

## Breeding for Outcrossing Ability in Rice, to Enhance Seed Production for Hybrid Rice Cropping

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### Abstract

**Background:** Adoption of the hybrid rice varieties by farmers is often impaired by the high price of hybrid seed, due to low yields in hybrid seed production fields. Female outcrossing ability (FOA) and female hybrid seed production ability (FHSPA), defined as the rate of filled spikelets of the male sterile (MS) line and as its grain yield under outcrossing, respectively, determine plant traits for hybrid seed yield. Breeding for FOA and FHSPA in rice has suffered from the lack of a high throughput phenotyping method and the inbred breeding approach used for the development of MS lines. We developed an innovative hybrid rice breeding strategy that uses the monogenic recessive male-sterility gene *ms-IR36* for the reciprocal recurrent improvement of maintainer and restorer populations.

**Results:** High throughput screening for FOA and FHSPA can be achieved by scoring the grain weight of MS plants and the grain yield of fertile plants of progenies extracted from breeding populations segregating for the *ms-IR36* gene. Using this phenotyping method in seven field trials, each involving several hundred entries, we revealed a very broad diversity for FOA (ranging from zero to 89%) and FHSPA, within the F<sub>3</sub> progenies of bi-parental crosses and within S<sub>1</sub> and S<sub>2</sub> progenies extracted from different breeding populations. The seven experiments produced convergent results and heritabilities of 0.59-0.90 for FHSPA and 0.45-0.72 for FOA. Correlations between FHSPA and FOA were tight and highly significant. Correlations were looser between FHSPA and grain yield of the selfed fertile sibling (GW-MF). Correlations between FOA and GW-MF were not significant. Tight significant correlation was also observed between FHSPA of S<sub>1</sub> lines and S<sub>2</sub> lines extracted from the former.

**Conclusion:** Population breeding through recurrent selection, using the *ms-IR36* gene as a tool for both recombination and seed production for testcrossing, is a favorable framework for harnessing rice genetic diversity for FHSPA. Rapid and cost-effective genetic gain for hybrid seed production can be achieved using results of the *ms-IR36* gene mediated test cross seed production process as FHSPA early screening.

**Keywords:** Rice; *O. sativa*; F1 hybrid variety; Outcrossing ability; Hybrid seed

### Introduction

Hybrid rice is a proven and successful technology for rice production. Compared to inbred lines, hybrid rice varieties have an average yield advantage of about 20%, due to the accumulation of more biomass before flowering and a higher harvest index at maturity [1-4]. Over the past 35 years, the use of hybrid rice has significantly increased rice productivity and farmers' incomes, as well as food security [5]. First developed and widely adopted in China, almost all major Asian rice growing countries and more recently the American continent (USA, Brazil, Colombia, Uruguay and Argentina) have invested in the hybrid rice technology with the involvement of the seed industry [1,6,7]. However, due to seed production constraints, seed production costs remain high, a major obstacle for wider and faster adoption of hybrid rice [7].

In spite of the partial allogamy of its progenitor *O. perennis* [8], *O. sativa* is a highly autogamous species and self-pollination has probably been reinforced by the process of pedigree breeding extensively used for the development of the modern inbred rice varieties. The presence of the male and female organs in the same small spikelet and their simultaneous maturation prevent cross-pollination. The production of hybrid seeds consequently requires a male-sterile (MS) line. The development of the first hybrid rice varieties relied on the genocyttoplasmic male sterility (CMS), or three-line system. This system requires a cytoplasmic MS or A line; a maintainer or B line (genetically identical to the A line except for the cytoplasm and hence fertile) that, when crossed with the A line, produces MS offspring; and a restorer or R line that, when crossed with the A line, produces fertile hybrid seeds [9]. In the mid-1990s, a new male sterility system called two-line was developed [10]. It is based on male sterility resulting from the interaction of nuclear gene(s) with environmental conditions such as photoperiod (PGMS) and/or temperature (TGMS). With this system, any line can be used as pollinator parent to obtain a fertile hybrid [6].

The two-line system has several advantages but the dependence of male sterility on temperature and day length makes implementation tricky and imposes temporal and geographical limits on hybrid seed production [11].

Whatever the female sterility system used, hybrid seed yield in a hybrid seed production field depends on the yield potential, or the total number of spikelets per surface unit, of the female MS line, and the rate of fecundation of the MS plants by pollen of the pollinator line. Techniques for achieving a high fecundation rate of MS plants include: (i) differential treatments of the two parents to synchronize their flowering time (crop establishment dates, applications of fertilizer and/or growth hormones, etc.); (ii) specific cropping designs to optimize the distribution of pollen in the field (quantitative ratio of the two parents as well as their relative geographical position); (iii) physical actions during flowering (mechanical shaking of the male parent plants or wind blowing) to enhance pollen dispersal [12]. However, the efficiency of these techniques depends on the intrinsic outcrossing ability of the parental lines, and more specifically on the outcrossing ability of the female line. Therefore, as early as the mid-1980s, the outcrossing ability of MS lines, or female outcrossing ability (FOA), has been a major target in hybrid rice breeding [13-15].

The extent of natural outcrossing in cultivated rice varieties, as reported in the literature, ranges from zero to 6.8% [16]. Data from recent investigations into the pollen-mediated gene flow between genetically modified (GM) rice and non-GM rice or between GM rice and weedy rice, confirmed the low rate of outcrossing (almost always less than 1%) between male fertile (MF) rice plants [17]. However, FOA varies considerably among MS lines developed for hybrid rice breeding. The percentage of filled spikelets ranges from zero to 45% depending on the genotype, the growing conditions, and the pollinating conditions [18]. In China, the outcrossing seed set often surpasses 50% and reaches 85% in some high yielding plots under the most favourable crop management systems [15]. Varietal differences in floral behaviour and in the floral traits that influence outcrossing in rice are also well documented [13,19]. Inheritance studies [20,21], for floral traits such as anther length, stigma length, and stigma exertion in rice indicated that these traits are determined by polygenic inheritance. Both additive and non-additive effects are important for their inheritance [22].

Using such knowledge, major progress has been made in improving the yield of hybrid seed production fields. For instance, in China, while in the mid-1970s, hybrid seed production yield was 324 kg ha<sup>-1</sup> (representing an area ratio between seed production and hybrid rice cultivation of only 1:15), by 2007, it had reached an average of 2,550 kg ha<sup>-1</sup> representing an area ratio of 1:120 [22]. However, to our knowledge, no practical method has yet been proposed for large scale FOA screening that can be implemented during the early generations of breeding for MS female lines. Likewise, less attention has been paid to the overall female hybrid seed production ability (FHSPA), which results from the combined effects of the FOA and the intrinsic yield potential of the MS line. Last, technologies developed in Asia cannot be transferred directly to the American continent where, due to labor scarcity, rice is direct-seeded and the area ratio between hybrid seed production and commercial rice cultivation is estimated to be 1:50, as a sowing density of less than 40 kg/ha is difficult and yields in hybrid seed production field rarely exceed 2,000 kg/ha.

We developed an innovative hybrid rice breeding program based on the improvement of maintainer and restorer populations through

reciprocal recurrent selection [23]. The program is based on the use of a monogenic recessive gene *ms-IR36* that does not interact with environmental factors and displays stable expression and Mendelian segregation [24-26]. The homozygous plants for the recessive allele (*ms,ms*) are fully male sterile while the heterozygous (*Ms,ms*) and homozygous (*Ms,Ms*) plants for the dominant allele are fully male fertile. Segregation of *ms-IR36* gene within the breeding population facilitates the recombination phase of the recurrent selection schemes [27] and the testcross seed production stage of progenies extracted from the breeding populations [23]. In this paper we (i) present a method for the large scale evaluation of FOA and FHSPA in the early generation of segregating progenies extracted from the breeding populations that relies on the use of the *ms-IR36* gene; (ii) report the genetic variability observed for FOA and FHSPA within different categories of segregating material and (iii) discuss breeding strategies to harness rice genetic diversity for FOA and FHSPA.

## Material and Methods

### Plant material and experimental designs

Seven field experiments were conducted in Colombia (experimental farm of the El Aceituno seed company, Ibagu , 4°20'59.28"N, 74°59'59.65"W, elevation 636 m asl.) and in Brazil (experimental station of EMBRAPA, Goianira, 16°25'58.45"S, 49°24'02.32"W, elevation 736 m asl.), between 2010 and 2016. Among these experiments, four were specifically designed to evaluate both FHSPA and FOA in sets of F<sub>3</sub> or S<sub>1</sub> lines, with 2-3 replicates under a randomized complete block design or an alpha lattice design (Table 1).

The plant material was composed of F<sub>3</sub> lines extracted from two bi-parental crosses, and S<sub>1</sub>/S<sub>2</sub> progenies extracted from four populations that had undergone several cycles of recurrent selection (Tables 1 and 2).

Results of three sets of testcross seed production plots were also used to study the FHSPA of S<sub>1</sub> and S<sub>2</sub> progenies (Table 2).

Each bi-parental cross involved two B lines endowed with the male sterility recessive gene *ms-IR36*, so as to observe within the population of F<sub>2</sub> progenies, the Mendelian distribution of ¼(*ms,ms*), ½(*ms,Ms*) and ¼(*Ms,Ms*) genotypes, corresponding to ¼MS and ¾MF phenotypes and a proportion of ¼MS plants within each F<sub>3</sub> line descending from the selfing of individual (*ms,Ms*) F<sub>2</sub> plants. The three B lines involved in the two bi-parental crosses are characterized by contrasted FOA and FHSPA (Table 3).

The four recurrent populations (P14, P20, P24, P29) segregate for the *ms-IR36* gene. P14 is an *indica* population with a wide genetic base currently used in the El Aceituno/Cirad hybrid rice breeding program in Colombia. P20, P24 and P29 are three populations currently used by the Embrapa/Cirad hybrid rice breeding in Brazil. P20 is of *indica* background with a small *japonica* introgression. P24 is the product of a bi-parental cross between Cirad 450B and Cirad 464B and P29 was obtained by combining an *indica* population with a *japonica* inbred line. Using the *ms-IR36* gene mediated recurrent selection process described by Taillebois et al. [23] and summarized in (Figure S1), all four populations have undergone one or two recurrent selection cycles for grain yield and grain quality combining ability.

	Origin	Progenies tested	Number of progenies	Experimental design	Variables measured in each plot	Year and place of the experiment
FE1	Biparental crosses Cirad464Bms/ Cirad450B And	F <sub>3</sub> lines obtained by selfing of ( <i>Ms,ms</i> ) & ( <i>MS,MS</i> ) F <sub>2</sub> plants	99/64 <sup>(1)</sup> F <sub>3</sub>	Randomized blocks with 3 replicates;	GW of all MS plants GW of 5 MF plants	2010 Ibague, Colombia
FE2	Cirad450Bms/ Cirad464B.	F <sub>3</sub> lines obtained by selfing of ( <i>Ms,ms</i> ) F <sub>2</sub> plants	9 <sup>(2)</sup> F <sub>3</sub>	Plot=2 rows of 10 plants	GW of 20 MS plants GW of 30 MF plants	2011 Ibague Colombia
FE3	Biparental cross Cirad464Bms/ Cirad468B.	F <sub>3</sub> lines obtained by selfing of ( <i>Ms,ms</i> ) & ( <i>MS,MS</i> ) F <sub>2</sub> plants	376/294 <sup>(1)</sup> F <sub>3</sub>	4 trials of randomized blocks with 2 replicates(3)Non-replicated Plot=500 plants	GW of all MS plants GW of 5 MF plants	2014 Ibague Colombia
FE4	P29 population	S <sub>1</sub> lines obtained by selfing of ( <i>Ms,ms</i> ) S <sub>0</sub> plants	251 S <sub>1</sub>	Alpha-lattice of two replicates and 10 plots/block(4) plot=1 row of 25 plants	GW of all MS plants GW of 5 MF plants	2016 Goiânia Brazil

(1): The first number is the number of F<sub>3</sub> lines planted and the second number is the number of F<sub>3</sub> lines segregating for *ms-IR36* gene. (2): The 9 F<sub>3</sub> lines were chosen on the basis of their FHSPA in FE1 experiment: one with the lowest and 8 with high FHSPA. (3): Connection between the 4 alpha lattice designs was ensured by 8 F<sub>3</sub> lines and 2 checks present in each trial. The 2 checks Cirad464 and Cirad 468 were alternated every 10 lines tested; each check was composed of 25% CMS sterile (A) plants and 75% of maintainer (B) plants. (4): Seven check varieties composed of 25% CMS A plants and 75% maintainer (B) plants of five A/B lines.

**Table 1:** Main characteristics of the field experiments (FE) conducted for the evaluation of female hybrid seed production ability (FHSPA) and female outcrossing ability (FOA).

	Origin	Progenies tested	Number of progenies	Mating system for FHSPA evaluation	Variables measured in each plot	Year and place of the experiment
FE5	P14 population ( <i>indica</i> large base population).	S <sub>1</sub> lines obtained by selfing of ( <i>Ms,ms</i> ) S <sub>0</sub> plants	120	Crossing with 4 testers (3 inbred varieties and one population)	GW of all MS plants among 58 S <sub>1</sub> plants.	2014 Ibague Colombia
FE6		S <sub>2</sub> lines obtained by selfing of ( <i>Ms,ms</i> ) S <sub>1</sub> plants.	79 <sup>(1)</sup>		GW of all MS plants among 48 S <sub>2</sub> plants	2014 Ibague Colombia
FE7	P20 population ( <i>indica</i> large base population).	S <sub>1</sub> lines obtained by selfing of ( <i>Ms,ms</i> ) S <sub>0</sub> plants	118	Crossing with 2 inbred testers.	GW of all MS plants among 30 S <sub>1</sub> plants	2016 Goiânia Brazil
	P24: biparental population from Cirad 464Bms/Cirad 450B and Cirad 450Bms/Cirad 464B		50			

(1): The 79 S<sub>2</sub> lines were derived from 79 different S<sub>1</sub> lines.

**Table 2:** Main characteristics of testcross seed production experiments (FE) used to evaluate FHSPA of S<sub>1</sub> and S<sub>2</sub> lines.

Name of B line	Origin / Genetic background	Tillering ability	FHSPA	FOA
Cirad 450	Asian	++	+	-
Cirad 464	Latin American	-	-	+
Cirad 468	V41B x IR58025	+/-	+	++

**Table 3:** Characteristics of the A lines in biparental crosses and in test crosses.

### Method to evaluate FHSPA and FOA

The method is based on the following steps (Figure 1): (i) selection and selfing of fertile F<sub>2</sub> or S<sub>n</sub> plants heterozygous for the *ms-IR36* gene,

derived from a biparental cross or a recurrent population, respectively; (ii) cultivation of F<sub>3</sub> or S<sub>n+1</sub> lines and tagging, within each of these lines, of MS plants right at the beginning of flowering (trained eyes can distinguish fertile and sterile spikelets very easily, sometimes before

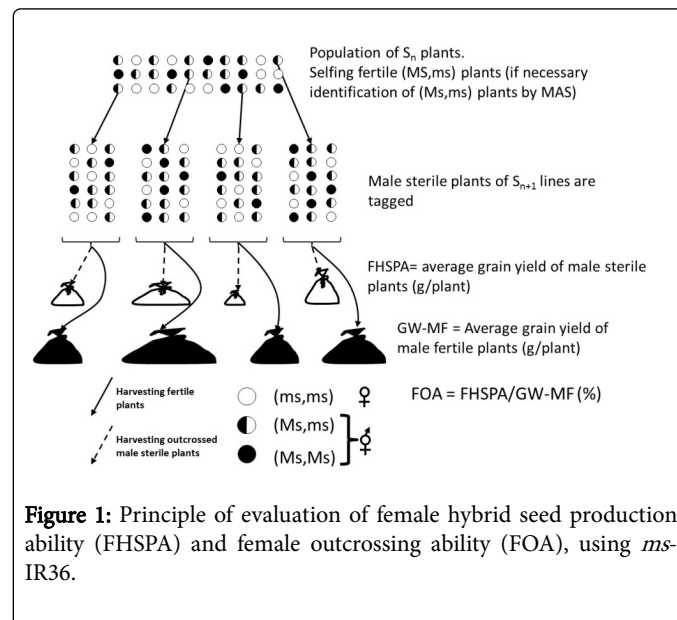
spikelet opening, as male sterile plants have slender white anthers whatever their genetic background and fertile plants have bigger yellow anthers); (iii) letting the MS tagged plants be naturally outcrossed by fertile neighboring plants; (iv) measurement of the grain weight of MS plants (MS-GW) by harvesting and weighing of tagged individuals of  $F_3$  or  $S_{n+1}$  plants; (v) measurement of the grain weight of male fertile plants (GW-MF) by harvesting and weighing a given number of  $F_3$  or  $S_{n+1}$  fertile plants; (vi) for each  $F_3$  or  $S_{n+1}$  line, computing the FHSPA as the average of the individual MS-GW; (vii) for each  $F_3$  or  $S_{n+1}$  line, computing the FOA as the ratio of FHSPA and the average MF-GW.

The FHSPA parameter can also be evaluated in the framework of seed production for testcrossing in the following steps (Figure S2): (i) Selection and selfing of fertile  $S_n$  plants heterozygous for *ms-IR36* gene; (ii) cultivation of  $S_{n+1}$  lines alternately with lines of tester varieties; (iii) identification and removal of male fertile plants in each  $S_{n+1}$  line right at the beginning of flowering; (iv) harvesting and weighing the MS plants of each  $S_{n+1}$  that have been naturally outcrossed by the neighbouring tester plants; (v) computing the FHSPA of each  $S_{n+1}$  as the average grain weight of the MS plants.

### Data analysis

For each experiment, first the raw FHSPA and FOA data were subjected to cubic root transformation to obtain normal distribution of the data and of the residues. This transformation is appropriate because FHSPA and FOA are probably volume-dependent (volume of the stigma, the panicle, the plant). Second, using the transformed data, adjusted means were calculated for each entry using a linear mixed

model in which the entry effect was considered as fixed and the effects of replicate and experimental designs were considered as fixed or random depending on the experiment (Table 4). Third, a linear mixed model, in which the entry effect was considered as random, was used to calculate trait broad-sense heritability [28]. These analyses were implemented using the Proc mixed procedure of S.A.S software v9.2 (SAS Institute, Cary NC, USA).



**Figure 1:** Principle of evaluation of female hybrid seed production ability (FHSPA) and female outcrossing ability (FOA), using *ms-IR36*.

	Models for computing adjusted mean for entries			Models for computing trait broad sense heritability	
	Fixed effects	Random effects		Fixed effects	Random effects
FE1	Block, Progeny			Block	Progeny
FE3	Trial, Replicate/Trial, Progeny, Check			Trial, Replicate/Trial, Checks	Progeny
FE4	Replicate, Progeny, Check	Block/Replicate		Replicate, check	Block/Replicate, Progeny
FE5	Tester, Progeny			Tester	Progeny
FE6	Tester*Distance, Progeny			Tester*Distance	Progeny
FE7	Tester*P24 <sup>(1)</sup> , Progeny*P29	Tester*P29 <sup>(1)</sup>	Progeny*P24,	Tester*P24, Tester*P29	Progeny*P24, Progeny*P29

(1) P24 and P29 are two dummy variables: P24 with 1 value when P24 is used and 0 when P20 is used; P29 with 0 value when P24 is used and 1 when P20 is used.

**Table 4:** Linear model used to calculate progenies' adjusted mean and broad sense heritability in each field experiment (FE).

## Results

### FHSPA and FOA in the progenies of bi-parental crosses

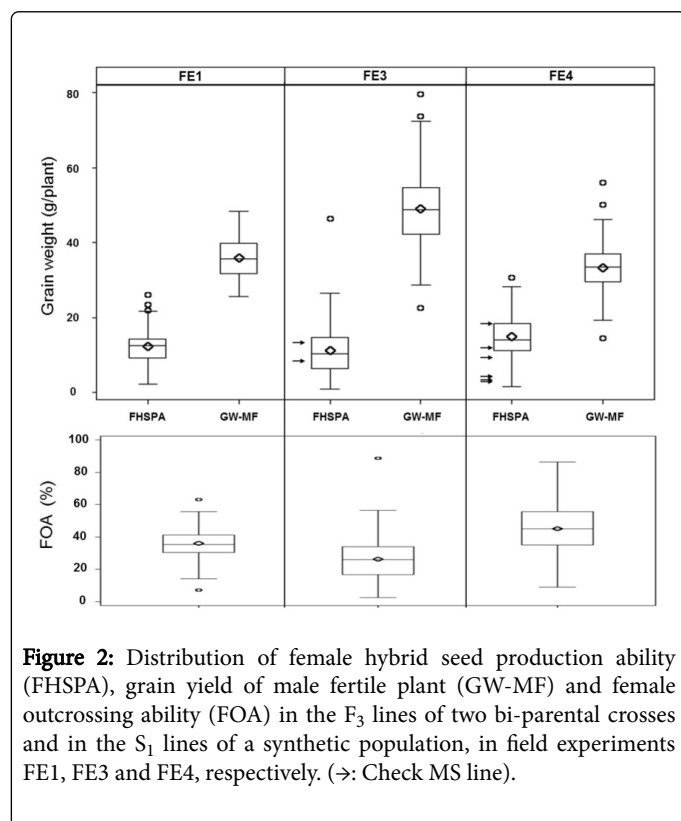
Among the 99  $F_3$  lines obtained by selfing of (*MS,ms*), (*MS,MS*)  $F_2$  plants of the Cirad 450/Cirad 464 cross, evaluated in experiment FE1, 64 showed segregation for the *ms-IR36* gene, which was in agreement with the segregation of 1/3 of (*MS,MS*) plants and 2/3 (*ms,MS*) plants in the  $F_2$  population. The average number of MS plants among the 64  $F_3$  lines segregating for the *ms-IR36* gene (4.7) was close to the expected percentage of 25% or 5 plants out of 20 plants per plot. The FHSPA averaged 12.2 g/plant and the MF-GW averaged 36.9 g/plant,

giving an average FOA of 36% (Figure 2). The line effect was highly significant for both FHSPA and FOA, but not for MF-GW (Table 5). The broad-sense heritability of FHSPA and FOA was 0.74 and 0.72, respectively. The performances of the 8  $F_3$  lines with the highest FHSPA and the one  $F_3$  line with the lowest FHSPA in experiment FE1, were evaluated anew with a larger number of plants per line in experiment FE2. The mean FHSPA of the 8 best  $F_3$  lines was 68 g/plant, while the FHSPA of the worse  $F_3$  lines was 24 g/plant only. When the worst  $F_3$  line was discarded, the correlation between estimates of FHSPA in FE1 and FE2 was rather tight ( $r=0.67$ ,  $p=0.07$ ).

Experiment	Traits	Num DF	Den DF	F Value	Pr>F	Broad-sense heritability (1)
FE1	MF-GW	63	122	1.3	0.137	0.22 ± 0.17*
	FHSPA	63	123	3.9	<0.0001	0.74 ± 0.06
	FOA	63	122	3.5	<0.0001	0.72 ± 0.06
FE3	MF-GW	293	400	1.5	0.003	0.31 ± 0.07
	FHSPA	293	401	6.3	<0.0001	0.84 ± 0.02
	FOA	293	400	3.6	<0.0001	0.71 ± 0.03
FE4	MF-GW	250	230	2.1	<0.0001	0.52 ± 0.06
	FHSPA	249	225	2.5	<0.0001	0.61 ± 0.05
	FOA	249	225	1.8	<0.0001	0.45 ± 0.07

(1) Heritability ± standard error of estimation; heritability of FHSPA and FOA were estimated using cubic root transformed data.

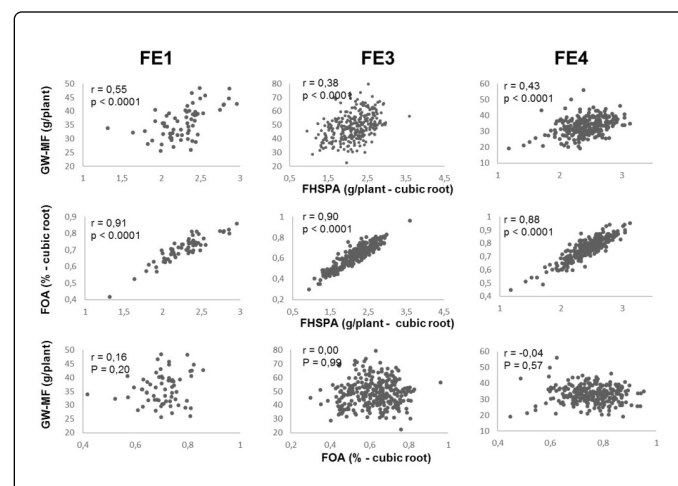
**Table 5:** Results of analysis of variance for MF-GW, FHSPA and FOA and the estimates of their heritability.



Similar features were observed in experiment FE3, which was designed to evaluate FHSPA, FOA and MF-GW in F<sub>3</sub> lines of the Cirad 464/Cirad 468 cross. Among the 374 F<sub>3</sub> lines sowed, 294 segregated for the *ms-IR36* gene. An average of 9.4 MS plants were observed in each F<sub>3</sub> line segregating for *ms-IR36* gene, a number in accordance with the expected 25% of the 40 plant per plot. The averages of adjusted means were 10.8 g/plant for FHSPA, 47.2 g/plant for MF-GW and 26.6% for FOA (Figure 2). The line effect was highly significant for all three traits (Table 5). At trial level, broad-sense heritability was 84% for FHSPA, 71% for FOA and 31% for MF-GW. The distributions of FHSPA showed both positive and negative transgressive segregation compared to both progenitors Cirad 464 and Cirad 468 used as checks (Figure 2). In both FE1 and FE3, the correlation was very high ( $r \geq 0.90$ ,  $p < 0.0001$ ) between FHSPA and FOA of the F<sub>3</sub> lines, rather loose but significant ( $r \leq 0.55$ ,  $p < 0.001$ ) between FHSPA and MF-GW, and not significant between FOA and MF-GW (Figure 3).

### FHSPA and FOA within the progenies of the recurrent populations

Among the 239 S<sub>1</sub> lines extracted from Msms-S<sub>0</sub> plants that were evaluated in field experiment FE4, all segregated for *ms-R36* gene. The average number of MS plants per line, 5.8, was slightly lower than the expected 6.3 plants, probably because the actual number of plants per plot was less than 25 in some plots and some MS plants had escaped tagging. The average of adjusted means was 14.5 g/plant for FHSPA, 33.4 g/plant for MF-GW and 44.0% for FOA (Figure 2). The line effect was highly significant for FHSPA, MF-GW and FOA (Table 5). Some S<sub>1</sub> lines exhibited significantly higher FHSPA than the seven A/B lines used as checks (Figure 2), suggesting a margin exists for further improvement of the trait. The correlation between FOA and FHSPA of the 239 S<sub>1</sub> lines was very high ( $r = 0.87$ ,  $p < 0.0001$ ), loose but significant between FHSPA and MF-GW ( $r = 0.42$ ,  $p < 0.001$ ), and not significant between FOA and MF-GW (Figure 3).

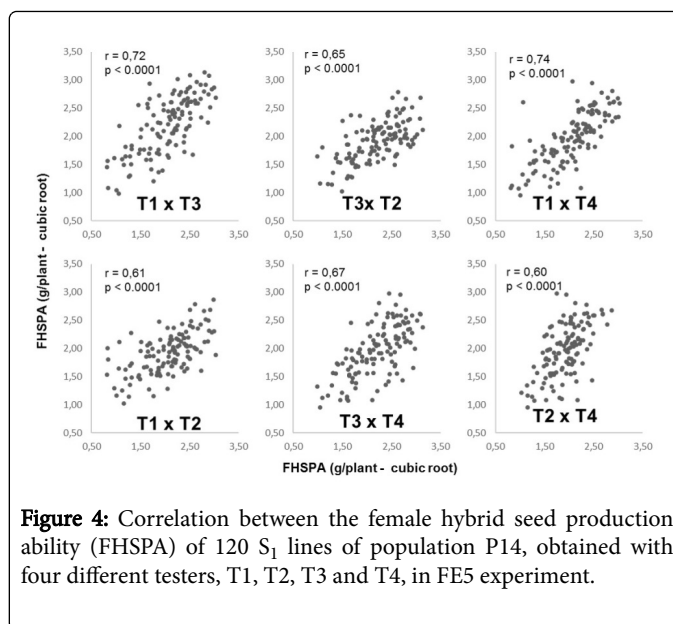


### FHSPA evaluated in the framework of seed production for testcrosses

The average number of MS plants harvested for each of the 480 plots of S<sub>1</sub> lines of P14 population (120 S<sub>1</sub> lines testcrossed by 4 testers), in the testcross seed production experiment FE5 was 13.3. This number is compatible with the expected number of 14.5 MS plants for each line theoretically represented by 58 plants. In practice, some lines were represented by fewer plants and some MS plants were miss-tagged.

The average FHSPA was 11.9 g/plant and highly significant differences were found between S<sub>1</sub> lines (Table 6). The tester effect was also highly significant, suggesting differences in pollinating ability, and/or in flowering synchronization, or/and in the combined effect of the two factors. The highest mean FHSPA (14.9 g/plant) was found in the tester Cirad 468 and the lowest mean FHSPA (9.0 g/plant) in the tester Cirad 464. Individual S<sub>1</sub> lines' FHSPA with each of the four testers were significantly correlated (Figure 4). Broad-sense heritability for FHSPA among the S<sub>1</sub> lines was 0.88.

Similar features were observed in FE6, using 79 S<sub>2</sub> lines of the same P14 population for hybrid seed production. The average number of MS plants was 9.6, markedly lower than the expected 12.5 plants. This was because the actual number of plants per S<sub>2</sub> line was often less than the originally planned number (50). The presence of a windbreak made of trees in the vicinity of the experiment resulted in a strong gradient of pollen dispersal in a number of plots. To take this gradient into account, the distance of each plot from the windbreak was added as a co-variable in the mixed model. The broad-sense heritability estimated with this model, 0.90, was of the same order of magnitude as the one observed with S<sub>1</sub> lines of FE5. Interestingly, a significant correlation (r=0.50 p<0.0001) was observed between the BLUPs of S<sub>2</sub> lines and BLUPs of corresponding S<sub>1</sub> lines.



**Figure 4:** Correlation between the female hybrid seed production ability (FHSPA) of 120 S<sub>1</sub> lines of population P14, obtained with four different testers, T1, T2, T3 and T4, in FE5 experiment.

In FE7, the average number of MS plants per plot was 7.3, very close to the expected 7.5 (25% of the 30 transplanted plants). The mean FHSPA of S<sub>1</sub> lines extracted from the P20 and P24 populations were 6.9 g/plant and 8.3 g/plant, respectively. Large intra-population variability was observed (Figure S3), and line and tester effects were highly significant (Table 6). Broad-sense heritability of FHSPA was 0.59 and 0.66 for populations P20 and P24, respectively. The FHSPA of S<sub>1</sub> lines under the two testers was significantly correlated in both populations (r=0.63, p<0.0001 for P20 population and r=0.36, p<0.0001 for P24 population) (Figure S4).

Experiment	Traits	Num DF	Den DF	F Value	Pr>F	Broad-sense heritability (1)	
FE5	FHSPA	123	369	8.6	<0.0001	0.88 ± 0.02	
	Tester effect	3	369	26.8	<0.0001		
FE6	FHSPA	77	229	10.2	<0.0001	0.90 ± 0.02	
	Tester effect	3	229	18.0	<0.0001		
FE7	P20	FHSPA	117	163	2.9	<0.0001	0.59 ± 0.07
		Tester effect	1	163	5.8	0.017	
	P24	FHSPA	49	163	2.9	<0.0001	0.66 ± 0.08
		Tester effect	1	163	18.8	<0.0001	

(1) Heritability ± standard error of estimation; heritability of FHSPA were estimated using cubic root transformed data.

**Table 6:** Results of analysis of variance for FHSPA and the estimates of its heritability in the testcross experiments.

### Discussion

Low yields in hybrid seed production fields is one of the limiting factors for the sustainable development of value chains based on hybrid rice. Thus, in hybrid rice breeding programs, the same attention should be paid to parental line traits that contribute to hybrid seed production ability as that paid to combining ability for grain yield or grain quality.

Under a given crop management system (layout of the MS and MF lines, plant density, synchronization of flowering, fertilizers, etc.), the yield of a hybrid seed production field depends on the total number of spikelets per plant (or surface unit) of the MS line, and the percentage of spikelets actually fertilized by the pollen of the MF line. The former parameter depends on the intrinsic tillering ability of the MS line and the associated number of spikelets per panicle, while the latter parameter mainly depends on the outcrossing ability of the female MS

line (FOA), and the amount of pollen produced by the MF parent. Breeding for hybrid seed production ability should consider either these three component-traits separately or a more synthetic parameter, female hybrid seed production ability (FHSPA), which is the grain yield of the outcrossed MS plants.

Whatever the option chosen, at least three factors determine the feasibility of breeding for hybrid seed production ability: the extent of genetic diversity for the component traits, the availability of a high throughput phenotyping method and the general structure of the breeding program.

### Genetic diversity for FOA and FHSPA

Our analysis of FOA within the  $F_3$  progenies of bi-parental crosses and within the  $S_1$  and  $S_2$  progenies derived from populations with a narrow genetic base revealed a very transgressive distribution of the trait. The extent of these distributions (ranging from zero to 89%) was as wide as that reported in the literature for the whole set of existing rice MS lines. Indeed, natural outcrossing of MS plants (i.e. not assisted by techniques that enhance the outcrossing rate in hybrid seed production plots) is reported to vary between zero and 44% [13,19]. Azzini and Rutger [29] observed a 5-32% outcrossing in the Birco CMS line. More recently, Cheng et al. [10] reported an outcrossing ability of 76% for the very popular CMS line, Zhong 9A. High diversity was also observed for FHSPA within both  $F_3$  progenies of biparental crosses and within  $S_1$  and  $S_2$  lines derived from populations, regardless of the extent of their genetic base. The rather tight correlation between the FHSPA of  $S_1$  lines and the FHSPA of  $S_2$  lines extracted from these  $S_1$  lines, demonstrates the high heritability of the trait. Such high heritability, despite extraction of only one  $S_2$  for each  $S_1$ , points to the possibility of efficient breeding for FHSPA in an early generation.

The high diversity for FOA and FHSPA within the very limited share of *O. sativa* genetic diversity used in this study, suggests that access to genetic diversity for FOA and FHSPA should not be a major problem for hybrid rice breeding programs dealing with *indica* germplasm (the progenitors of the bi-parental crosses and of synthetic populations used in the present study belonged to the *indica* group).

As low FOA is often considered to be a trait related to domestication, one can hypothesize that breeding for FOA may also affect yield potential and other traits of agronomic interest. However, the absence of a correlation between FOA and MF-GW we observed does not support this hypothesis.

Large and significant tester effects were observed for the mean-FHSPA of the  $S_1$  and  $S_2$  lines of different populations in experiments FE5, FE6 and FE7 conducted in the framework of test-cross seed production purpose. These significant effects confirm that the seed set of a MS plant is the result of an interaction between its own FOA and the outcrossing ability of the pollinator line.

### High throughput phenotyping method for hybrid seed production ability

Counting the number of grains per panicle and the seed set in a large number of samples to estimate FOA is often beyond the reach of small and medium scale breeding programs, especially in countries where labour is scarce. Instead of counting, weighing the filled spikelets of MS and MF plants, and using the ratio of these two parameters as a proxy for FOA, greatly improves the throughput of phenotyping. Differences in grain weight between fertile and sterile

plants of a given genotype (for instance CMS A line and maintainer B line, in a three-line hybrid system) might, in some cases, lead to an underestimated FOA. However, this is a minor risk in the context of screening for FOA where selection decisions are based on the relative scores of the breeding material, and not on absolute FOA scores.

We found very high significant correlations ( $r \geq 0.87$ ) between FHSPA and FOA of  $F_3$  lines extracted from two bi-parental crosses and of  $S_1$  lines extracted from a maintainer population. One can thus consider that breeding for FHSPA also makes it possible to breed for FOA, with the advantage of much easier measurement. Indeed, FHSPA can be evaluated in the framework of hybrid seed production for testcrossing, i.e. without dedicated experiments and resources.

But the success of such high throughput phenotyping methods for FOA and FHSPA relies on the availability of a large number of MS germplasms endowed with FOA and FHSPA variability, which is difficult to achieve in the framework of conventional breeding approaches for autogamous crops.

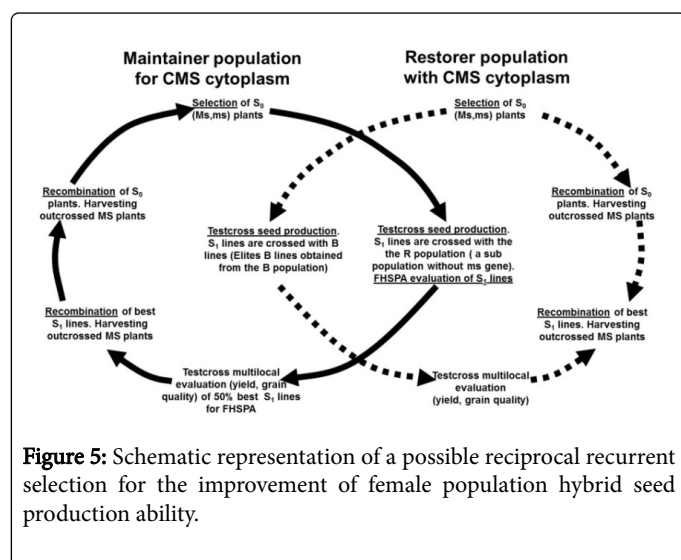
### Breeding strategies to harness rice genetic diversity for FOA and FHSPA

Xie (2009) [7] reported that when 92 IRRI-bred CMS lines were grown side-by-side in the field with their corresponding maintainer lines, the average seed set of CMS lines was 10.7%, and most (86%) lines had less than 20% seed set, compared with two Chinese commercial CMS lines that were averaging 47% seed set. Xie (2009) [7] also reported that IR58025A, the most popular CMS line in Asian countries, had a seed set of only 14.7%. He attributed the low outcrossing ability of IRRI-bred CMS lines to the fact that all CMS B lines were directly derived from inbred breeding programs. In fact, this was also the case of the early Chinese CMS B lines. However, in the 1990s, Chinese scientists extensively transferred traits that enhance FOA (especially stigma exertion) from exotic donors into CMS B lines of high general combining ability (GCA), using a backcrossing approach [30].

These reports are evidence that the evaluation of FOA and FHSPA of female lines often takes place at the end of their development process. Indeed, during the first stage of the development of female lines, the main target trait is the GCA. Due to the autogamous reproductive regime of *O. sativa*, limited quantities of hybrid seeds are produced for testcross purposes, through manual crossing of the female lines with one or more testers, which does not enable FOA and FHSPA estimation. The evaluation of FOA and/or FHSPA takes place during or after the long process of fixation and, with the three-line system, only after cytoplasm conversion. As the production throughput of such CMS A lines is low (a few lines per year), the chances of obtaining high FOA and FHSPA is limited. We are running an innovative hybrid rice breeding program that offers a more efficient framework for harnessing genetic diversity for FOA and FHSPA. It is structured in such a way that screening of B lines for GCA takes place after the screening for FHSPA. It is based on *ms*-IR36 MS-facilitated recurrent selection (RS), which has been extensively used to breed inbred lines [27,31-33]. The *ms*-IR36 gene cloned by Frouin et al. [26], facilitates marker-assisted screening of *ms*,*Ms* plants during the vegetative stage. Recurrent selection is a widely used breeding procedure for the improvement of allogamous crops [34,35].

We developed base populations for breeding female and male lines, and, in order to progressively move toward heterotic populations, these base populations are bred for their combining ability with each other

using a reciprocal recurrent selection (RRS) scheme [35-38]. The routine application of RRS for a female population consists (Figure 5) in: (i) selection and selfing of a few hundred plants among fertile plants of  $S_0$  or  $S_1$  progenies (all fertile plants are heterozygous for *ms-IR36* gene if  $S_0$  plants come from recombination of a population, but for  $S_1$  plants MAS or progeny tests, it is necessary to identify heterozygous plants with the *ms-IR36* gene); (ii) Testcross seed production process: after elimination of fertile plants, the remaining male sterile plants of  $S_1$  or  $S_2$  lines are pollinated with the reciprocal male population (a sub population without the *ms-IR36* gene), (see Materials and Method section; Figure 1 and Figure S2). Half to one third of the best progenies for FHSPA, evaluated through the yield of testcross seed production, are selected, and the corresponding testcrosses are evaluated for yield and grain quality; (iii) the best 10 to 15 progenies, selected using a selection index that take into account FHSPA and combining ability for yield and grain quality, are recombined to form a new population. Those progenies are also used, in parallel, to breed new female lines through pedigree selection. During the pedigree selection, FHSPA can be continuously tested by monitoring the *ms-IR36* gene using the associated SNP marker. Thus, the use of *ms-IR36* sterility gene makes it possible to enhance genetic gain simultaneous for combining ability and hybrid seed production ability.



**Figure 5:** Schematic representation of a possible reciprocal recurrent selection for the improvement of female population hybrid seed production ability.

## Conclusions

The extent of FHSPA genetic diversity observed within our breeding populations confirms the existence of high genetic diversity for this trait within the *indica* group of *O. sativa*. Population breeding through recurrent selection offers a favorable framework for harnessing this genetic diversity. The *ms-IR36* genic male sterility tool integrated in recurrent population breeding schemes, enables early ( $S_0$ ,  $S_1$ ,  $S_2$ ) screening for FHSPA, during the testcross seed production process at no additional cost. Given the available genetic diversity for FHSPA, the tight correlation of the trait with FOA, and its high heritability, rapid genetic gain can be expected for increased yield in hybrid seed production fields, and lower hybrid seed prices, paving the way for the wider and faster adoption of hybrid rice technology by farmers.

## Availability of Data and Materials

Phenotypic data is available in the Cirad database at <http://tropgenedb.cirad.fr/tropgene/JSP/interface.jsp>

module=RICE, (Choose Tab Studies) as Hybrid\_Rice.

## Competing Interests

The authors declare they have no competing interests regarding the present manuscript.

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## Authors Contributions

JT: Conceived and designed the experiments; JT, JD and NPC supervised the implementation of the field experiments; JT, T-VC, NPC and NA analyzed the data. JT and NA wrote the manuscript. NPC and JD revised and approved the draft.

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## Authors Information

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