seeds via colour sorting and seems promising for decreasing labour inputs in hybrid seed production.

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1. Introduction
Although remarkable breakthroughs have been made in breeding hybrid rice with improved heterosis (G. Zhou et al., 2012) and ideal plant architecture (Jiao et al., 2010), the total area planted with hybrid rice in China declined from nearly 2089.78 million hectares in 1995 to 1617.87 million hectares in 2013 (Hu et al., 2016). This decline resulted from low seed productivity combined with a high seed cost (Peng, 2016). The production of hybrid seed is in essence a cultivation process involving a male sterile line (seed parent)
outcrossed with a restorer line (pollen parent). In a conventional system, this cultivation involves sowing and transplanting the two parental lines separately (‘separate seeding and transplanting’, hereafter referred to as SST) (C. X. Zhou et al., 2009). SST typically includes four steps (Figure S1(a)). First, the seed and pollen parents are sown individually in a nursery bed. Second, parental seedlings with 3–4 leaves are transplanted as neighbours at a certain density in the field. Third, the seed parent is crosspollinated with the pollen parent via artificial assistance at flowering. Fourth, the pollen parent is removed before maturity, and mature hybrids are collected from the seed parent (C. X. Zhou et al., 2009). To reduce labour inputs and increase seed production, this cultivation should be simplified, especially the processes of sowing and transplanting.

Direct seeding of rice, which allows germinated seeds to be grown to maturity in situ without transplanting, can greatly reduce the labour input in field management (Bhatt & Kukal, 2015). Inspired by this concept, agronomists have developed a strategy of directly seeding the female and male parents as a mixture (‘mixed direct seeding’, hereafter referred to as MDS) to produce hybrid rice seeds (Xu & Huang, 2010). However, MDS requires that the parents differ in at least one trait other than flowering time (which must be synchronized), which allows them to be distinguished from each other (Xu & Huang, 2010).

To date, several models have been designed for MDS. The most common model includes a pair in which the female parent is resistant to an herbicide while the male parent is sensitive to the herbicide. If the parents are sprayed with the herbicide after pollination, the pollen parents wither and die, but the seed parents bearing hybrids live to maturity (Fu et al., 2001; Sung et al., 2007). Another model sought to make use of a parental difference in grain size. The greater the difference, the more effective the system is. As previously reported, a seed parent with a thousand-grain weight (TGW) of less than 12 g can be easily physically separated from a pollen parent with normally sized grains on a machine (E. B. Xu et al., 2015; Wu et al., 2010; Yu et al., 2007). However, there are still concerns regarding these models. The herbicide-resistant lines were originally gained via transgenic approaches (Fu et al., 2001; Tian et al., 2015), thereby posing a risk to public safety and potentially facing a long path to commercial development (Dale, 1999). In contrast, most small-grain-sized seed parents are commonly weakened in biomass and seed yield, perhaps due to pleiotropic effects for dwarf/semi-dwarf gene(s) (Yu, 2010; Yu et al., 2007). Hence, a more viable model should be developed for application.

Previously, we identified a red-hull mutant named RG-1. The mutant shows an obvious phenotype after heading, allowing its mature seeds to be easily separated from those of the wild type by colour (K. Xu et al., 2018). Here, we propose a new model based on a ‘yellow-hull/red-hull’ pair for MDS (depicted in Figure S1(b)) and investigate its performance in terms of seed production.

2. Materials and methods

2.1. Materials

The seed parents P88S (X. N. Wang et al., 2008) and Y58S (Deng, 2005) are photo-/temperature-sensitive male sterile lines (PTGMS lines) with a normal yellow hull that are widely used in southern China. The pollen parents, namely, G-4 and G-15, are stable indica red-hull lines that were obtained by crossing the red-hull mutant RG-1 (K. Xu et al., 2018) with the restorer lines ZC176 and ZC151, respectively.

2.2. Pigment content analysis

Mature seeds from P88S, Y58S, G-4, G-15 and RG-1 were collected in Sanya, China, in April 2015, and they were ground to measure their anthocyanin and chlorophyll contents. Following the method of Rabino and Mancinelli (1986), anthocyanins were first extracted from hulls (~1.5 g) with acidic (1% HCl, w/v) methanol at 4 °C for 24 h. Then, the extracts were cleaned by filtration. The value of A530 – 0.25*A657 was used to calculate the anthocyanin content (absorbance values were normalized to the fresh weight of the hull samples). Chlorophyll was extracted from the hull samples with the same volume of 80% acetone or methanol. The Chi a and Chi b contents were determined as described by Porra et al. (1989). Each line was tested by using 3 samples, and its mean±sd was compared with that of RG-1.

2.3. Field arrangement for an MDS test

Two pairs, i.e., P88S/G-4 and Y58S/G-15, were tested as part of MDS and SST (as a control) in Changsha, China, from May to September in 2015 and 2016. Each pair was tested in 3 randomly arranged plots, and each plot covered an area of 9.0 m² (a breadth of 1.8 m plus a length of 5.0 m). In each MDS plot, ~750 germinated female seeds were mixed with ~150 male seeds (at a ratio of 5:1) and then seeded directly. For SST, the two parental lines of each pair were sown separately in a nursery bed with the same number of seeds used for MDS. In addition, approximately 30 days later, seedlings were transplanted as neighbours within a plot, following the pattern of 10 inner rows (with a plant spacing of
16.5 cm) of females and 2 outer rows of males. The field management was kept the same for both systems.

Before seeding, the field was treated with herbicides to clean off the exogenous plants and fertilized with compound fertilizer (N: P: K = 15:15:15) at 600 kg/ha as a base. The additional urea fertilizer was used twice with 120 kg/ha and 50 kg/ha, when the seedlings return green and initiate tilling, respectively. Given that the base part of panicle used to be enclosed in leaf sheath for male sterile lines, we sprayed 0.3% gibberellin chemical to maintain node elongated and panicle exposed. This treatment was repeated twice for a usage of 4 g/ha at the first day and 8 g/ha at the second day of heading. Moreover, an aid pollination with bamboo pole was continuously performed at 11:00 am for 9 day (begun at the third day of heading).”

2.4. Observation of the developmental period and stigma exertion rate

We recorded the initially heading date (IHD) when the proportion of headed tillers reached 10.0%, the fully heading date (FHD) when the proportion reached 85.0%, and the maturity date (MD). At flowering, 30 panicles from the seed parent were selected to measure the single-side stigma exertion rate (SSER) and bilateral-side stigma exertion rate (BSER). The total stigma exertion rate (TSER) was calculated as the sum of the SSER and BSER.

2.5. Measurement of correctness for colour-selected hybrids

The colour-sorting machine ‘Anke-CF1’ (Meyer Photoelectric Technology Co., Ltd., Hefei, China), equipped with a photoelectric sensor, was used to sort the seeds based on hull colour. At maturity, the hybrids generated on seed plants in each plot were harvested and mixed with their corresponding male seeds. Then, ~2.5 kg dried samples were weighed to conduct a sorting test on the ‘Anke-CF1’ machine. The seeds at the recycling outlet were sorted twice, and the correctness rate (Cr) for hybrid seeds with yellow hulls was calculated as follows: Cr = No. of yellow-hull seeds/Total no. of seeds at the recycling outlet ×100%.

2.6. Assessment of the productivity of hybrid seeds

Seed yield components, namely, the total panicle number (TPN), grain number per panicle (GNPP), outcrossing rate (OCR) and TGW, were quantified for each pair. The TPN was measured for all the seed plants within a plot, while the other three components were estimated based on 30 panicles collected from seed plants. The theoretical yield per plot (TYP) was calculated by multiplying the TPN, GNPP, OCR and TGW, while the actual yield per plot (AYP) was measured based on the total number of harvested hybrid seed of 9 m². The mean value over 3 plots was recorded and compared between MDS and SST.

2.7. Statistical analysis

The datum in floral traits and yield components between MDS and SST were analyzed using the SPSS statistics 26.0 with Tukey test at a probability level of 0.05 by two-way ANOVA with Year and Treatment as the fixed effects.

3. Results

3.1. Pigment analysis of parental lines

Based on the phenotype of hull color, the seed parents, i.e. P88S and Y58S, could be easily distinguished from the pollen parents, i.e., G-4 and G-15, by eye, while G-4 and G-15 were hardly differentiated from their common donor parent RG-1 (Figure 1(a)). Moreover, both the anthocyanin and chlorophyll contents differed between the seed and pollen parents. The anthocyanin content of the pollen parents G-4 and G-15 was significantly greater by 97.36% and 89.47% than that of P88S, and the chlorophyll content was 41.33% and 38.67% lower, respectively. However, there were no differences among G-4, G-15 and RG-1 (Figure 1(b)). The difference in hull color was a suitable trait for differentiating seeds of different parents in MDS.

3.2. Observation of growth and developmental period

To achieve a high seed yield, the seed and pollen parents must flower synchronously. In the test of MDS and SST, the two parents for each pair, i.e., P88S/G-4 and Y58S/G-15, flowered almost synchronously within a period of less than 2 days in 2015 and 2016. The female parent (i.e., P88S) headed 1 or 2 days earlier than its corresponding male parent (i.e., G-4) (Table 1), which should allow sufficient acceptance of the male’s pollen by females. In particular, PTGMS lines P88S and Y58S are sterile at high temperature with a threshold of ~24°C (Deng, 2005; X. N. Wang et al., 2008). They had suffered a high temperature stress of 30.5°C~31.4°C in 2015 and 29.8°C~31.1°C in 2016 during the sensitive period of fertility transition, thus produced non-pollen grains and set no self-pollinated seeds.
The IHD and FHD of the parental lines in MDS were earlier than those in SST by 2–6 days. For example, in 2015, P88S and G-4 initially headed on August 9th and August 11th in MDS, but they were delayed by approximately 5 days in SST. Similar cases were also observed in 2016. Additionally, all the tested lines reached maturity on the same day or just one day apart. Therefore, the tested pairs are suitable for use in MDS based on parental synchronization of growth and development (Table 1).

### 3.3. Investigation of stigma exertion rate

The TSER is a limiting component of hybrid seed yield. The seed parents P88S and Y58S exhibited similarly high TSER between MDS and SST in 2015 and 2016, and the TSER in 2016 was slightly higher than that in 2015. For example, the TSER was 86.21% for P88S and 77.66% for Y58S in 2015 and reached 93.27% and 86.94% in MDS in 2016. Furthermore, the BSER of these parents was approximately 2-fold higher than the SSER, which would promote a desirable OCR (Figure 2).

**Figure 1.** Phenotypes on hull colors (a) and pigment content analysis (b) for parental lines. Different letter shows significant difference at 0.01 probability level by Tukey test.

**Table 1.** Observation on development stage under MDS by contrast of SST.

| Year | Treatment | Pair    | Parent  | SD (Month-date) | IHD (Month-date) | DSH (day) | FHD (Month-date) | MD (Month-date) |
|------|-----------|---------|---------|-----------------|------------------|-----------|------------------|-----------------|
| 2015 | SST (control) | P88S/G-4 | P88S | 06–04 | 08–15 | 72 | 08–22 | 09–15 |
|      |           | G-4 | 06–04 | 08–16 | 73 | 08–21 | 09–14 |
|      |           | Y58S/G-15 | Y58S | 06–04 | 08–17 | 74 | 08–24 | 09–16 |
|      | MDS       | P88S/G-4 | P88S | 06–04 | 08–09 | 66 | 08–17 | 09–10 |
|      |           | G-4 | 06–04 | 08–11 | 68 | 08–17 | 09–09 |
|      |           | Y58S/G-15 | Y58S | 06–04 | 08–15 | 72 | 08–22 | 09–12 |
| 2016 | SST (control) | P88S/G-4 | P88S | 06–10 | 08–23 | 74 | 08–31 | 09–25 |
|      |           | G-4 | 06–10 | 08–25 | 76 | 08–31 | 09–23 |
|      |           | Y58S/G-15 | Y58S | 06–10 | 08–26 | 77 | 09–03 | 09–27 |
|      | MDS       | P88S/G-4 | P88S | 06–10 | 08–28 | 79 | 09–03 | 09–25 |
|      |           | G-4 | 06–10 | 08–28 | 79 | 09–03 | 09–25 |
|      |           | Y58S/G-15 | Y58S | 06–10 | 08–22 | 73 | 08–30 | 09–24 |

SD: sowing date; DSH: duration from sowing date to initially heading date.

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**Figure 1.** Phenotypes on hull colors (a) and pigment content analysis (b) for parental lines. Different letter shows significant difference at 0.01 probability level by Tukey test.
3.4. Effects on hybrid sorting

In MDS, the seeds from seed and pollen parents were harvested in a mixture at maturity, and the normal yellow-hull seeds were selected as hybrids via color sorting on a machine (Figure 3(a)). The Ci of hybrids was 99.93% for P88S/G-4 and 99.95% for Y58S/G-15 in the first sorting, and it reached 100.00% for both pairs in the second sorting (Figure 3(b)), suggesting that our hull-colour-sorting-based MDS model produces seeds that meet purity requirements for commercial use.

3.5. Preliminary evaluation of hybrid seed production

No significant interaction between year and treatment (P > 0.05) was detected (Table 2). Except for TGW, the yield components for P88S/G-4 and Y58S/G-15 in MDS were significantly higher or lower than those in SST in the two consecutive years (Table 2). On average, the OCR in MDS exceeded that in SST in 2015 and 2016, with an increase of 8.59% for P88S/G-4 and 7.61% for Y58S/G-15. Each pair exhibited a higher OCR in 2016 than in 2015 in MDS: by 4.15% for P88S/G-4 and 7.18% for Y58S/G-15 (Table 2). The TPN in MDS was higher than that in SST, with an increase of 4.78% for P88S/G-4 and 5.80% for Y58S/G-15. However, the GNPP for the two pairs was lower in MDS, with a decrease of 6.46% for P88S/G-4 and 5.88% for Y58S/G-15 (Table 2).

As a consequence, the total grain number per unit area (TPNxGNPP) was the same between MDS and SST. The TYP increased in MDS by 11.64% for P88S/G-4 and 11.96% for Y58S/G-15 because of an improved OCR (Table 2). In contrast, in terms of the AYP, the two pairs both performed better in MDS, with an average increase of 12.56% for P88S/G-4 and 8.95% for Y58S/G-15, which agreed well with the trend of TYP (Table 2). This result indicated that the proposed MDS model was good at improving seed yield.

4. Discussion

Direct seeding, a labour-saving cultivation technology, has been widely applied in rice (Bhatt & Kukal, 2015). An MDS system integrated with whole process mechanization operation is regarded as an approach for reducing labor inputs and improving seed production in hybrid rice. However, the existing MDS models are defective in practice, either because of poor purity, as in the herbi-cide-killing model, or an inferior OCR, as in the size-sorting model (E. B. Xu et al., 2015; Fu et al., 2001; Yu, 2010).

The colour-sorting model is another alternative. This model relies upon two parents that differ in hull colour. Calub (1988) first described this idea in a US invention patent (No. 4,764,643) for producing hybrid seeds, which utilized a gold-hull cytoplasmic male-sterile line (CMS line) and a straw-hull pollen parent. Later, He et al. (2001) developed a brown-hull CMS line named ‘Se-Xuan A’ and found that it could be separated from normal yellow-hull seeds by colour sorting. However, whether these trials worked effectively for MDS is unknown. An ideal hull-colour trait would be obvious and genetically recessive. In this study, a red-hull pollen parent originating from RG-1 (inherited via a recessive gene) (K. Xu et al., 2018) was employed in combination with a yellow-hull seed parent for an MDS test. The two parents could be distinguished from each other by eye (Figure 1) and were well separated by a machine, even to
Table 2. Performance of hybrids yield and yield components under SST (separate seeding and transplanting, control) and MDS (mixed direct seeding).

| Pair     | Year | Treatment | OCR (%) | TGW (g) | TPN (m²) | GNP (g/m²) | TYP (g/m²) | AYP (g/m²) |
|----------|------|-----------|---------|---------|----------|------------|------------|------------|
| P88S/G-4 | 2015 | SST       | 62.75 ± 2.36 d | 21.17 ± 0.31 a | 185 ± 3 b | 142 ± 4 a | 349.10 ± 24.46 b | 338.89 ± 13.10 d |
|          |      | MDS       | 71.86 ± 3.13 b | 21.33 ± 0.35 a | 195 ± 4 a | 134 ± 4 b | 400.27 ± 36.33 a | 385.56 ± 15.40 b |
| Y58S/G-15| 2016 | SST       | 67.98 ± 2.34 c | 21.50 ± 0.26 a | 188 ± 3 b | 136 ± 4 ab | 373.47 ± 27.87 ab | 372.59 ± 16.72 c |
|          |      | MDS       | 76.02 ± 2.88 a | 21.47 ± 0.31 a | 195 ± 4 a | 128 ± 5 c | 410.46 ± 40.17 a | 414.44 ± 19.47 a |
| P88S/G-4 | Significance of factors | Year | ** | NS | NS | * | NS | ** |
|          |      | Treatment  | ** | NS | ** | ** | ** | ** |
| Y58S/G-15| Significance of factors | Year | ** | NS | ** | * | ** | ** |
|          |      | Treatment  | ** | NS | ** | * | ** | ** |
|          |      | Year × Treatment | NS | NS | NS | NS | NS | NS |

Outcrossing rate is for OCR, thousand seed weight for TGW, the total panicle number for TPN, grain number per panicle for GNP, theoretical yield for TYP, and actual yield for AYP. Significant difference among varieties is shown by different alphabetical letter. NS means non-significant differences; * and ** indicate significant differences at 0.05 and 0.01 probability levels, respectively.

Figure 3. Effects on color sorting for hybrid seeds. (a) An MDS test for producing hybrids of Y58S/G-15. (b) The correctness rate for color-sorted hybrids of P88S/G-4 and Y58S/G-15.

a correctness greater than 99.90% (Figure 3). Hence, we inferred that our model would be more user-friendly in utilizing a valuable germ resource, RG-1. Although RG-1 seems to function in regulating anthocyanin biosynthesis (Figure 1), the target gene and the mechanisms underlying hull pigmentation remains unknown.

Most noteworthy, an MDS system requires more strict criteria than SST for other agronomic traits. To obtain a high seed yield, the parental lines should flower no more than 6 days apart and be highly compatible in terms of outcrossing and pollination (Liao et al., 1999). In this study, the seed and pollen parents for P88S/G-4 and Y58S/G-15 showed synchronized flowering with an interval of less than 2 days (Table 1). The female OCR is a decisive index for yield and is determined by many components, including the TSER, stigma/pollen vigour, pollen amount and parental compatibility (Kato & Namai, 1987; Marathi & Jena, 2015). The seed parents P88S and Y58S presented a high TSER ranging from 75.82% to 93.27% (Figure 1) and exhibited an average OCR ranging from 60.75% to 73.94% in MDS (Table 2). In addition, we chose a PTGMS line rather than a CMS line as a seed parent, in contrast to previous studies (Calub, 1988; He et al., 2001). The key reason for this choice was that PTGMS lines, i.e., Y58S, usually harbor an ideal plant architecture such as tight tillers, erect leaves and smaller
leaf angles, which would be helpful for accepting more pollen and thus improve the OCR (X. N. Wang et al., 2008).

Previous studies revealed that rice yield and yield components might be affected by direct seeding because of complex genotypic and environmental interactions (Chen et al., 2009; Dingkuhn et al., 1991; Huang et al., 2011). However, little is known about such effects on the seed yield of hybrid rice under MDS. The tested parental line in MDS headed earlier than that in SST, and the theoretical yield as well as actual yield for each pair was significant higher, mainly because of an increased OCR (Table 2). The increased OCR would be mainly resulted from a more closer space between parental lines. In a SST system, the two parents are separated as blocks, and it makes seed plants distant from pollen plants have less chances to be pollinated. Whereas in a MDS system, the parental lines seemed more closer each other in the distance, and it would facilitate pollen grains to be equally accepted for the seed plants so as to improve OCR. Other yield components such as TPN and GNPP for females are reportedly influenced by plant density (Huang et al., 2013; Jones & Snyder, 1987). At a fixed female/male seed ratio of 5:1, the TPN increased but the GNPP decreased in MDS, thus leading to the same level of total grains per plot between MDS and SST (Table 2). A higher seed yield of hybrids was also expected in response to adjusting the female/male ratio, but determining a suitable ratio requires additional research.

For optimization of our colour-sorting MDS system, other works during cultivation should be improved in the future, including developing presowing treatments against abiotic stress (Liu et al., 2015; W. Q. Wang et al., 2016), simplifying pollination operations (Tang et al., 2012) and enhancing weed control (Chauhan & Opeña, 2013) with mechanized techniques. In addition, breeding for new red-hull parents suitable for MDS as well as cloning RG-1 should be advanced.

5. Conclusion

We developed a colour-sorting-based model for producing seeds of hybrid rice effectively via direct seeding of its two parents in a mixture. The model requires two parents that differ in hull colour, including an elite yellow-hull seed parent (PTGMS line) and a red-hull pollen parent. Field experiments revealed that the parental lines flowered at the same time, and their hybrids could be accurately selected by colour sorting. More importantly, the productivity of the hybrid seeds in our model was substantially higher than that obtained by conventional means under the same conditions. Therefore, this colour-sorting-based model employing elite parents expressing agronomically useful traits can be used to obtain high seed yield in hybrid rice.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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