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Efficiency of different hybridization methods in single crosses of rice for pure line breeding

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Abstract

Hybridization efficiency of two crossing methods (involving emasculation by hot water or by anther aspiration), was determined in rice. Quantitative comparisons between crossing methods for pure line breeding are scarce and the objectives of this work were to evaluate the advantages and disadvantages of emasculation methods, and to determine an adequate temperature for hot water emasculation. Hot water emasculation at a temperature suitable to each variety (followed by panicle pollination) achieved a higher percentage of hybrid F1 plants (54.6%) than anther aspiration (8.4%), but significantly more seed set was obtained after anther aspiration (31.5%) than after hot water immersion (16.5%). The easiest and quickest emasculation method was the immersion in hot water of panicles from detached rooted tillers.

Additional key words: crop improvement, crossing, emasculation techniques, *Oryza sativa* L.

Resumen

Eficiencia de distintos métodos de hibridación en cruzamientos de arroz para obtención de líneas puras

Se determinó la eficiencia de dos métodos de cruzamiento en arroz, implicando emasculación (castración) por agua caliente o por aspiración de anteras. Las comparaciones cuantitativas entre métodos de cruzamiento para obtención de líneas puras son escasas y por ello los principales objetivos de este trabajo fueron evaluar las ventajas y desventajas de ambos métodos de emasculación, así como determinar una temperatura adecuada de castración con agua caliente. La emasculación por agua caliente a una temperatura adecuada para cada variedad (seguida de polinización con panícula) obtuvo un mayor porcentaje de plantas F1 híbridas (54,6%) que la aspiración de anteras (8,4%), pero se obtuvieron significativamente más semillas tras aspiración de anteras (31,5%) que tras inmersión en agua caliente (16,5%). El método más fácil y rápido de emasculación fue la inmersión en agua caliente de las panículas de tallos con raíz, pero separados de la planta madre.

Palabras clave adicionales: cruzamientos, mejora vegetal, *Oryza sativa* L., técnicas de emasculación.

Introduction

Single crosses are usually made for combining different characters from two varieties or species, at the F1 generation in a crop breeding program. Afterwards, F1 plants have to be checked out for hybridity.

In rice (*Oryza sativa* L.), Baldi (1967) found that hand emasculation followed by pollination with anthers had a lower seed set than emasculation with water at 41-43.5°C for 5 or 8 min (which opens mature florets), followed by bagging two panicles together for pollination. This author reported varietal differences in seed set but he did not determine self-pollination rates. Water temperature should be high enough to kill pollen, but not so high as to kill ovaries. In contrast, Jennings *et al.* (1979), at IRRI (International Rice Research Institute, Philippines), claimed that emasculation at hot temperatures was slower and had lower seed set than hand emasculation or anther aspiration, apparently because culms were bent to introduce panicles

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Abbreviations used: CIAT (Centro Internacional de Agricultura Tropical), CRR (Centro Ricerche sul Riso), IRAT (Institute de Recherches Agronomiques Tropicales), IRRI (International Rice Research Institute), LSD (least significant difference), NS (not significant).

in a thermos bottle and often got broken, and because florets open only for a short time, when pollen must be available; however, methods were not quantitatively compared. Since then, emasculation at IRRI is made by anther aspiration (Datta *et al.*, 2002). They get 40-50% hybrid F1 plants in the dry season, 20-30% in the wet season (Pamplona, personal communication).

Sarkarung (1996) reported that at IRAT (Institute de Recherches Agronomiques Tropicales, France) and CIAT (Centro Internacional de Agricultura Tropical, Colombia), the emasculation methods based on whole plants were more laborious than those for detached tillers maintained in water, but no quantitative data were shown. Rawson (1998) tried to replace manual emasculation in rice by growing it in a rooting medium with boron restriction, in order to induce male sterility. However, he was not succesful.

We are not aware of recent published comparisons between hybridization methods for pure line breeding, probably because current publications on plant crossing are focused on large scale male sterility for hybrid production. It seems that pedigree rice breeders use the method they think is best for their progenitors and prevailing environments.

The present work was made at Departamento del Arroz (Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain), where a breeding program is conducted, doing crosses at a small scale. In our department, hand emasculation followed by brush pollination of crushed anthers achieved in the past an average of 48.2% hybrid F1 plants (unpublished data), but it has been replaced by quicker methods: anther aspiration and hot water emasculation. This study was carried out comparing the latter two crossing methods, in order to assess their advantages and disadvantages (including self-pollination rate) and to determine the optimal temperature for emasculation with hot water, in several varieties. The study is relevant because we are not aware of other published quantitative comparisons between hot water emasculation and vacuum emasculation in rice, and because published works only compare seed set, and not hybrid F1 plants.

Material and Methods

This work included emasculation tests and single crosses between different varieties. The origin of the plant varieties used was Spanish (Sarcet, JSendra, Sivert, Clavel, Albufera, Montsianell, Susi, Carmen, the inbred line L202 × YL33), Italian (Giovanni Marchetti, Drago, Cesare, Romolo) and Korean (Jinbubyeo). Parent seeds were sown in a greenhouse. Mother plants for hot water emasculation were transplanted outdoors to pots filled with clayey soil. Pollen donor plants and mother plants for anther aspiration were transplanted to concrete basins filled with the same soil. Concrete basins and pots had the same soil depth and surface per plant. Basins and pots received the same fertilization (N equivalent to 175 kg ha^{-1} in the form of ammonium sulphate, P_2O_5 equivalent to 112.5 kg ha^{-1} in the form of calcium superphosphate and K_2O equivalent to 208.3 kg ha^{-1} in the form of potassium sulphate). Average monthly temperatures during growth were: 22.3°C (May), 24.8°C (June), 26.2°C (July), 26.7°C (August). Mother plants were emasculated when one third to one half of the panicle was exerted, at the beginning of August, from 8:00 to 10:00 am. Self-pollination can occur by emasculating florets which are too mature. In order to minimize this risk, emasculation was effected in florets at the middle part of the panicles, while florets at the top and at the bottom were discarded. The two methods used were:

1. Anther aspiration (Van der Meulen, 1933, cited by Chandraratna, 1964; Baldi, 1967; McDonald, 1994), removing anthers with a glass connected by a plastic tube to a motor which acts as vacuum aspirator. Emasculation tests were performed in one panicle of each of two varieties in 2005. Hybridizations were made on several panicles of each mother plant variety.

2. Panicle emasculation with hot water (thermal emasculation): Matsubayashi *et al.*, 1965, cited by Tong and Yoshida, 2008; Baldi, 1967. Preliminary tests of hot water emasculation in our department showed that emasculation at 41°C produced about 70% self-pollinated seed and that emasculation at 47°C was lethal for the ovary in most cases. Panicles were introduced in a thermos bottle, after removing flag leaves. Before 2005, potted plants were placed upside down with a hand machine (to avoid culm breaks while inserting panicles in the thermos). One panicle of each variety was emasculated at each temperature. In 2005 and 2006, rooted tillers were detached, defoliated and preserved in fresh water after emasculation, which was renewed twice a week. This latter technique is a combination of the methods of Baldi (1967) and Sarkarung (1996), and is currently used at CRR (Centro Ricerche sul Riso, Italy). In both years, emasculation was tested with several varieties at three temperatures (43, 44 and 45°C), in order to discriminate the optimal value. In

Table 1. Effect of water temperature on the number of self-pollinated seeds with respect to emasculated florest (direct measure) in five rice varieties (whole plant emasculated in pots)

Variety	Immersion temperature[1]					
	43°C			45°C		
	f[2]	s[3]	s/f (%)	f	s	s/f (%)
Giovanni Marchetti	82	27	32.9	89	6	6.7
Sarcet	88	0	0	126	0	0
JSendra	70	1	1.4	45	0	0
Sivert	73	3	4.1	43	0	0
Clavel	84	0	0	129	3	2.3
Mean percentage (s/f)			7.7			1.8
Comparison between mean percentages			$t_6 = 0.91$ NS[4]			

[1] Five minutes of inmersion. [2] Number of florets emasculated. [3] Number of seeds produced. [4] NS: not significant.

2005, three immersion times (3, 4 and 5 min) were tested at each temperature, one panicle in each combination. In 2006, two panicles of each variety were emasculated at each temperature.

Pollination was carried out around midday. Florets can be pollinated during the time where they open or after clipping. Brush pollination (Font de Mora, 1939; Carrasco, 1952) was used after anther aspiration. Panicle pollination, by bagging two panicles together (Baldi, 1967), was used after hot water emasculation; in two crosses, it was performed in two consecutive days. Varieties differing in flowering date were sown at different dates.

A manageable number of F1 seeds from each cross were sown in the next spring and hybridity was checked morphologically by comparing height, leaf width, apex colour, hairyness or grain dimensions with the parents (Jennings *et al.*, 1979) or, in some cases, using DNA microsatellite markers.

Self-pollination percentage was measured in two ways: 1) directly: the proportion of florets that produce seed when panicles are emasculated but not pollinated; this value is inversely related to emasculation efficiency; 2) indirectly: the self-pollination percentage in a cross is the proportion of F1 plants which are not hybrid. In exceptional cases, there is pollen contamination from another variety. Pollination efficiency in a cross was measured as percentage of seed set with respect to florets pollinated. This estimate is biased by the self-pollination percentage (florets not completely emasculated). The global hybridization efficiency (pollination efficiency minus self-pollination percentage) was measured by the proportion of emasculated florets that produce F1 hybrids after crossing. It is a better estimate.

Mean percentages were compared statistically with *t* tests, but the analysis of hot water emasculation was effected by a factorial analyses of variance (ANOVA), with variety, temperature and time as fixed factors, after standardising seed set percentages by the arcsin of the square root, to get a normal distribution (Gómez and Gómez, 1976). Means were separated by Fisher's LSD. ANOVA was effected with a MSTAT program (Michigan State University, 1988).

Results

Hot water emasculation

Thermic emasculation of whole plants in pots with no pollination at 45°C resulted in a lower selfed seed set than at 43°C in some varieties, although the difference was not statistically significant (Table 1). In var. Giovanni Marchetti, 45°C was clearly more efficient, but there were no clear differences between temperatures in the rest of varieties (Sarcet, JSendra, Sivert and Clavel).

In the trials made with detached defoliated tillers, selfed seed set after emasculation was lower at 45°C than at 44°C in 2005 and 2006 (Table 2). No differences were detected between 43 and 44°C.

In 2005, there were no significant differences in selfed seed set between immersion times, but the effect of emasculation temperature was better discri-

Table 2. Effect of water temperature on the self-pollination percentage (direct measure on tillers detached, defoliated and emasculated)

Temperature	2005	2006
43°C	23.82ab[1]	29.0a
44°C	27.92a	30.0a
45°C	9.38b	10.1b

[1] Means separated after transforming percentages by the arcsin of the square root. Means with different letter in each column are different (LSD at P = 0.05).

minated and more realistic after 5 min immersion (Table 3).

In 2005, hot water emasculation was more efficient (higher pollen mortality) in Sarcet and Albufera than in JSendra (Table 4). However, in 2006, the differences in pollen mortality were not statistically significant. The number of self-pollinated seeds in var. Sivert was higher after hot water emasculation than after anther aspiration, while that of Sarcet was lower after hot water emasculation (Table 4).

Comparison of methods in single crosses

Table 5 shows the efficiency of both hybridization methods in several crosses, comparing F1 seed set and F1 hybridity. It can be seen there that the mean seed set with respect to florets pollinated was significantly higher after anther aspiration (31.5%) than after hot water emasculation (16.5%). However, the cross JSendra × Giovanni Marchetti, which was emasculated with hot water and pollinated in two consecutive days, obtained a high seed set (47.3%). On the other hand, hot water emasculation (with temperature adjusted to each variety), followed by panicle pollination, resulted in an average of 54.6% hybrid plants in the F1, a proportion significantly higher than after anther aspiration (8.4%). Global hybridization efficiency (proportion of hybrid F1 plants with respect to florets pollinated) was 6.5% on average after hot water emasculation, against 1.7% after anther aspiration, but the difference was not significant. Four of the crosses made after hot water emasculation achieved 100% hybrid efficiency, but three crosses were completely unsuccessful.

Table 3. Effect of water temperature and immersion time on the self-pollination percentage (direct measure) in 2005 (tillers detached, defoliated and emasculated)

Temperature	Immersion time		
	3 min	4 min	5 min
43°C	21.5a[1]	16.1a	33.9a
44°C	37.5a	30.8a	15.5ab
45°C	12.4a	8.0a	7.8b
Global mean percentage[2]	23.8a	18.3a	19.1a

[1] Means separated after transforming percentages by the arcsin of the square root. Means with different letters in each column are different between temperatures (LSD at P = 0.05). [2] Global mean percentages are not significantly different between times.

Table 4. Effect of variety on the self-pollination percentage after emasculation (direct measure)

Variety	2005		2006
	Hot water	Vacuum	Hot water
JSendra	33.22a[1]	—	27.55a
Sivert	22.69ab	0	—
Albufera	13.83b	—	15.85a
Sarcet	11.73b	19.5	25.65a
Grand mean	20.4	9.75	23.0

[1] Means separated after transforming percentages by the arcsin of the square root. Means with different letters in a column are significantly different (LSD at P = 0.05).

Discussion

In the hot water emasculation tests made in 2005, the effect of water temperature was more clearly seen when panicle immersion time was 5 min (Table 3). Therefore, that was the time used in 2006. Hot water immersion acts simultaneously upon the whole panicle: when temperature and panicle stage are appropriate, emasculation is 100% effective (this was the percentage of hybrid F1 plants of four crosses), but if temperature is too low to kill the anthers of a particular variety, emasculation can fail completely (this is possibly the case of three crosses with no hybrids) (Table 5). Emasculation with water at 45°C achieved a lower self-pollination percentage than at lower temperatures (Table 2). Tong and Yoshida (2008) similarly reported effective rice emasculation at 45°C and 46°C. Therefore, if the breeder does not have time to test the best water temperature for emasculating a variety, it would be safer to use it. The hot water treatment applied to detached tillers (which thereafter were preserved in water), was the easiest and quickest of the methods reported here:

Table 5. Effect of emastulation technique and varieties crossed on hybridization efficiency: F1 seed set (number of seeds produced) and F1 hybridity

Cross	Seed set			Hybrid % of F1 plants			Global efficiency
	f[1]	s[2]	s/f (%)	F1	h[3]	h/F1 (%)	h/f (%)
Cross							
JSendra × Montsianell	175	27	15.4	12	3	25	1.7
Drago × Sarcet	176	30	17.0	8	5	62.5	2.8
Clavel × Susi[4]	160	62	38.8	0	50	0	0
Clavel × Carmen	127	13	10.2	2	10	20	1.6
JSendra × Giovanni Marchetti[4]	91	43	47.3	30	30	100	33.0
JSendra × Cesare	385	39	10.1	33	33	100	8.6
JSendra × Jinbubyeo	265	47	17.7	35	35	100	13.2
Sivert × Jinbubyeo	263	7	2.7	6	6	100	2.3
Albufera × Romolo	355	35	9.9	28	30	93.3	7.9
Susi × (L202 × YL33)	280	1	0.4	0	1	0	0
Giovanni Marchetti × Sarcet	228	27	11.8	0	14	0	0
Mean percentages			16.5			54.6	6.5
Vacuum							
JSendra × Montsianell	250	57	22.8	6	30	20	2.4
Drago × Sarcet	389	68	17.5	5	44	11.4	1.3
Clavel × Susi	180	40	22.2	2	33	6.1	1.1
Clavel × Carmen	118	32	27.1	0	28	0	0
JSendra × Giovanni Marchetti	331	73	22.1	3	40	7.5	0.9
JSendra × Cesare	121	64	52.9	0	49	0	0
JSendra × Jinbubyeo	127	65	51.2	8	50	16	6.3
Sivert × Jinbubyeo	95	24	25.3	3	20	15	3.2
Albufera × Romolo	90	25	27.8	2	24	8.3	2.2
Susi × (L202 × YL33)	126	75	59.5	0	41	0	0
JSendra × Sarcet	253	47	18.6	2	25	8	0.8
Mean percentages			31.5			8.4	1.7
Comparison between mean percentages (hot water vs *vacuum)*		t_{20}=2.38*			t_{10}=3.32**		t_{11}=1.15NS[5]

[1] Number of florets emasculated. [2] Number of seeds produced. [3] Number of hybrid F1 plants. [4] Pollination on two consecutive days. [5] NS: not significant. * Significantly different at p(α) ≤ 0.05. ** Significantly different at p(α) ≤ 0.01.

tillers can be taken from any plant (in pots or in soil), because there is no need to get pots upside down.

When selfing percentage was measured directly, it was higher after hot water emasculation than after anther aspiration (Table 4), but comparisons were too few to draw conclusions.

Crosses made after hot water emasculation achieved less seed set percentage than after anther aspiration (Table 5), as mentioned by Jennings *et al.* (1979). At IRRI, anther aspiration can achieve a higher proportion of hybrid F1 plants (40-50%) than in our crosses. There are two possible causes to explain it. 1) If anther aspiration is not complete, leaving some anthers unremoved, they are an additional source of self-pollination. At IRRI, panicles are inspected in the early next morning after emasculation, and those with left anthers hanging from the florets are discarded (Pamplona, personal communication). We will try to add this practice in our future crosses. 2) We emasculated in the early morning, while emasculation at IRRI is done in the late afternoon.

Baldi (1967) detected a genetic component in seed set after crossing, both among maternal parents and among pollinators. In our study, genetic differences were also detected: hot water emasculation in Sarcet and Albufera was more efficient than in JSendra (Table 1); hot water emasculation in Sarcet was more efficient than in Sivert (Table 4).

In the present study, the hybridization method that resulted in a higher proportion of hybrid F1 plants was hot water emasculation at a suitable temperature for each variety, followed by panicle pollination: an average of 54.6% (Table 5), slightly higher than the proportion obtained after hand emasculation in the past in our department (48.2%, data not shown). If pollination is carried out on two consecutive days, seed set can be improved: an average of 21.9% in 2007 crosses was obtained with different varieties after hot water emasculation. However, no method obtained hybrid plants in all crosses (data not shown). Therefore, F1 hybrid production at a small scale could be better insured by using both hot water emasculation and anther aspiration.

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