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# THE DEVELOPMENT OF POTENTIAL DROUGHT- AND SALINITY-TOLERANT RICE (*Oryza sativa* L.) THROUGH HETEROSIS BREEDING

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**Abstract:** Climate change and extreme conditions significantly reduce rice production. In order to improve food security in Malaysia, the development of high-yielding rice varieties with drought and salinity tolerance are of utmost importance. In this study, $F_1$ hybrids were developed using MR263 and MR219 (high-yielding rice) and IURON 6, IURON 14, IURON 18 and IURON 21 (upland rice) as female parents, with drought-tolerant variety Dular and salinity-tolerant variety Pokkali as male parents to generate different $F_1$ hybrid combinations in the field. The $F_1$ phenotyping screening was done based on the diameter of the stomata opening, chlorophyll content, plant height, length of flag leaf, diameter of flag leaf, length of seeds and width of seeds. Significant differences ($p < 0.05$) were observed for most traits when $F_1$ hybrids were compared with parental cultivars, indicating the deployment of drought- and salinity-tolerant genes from the male parents. A significantly ($p < 0.05$) smaller stomata opening at midday with a high grain width compared with the female plant (MR263) and high grain length compared with the male plant (Pokkali) were observed in $F_1$ hybrids M(i-iii) and M(iv). The development of doubled haploid plants is currently ongoing, thus would reduce cultivar development time.

Keywords: Drought tolerance, salinity tolerance, phenotyping screening, *Oryza sativa* L.

## Introduction

Rice (*Oryza sativa* L.) is one of the commodity crops that has been cultivated in Malaysia since ancient times. Worldwide, it is the second most important crop after wheat. It is grown in 115 countries, and on more than 150 million ha globally (Rajamoorthy *et al*., 2015). The recent economic crisis has forced more people in Asia to depend on the main diet due to the higher cost of varying diets (Najim *et al*., 2007). Therefore, increased productivity is the only viable option to meet growing demand and keep rice affordable for millions of people. Drought or water deficit is one of the abiotic stresses that has been the single largest factor limiting (Lesk *et al*., 2016) rice yield in approximately 46 million ha of rainfed rice fields in Asia (Li & Xu, 2007; Lesk *et al*., 2016). Furthermore, climate change has significantly affected rice production due to severe temperatures and levels of carbon dioxide, as well as severe water shortages (Spinoni *et al*, 2014). This subsequently affects the economic growth of Malaysia (Vaghefi *et al*., 2011).

The Dular variety has been reported to have a greater root length density at depth compared with other high-yielding but drought-susceptible varieties, such as IR64, and thus declared as a drought-tolerant variety (Henry *et al*., 2012; Munasinghe *et al*., 2017). Likewise, Pokkali had better growth than the Koshihikari variety under both saline and non-saline conditions (Abdelgadir *et al*., 2005) and has been used as a salt-tolerant variety in several molecular studies (Reddy *et al*., 2009). The IURON 6, IURON 14, IURON 18 and IURON 21 are upland rice varieties that harbour great genetic potential for rice improvement as they are subjected to subtle selection over a long period of time (Singh *et al*., 2013a). Moreover, IURON 6 has good features, like grain quality, zinc tolerance and blast disease resistance (Meng *et al*., 2016). Similarly, MR263 is a variety that was produced by the crossing of two mutants; SPM 156 (the Kurau Wangi mutation induction) and MR221 (the high quality Q31 mutation induction) (Zainudin *et al*., 2012; Asfaliza, 2013). Whereas MR219 is a famous high-yielding variety with excellent grain quality (i.e. shape and taste) (Alias, 2002).

Therefore, the combination of drought- or salinity-tolerant genes in high-yield rice cultivars could be the solution in reducing the impacts of drought and high salinity in water sources to ensure food security. Hybrids are produced from a single crossing of two genetically dissimilar parents, which tends to create varieties that mature earlier, produce higher yields and has a greater uniformity (Teo, 2007). They display heterosis or hybrid vigour, and the yield increase in hybrid rice is about 15 to 20% than existing high yielding varieties (Singh *et al*., 2013b).

Physiological parameters, like the opening of the stomatal pores in the epidermis, provide gates for photosynthesis and transpiration and ultimately improve water-use efficiency by reducing water use through mechanisms that regulate or determine the stomatal conductance of water vapour, which involves the stomatal number (density), stomatal aperture, the cuticle and the boundary layer (Yoo *et al*., 2009; Kusumi *et al*., 2012). The stomatal aperture and conductance in rice are strongly correlated to leaf photosynthesis (Ishihara & Saito, 1987; Hirasawa *et al*., 1988).

Heterosis or hybrid vigour is the superior performance of hybrids relative to their parents (Birchler *et al*., 2010; Goff & Zhang, 2013). Hybrid variety Nanyou No. 2 has good heterosis and high-yield potential and was the first commercialized hybrid variety, which was introduced to the world by China in late 1970s (Karplus & Deng, 2007). In Malaysia, Siraj 297 is one of the hybrid varieties that is resistant to rice blast diseases and boosts rice production. It was introduced by the Malaysian Agricultural Research and Development Institute (MARDI). Malaysia has developed high-productivity rice varieties and come up with high-yielding cultivars, such as MR84, MR219, MR263, MR220, MR297, including recent improved rice variety, PadiU PUTRA (Adedoyin *et al*., 2016; Afifi, 2018). However, many of these varieties are sensitive to drought and high temperature stresses.

Several grain characters, such as the number of panicles, grains per panicle, grain yield, weight and seed shape, are useful in identifying rice hybrids and are also valuable in the seed certification process to control field and seed standards (Geetha *et al*., 1994; Kalaichelvan, 2009). Rajanna *et al.* (2011) found that longer grains will produce narrower grains, while shorter grains will produce broader grains, and these characters can be used to group hybrids and parental lines into distinct classes.

The main purpose of this study is to assess the contribution of Dular and Pokkali cultivars to $F_1$ progenies in terms of different morpho-physiological traits in rice. The information obtained from this study may provide clues to the interest of hybrids as potential sources for new drought or salinity tolerance accessions that are tested in the field following doubled haploid production in the future.

## Materials and Methods

### *Plant Materia*ls

The varieties used to develop the hybrid is presented in Table 1. The cultivation of parental lines were done at the Bukit Kor campus (5° 12.64'N 103° 09.96'E), Universiti Malaysia Terengganu. Twenty-five (25) plants from every variety was cultivated randomly in 15.24 metre (m) of plant bed (each plant was 0.61m x 0.61m) that was cultivated and irrigated three times a day. Crosses were made between May to June 2018.

Table 1: List of cultivars used to develop hybrids in the field (May-June 2018)

| Cultivar | Origin | Ecosystem |
|---|---|---|
| Dular | India | Lowland |
| Pokkali | India | Lowland |
| IURON 14 | IRRI | Upland |
| IURON 21 | IRRI | Upland |
| IURON 18 | CIAT | Upland |
| IURON 6 | Indonesia | Upland |
| MR263 | MARDI | Lowland |
| MR219 | MARDI | Lowland |

IRRI: International Research Rice Institute;
CIAT: International Center for Tropical Agriculture

***Emasculation and Hybridization***

The top and bottom of one-third of the desired female panicles were cut the previous evening. The anther was removed before the pollen was extruded from the female flower using tweezers swabbed with 70 % ethanol. The emasculated spikelets were covered with butter paper to avoid cross pollination from an unintended pollen source. The plant was then tagged with the date of emasculation. The extruded pollen from desired males (cv. Dular or Pokkali) were taken and inserted into the stigma of each emasculated spikelet the next morning (around 7am to 10am). Following pollination, the whole panicle was covered again using butter paper to avoid undesired cross pollination. The newly pollinated flower was tagged, which noted the female parent, cross signed, the male parent and the date of the crossing. The tag was tied at the base of the panicle to identify the crossed ones.

***Phenotyping Screening***

All parental lines and potential hybrids were cultivated in the same environment (UMT greenhouse) on the same day. Hybrid confirmation through phenotyping or morphological screening, such as the diameter of the stomata opening, chlorophyll content, plant height, length and diameter of flag leaf, and length and width of seeds, were observed and compared for all parental lines and potential $F_1$ hybrids. The potential $F_1$ hybrids that showed a significant difference ($P < 0.05$) compared with the female parent might have inherited morphology characteristics from the male parent (Langevin *et al*., 1990; Scarascia-Mugnozza *et al*., 1997) in optimal greenhouse conditions. The purpose of this phenotyping screening was to evaluate $F_1$ hybrids and parental lines in optimal conditions for them to fully express their highest potential (Ma'arup *et al*., 2019). The $F_1$ hybrids were evaluated from different individual female parent plants to assess the genetic behaviour (for exploitation of heterosis) of easily observable characteristics and also to identify accidental self-fertilization plants, which are expected to resemble the female parents.

***Diameter of Stomata Opening***

The diameter of the stomata opening for each cultivar was measured from the flag leaf during a sunny day between 12 pm and 2 pm with a temperature of 31°C. Nail polish was applied on the underside of the leaf and the patch formed after it dried was peeled off with a tape. The tape was put on a microscope slide and observed under a microscope. The diameter of the stomata opening (µm) was observed under a digital compound microscope (BX43) at a 100x magnification and was documented by a digital camera (Olympus model DP21). In each cultivar (parental lines) and hybrid, three flag leaves in three replicates were used to record these measurements.

***Chlorophyll Content***

The chlorophyll content of leaves was measured using a handheld SPAD 502-Plus chlorophyll meter (Konica Minolta Sensing Inc, Osaka, Japan). In each cultivar (parental lines) and hybrids, three flag leaves in three replicates were used to record these measurements.

***Plant Height***

The height of the main tiller was recorded at maturity from the ground to the top of the spike. The measuring tape was placed flat on the soil surface and the height was expressed in centimetres. In each cultivar (parental lines) and hybrid, three replicates were used to record these measurements.

***Length of Flag Leaf***

The length of the flag leaf was measured from the point just above the main shoot to the tip of the flag leaf blade by using a measuring tape and expressed in centimetres. In each cultivar (parental lines) and hybrid, three replicates were used to record these measurements during the flowering stage.

***Diameter of Flag Leaf***

The diameter of the flag leaf was measured at the widest part of the flag leaf blade by using a measuring tape and expressed in centimetres. In each cultivar (parental lines) and hybrid, three flag leaves of three replicates were used to record these measurements during the flowering stage.

***Length of Grain***

The seed length was measured using an electronic digital caliper as the distance from the base of the lower most sterile lemma to the tip of the lemma or palea, and the mean seed length was expressed in milimetres. In each cultivar (parental lines) and hybrid, three seeds of three replicates were used to record these measurements.

***Width of Grain***

The seed width was measured using an electronic digital caliper as the distance across the lemma and palea (the widest point) and the mean seed width is expressed in mm. In each cultivar (parental lines) and hybrid, three seeds of three replicates were used to record these measurements.

***Stem Colour***

The stem colour was observed at the base of the plant stems. The observation was recorded during the tillering stage, when the colour of stem was most obvious (Appendix A).

***Heading Days***

The number of days to heading was recorded from the date of sowing to the date when 50% of the spike has emerged (i.e., middle of the spike at the flag leaf ligule) on 50% of all stems (3 replicates).

***Heterosis Over Mid-Parent***

Heterosis is the phenomenon where the progeny showed vigorous characteristics compared with the parents. A mid-parent heterosis indicated that a trait displayed a hybrid performance that was significantly better than the average (mid-parent) value of the two parental inbred lines (Hochholdinger & Hoecker, 2007). The heterosis over mid-parent was calculated using equation 1:

$$\text{Heterosis over mid-parent (H)} = \frac{\text{F1} - MP}{MP} \text{ X } 100 \quad (1)$$

where F1= Potential hybrid characteristic mean; MP = Mean of the parent characteristcs (i.e. plant height trait). The value of H above 100% indicated that the hybrid was superior to the highest parent, and presented a positive heterosis over the highest parent.

***Statistical Analysis***

All results were statistically compared using IBM SPSS version 20 (IBM Corp, Armonk, New York, USA). One-way ANOVA was used to analyse the data, followed by Tukey's

Multiple Comparison. Significant difference was considered at P < 0.05. The results were presented as means, and compared between parental lines and potential hybrids.

## Results and Discussion

### *Plant Height and Heading Days*

All hybrids were significantly ($p < 0.05$) taller compared with the female (MR219) parents, except S(ix) for plant height (Figure 1). The overall plant height was significantly ($p < 0.05$) different for hybrids compared with their parents (data not shown). The taller plants intercepted more light, had longer internodes, larger leaves and more rapid canopy closures (Dougher & Bugbee, 1997; Sankaran *et al*., 2019), and most of the hybrids (Si-Sviii) inherited male (Dular) traits in plant height (Figure 1). Thus, it did influence canopy temperature in most experiments. Potential hybrid M(iii) produced head early (72 days) compared with its parents Pokkali (82 days) and MR263 (86 days) (data not shown). Most of the accessions identified as being from drought-tolerant donors was early flowering (Torres *et al*., 2013; Basu *et al*., 2016). Plant height and heading days were the highest heritability that had been observed in grain (Zhou *et al*., 2016; Ma'arup *et al*., 2019).

### *Heterosis in Hybrids*

Heterosis refers to the superior phenotypes observed in hybrids relative to their inbred parents with respect to traits such as growth rate, reproductive success, and yield (Ben & Lavi, 2012). Generally, all parameters were moderate for both positive and negative heterosis values for potential $F_1$ hybrids, except for Kii and Kiii hybrids from IURON 21 x Dular crosses, which showed a heterosis value of more than 100 (Table 2) for the diameter of the stomata opening (Figure 2). This suggests the overdominance hypothesis, which assumes that the heterozygous combination of the alleles at a single locus is superior to either of the homozygous combinations of the alleles at that locus (Xiou *et al*., 1995). Mostly, the rest of the potential $F_1$ hybrids exhibited negative heterosis in other parameters.

### *Diameter of Stomata*

Significant differences ($p < 0.05$) were observed among all potential hybrids for the diameter (Figure 2) of the stomata opening when compared with their respective parents (Figure 3). Stomata are pores found in the epidermis of leaves and stems, where the pores are bordered

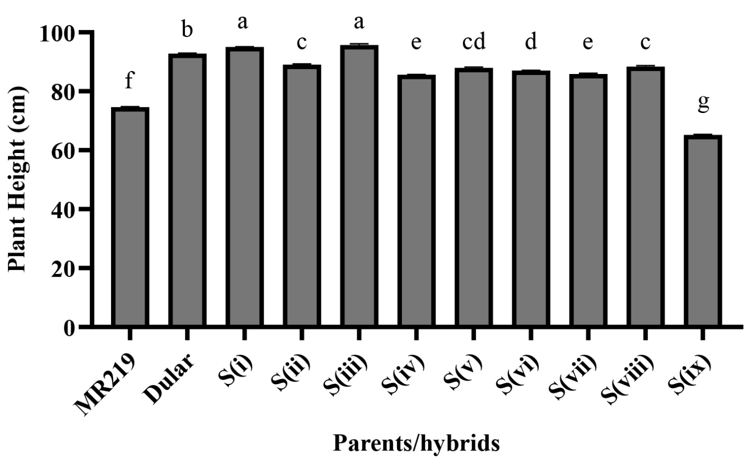


Figure 1: Comparisons between hybrids (MR219 X Dular) and their parents in plant height. Different letters indicate significance at $p < 0.05$ (replicates, n=3). The values are the means and the error bars are the standard error. S(i, ii, iii, iv, v, vi, vii, viii, ix) represent $F_1$ hybrids obtained from different individual female plants (MR219)

Table 2: The heterosis of potential $F_1$ hybrids over mid-parents

| Parents | Hybrids $F_1$ | Heterosis (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Diameter stomata | Chlorophyll content | Plant height | Length flag leaf | Diameter flag leaf | Length of Seeds | Width of seeds |
| IURON 18 x Dular | Aiii | -29.95 | -4.36 | -2.18 | 2.59 | 9.33 | 3.18 | -14.74 |
| | Aiv | 5.27 | 2.433 | -4.594 | -11.72 | 6.999 | 0.72 | -19.4 |
| | Av | 18.27 | -17.87 | -0.81 | -20.39 | -23.24 | - | - |
| IURON 18 x Pokkali | Ai | -32.21 | -17.99 | -15.34 | -1.48 | 9.52 | - | - |
| | Avi | 15.29 | 1.12 | 17.54 | -27.314 | 16.43 | -6.67 | -8 |
| IURON 21 x Dular, | Kii | 149.32 | -1.63 | 13.9 | -0.05 | -5.41 | -5.66 | -7.35 |
| | Kiii | 122.63 | 2.23 | -1.943 | 44.4 | -10.11 | 4.78 | -8.79 |
| IURON 21 x Pokkali | Ki | 8.45 | 2.03 | -30.88 | -17.76 | -14.97 | -2.5 | -21.81 |

by a pair of specialized cells known as a guard cell, which is responsible for governing the stomata opening size (Pirasteh-Anosheh *et al*., 2016). The stomata have two key functions, which are controlling transpiration, which supplies nutrients and regulates leaf temperature, and controlling the entry of $CO_2$ into the leaf (Martin-St Paul *et al*., 2017). The density of the stomata was not an appropriate index for the screening of almond seedlings for drought resistance, while a smaller stomatal size could potentially be an appropriate index for drought-tolerant seedlings (Pirasteh-Anosheh *et al*., 2016). In this study, most of all the potential hybrids have a smaller stomatal size compared with the mother plant. Thus, it could be said that they may have inherited the drought-tolerant genes from the male parents. Mohammed *et al*. (2019) found that overexpression of the Epidermal Patterning Factor 1 (*OsEPF1*) gene in rice plants would reduce stomatal densities and increase the root cortical aerenchyma formation, resulting in lowered leaf stomatal conductance and enhanced water-use efficiency. It is likely that the diameter and densities of stomata will contribute towards water loss in rice plants and further observation should be undertaken into root-to-shoot signalling events under two contrasting moisture regimes following doubled haploid production from these hybrids.

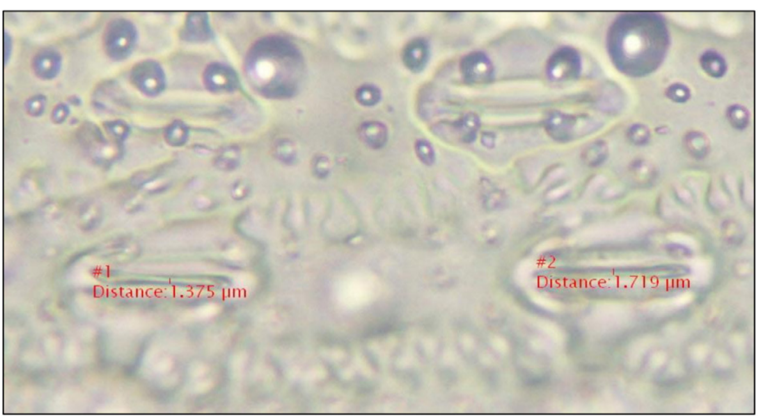


Figure 2: The diameter of the opening of the stomata observed under a compound microscope. (x100; replicates, n=3) in hybrid (Miv; MR263 X Dular; Figure 3b)

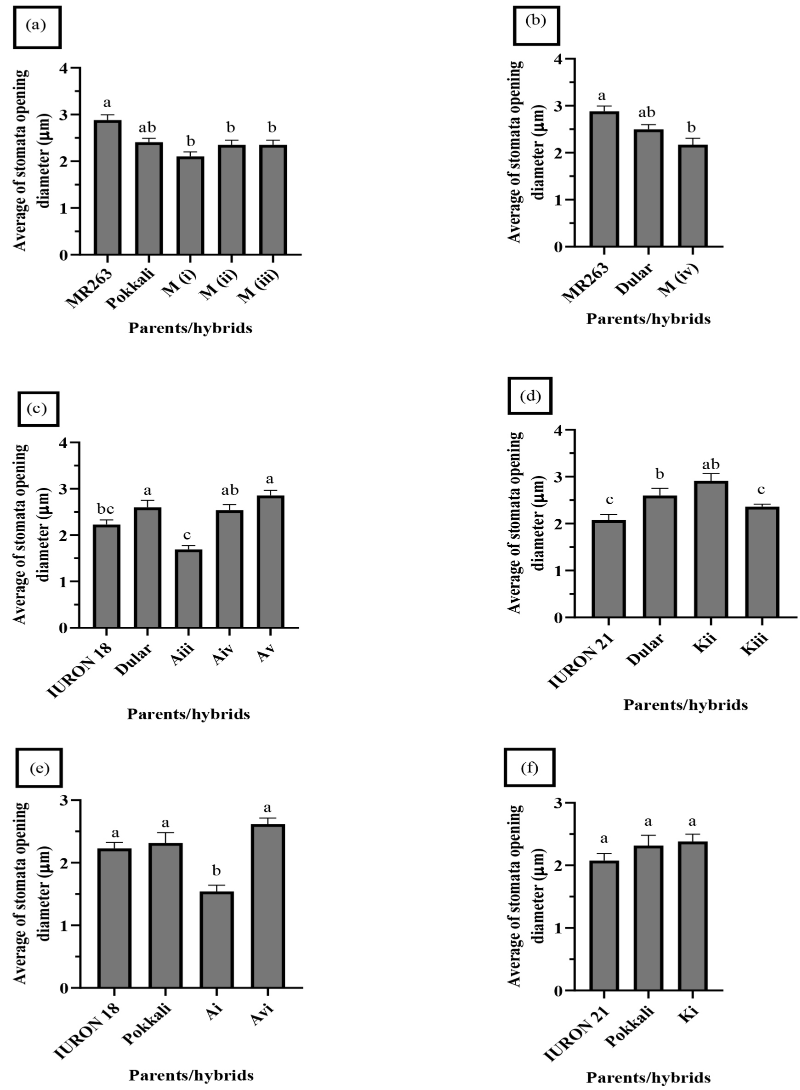


Figure 3: Comparison between potential $F_1$ hybrids of (a) MR263 x Pokkali, (b) MR263 x Dular, (c) IURON 18 x Dular, (d) IURON 21 x Dular, (e) IURON 18 x Pokkali, and (f) IURON 21 x Pokkali, and their parents in stomata opening diameter. Different letters indicate significance at $p < 0.05$ (replicates, n=3). The values are the means and the error bars are the standard error. M(i, ii, iii), A (iii, iv,v), K(ii, Kiii) and A (i,vi) represent $F_1$ hybrids obtained from different individual female plant

### *Length and Width of Grain*

Most of the hybrids exhibited significant differences at ($p < 0.05$) among all potential hybrids for the length and width of grain when compared with their respective mother plants (Figures 4 and 5). In short, most of all the potential hybrids have a shorter length of grain compared with their mother plants (Figure 4) and an intermediate width (Figure 5) of seeds compared with both parents. Therefore, most of the hybrids inherited these characters from their male parents (Dular or Pokkali). The seed size and shape have been used to differentiate the rice genotypes and Ramaiah and Rao (1953) suggested that the genes governing length also partly govern the seed shape. Rajanna *et a*l (2011) stated that the shape, length and width of grain were found useful for the identification and grouping of hybrids and parents to maintain genetic purity during seed production. Grain length and width were highly heritable to the $F_1$ generation (Rafii *et al*., 2014). Thus, grain size and shape are closely related to yields of head rice or unbroken grain.

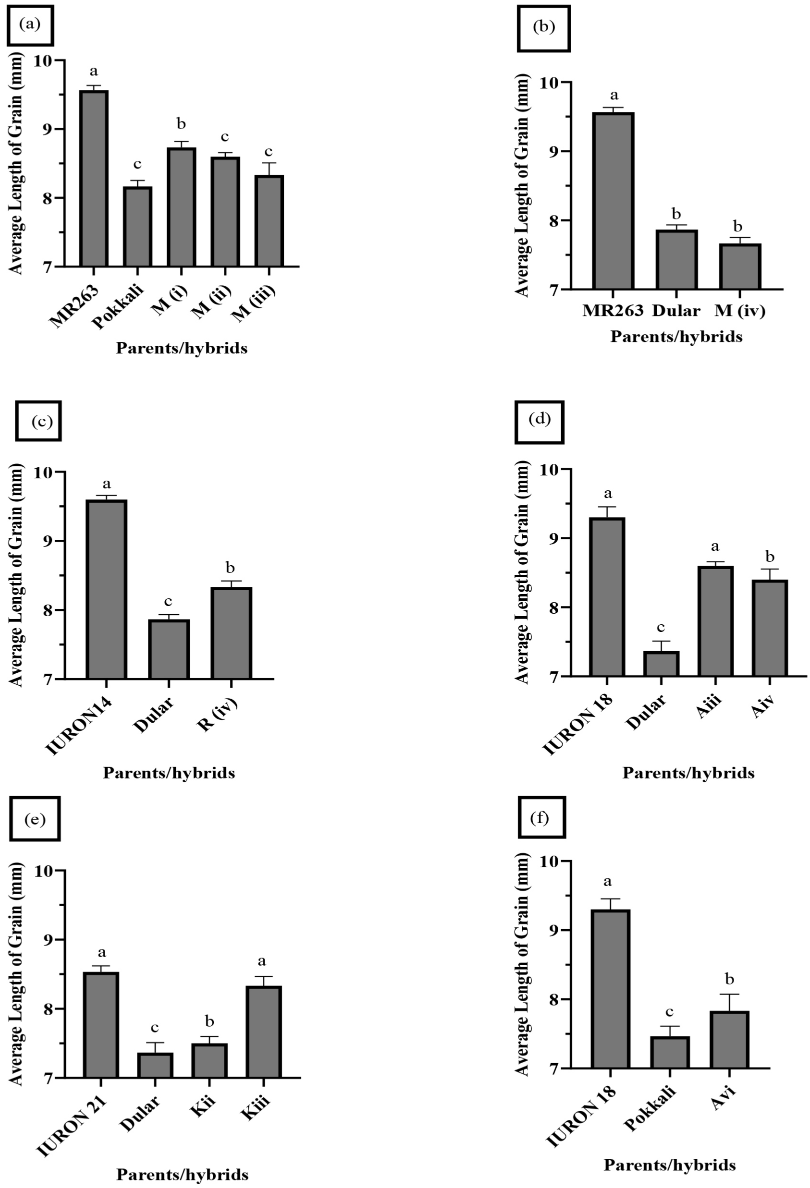


Figure 4: Comparison between potential $F_1$ hybrids of (a) MR263 x Pokkali, (b) MR263 x Dular, (c) IURON 14 x Dular, (d) IURON 18 x Dular, (e) IURON 21 x Dular, (f) IURON 18 x Pokkali and their parents in length of grains. Different letters indicate significance at $p < 0.05$. The values are the means and the error bars are the standard error (replicates, n=3). M(i, ii, iii), A (iii, iv) and K(ii, Kiii) represent $F_1$ hybrids obtained from different individual female plant

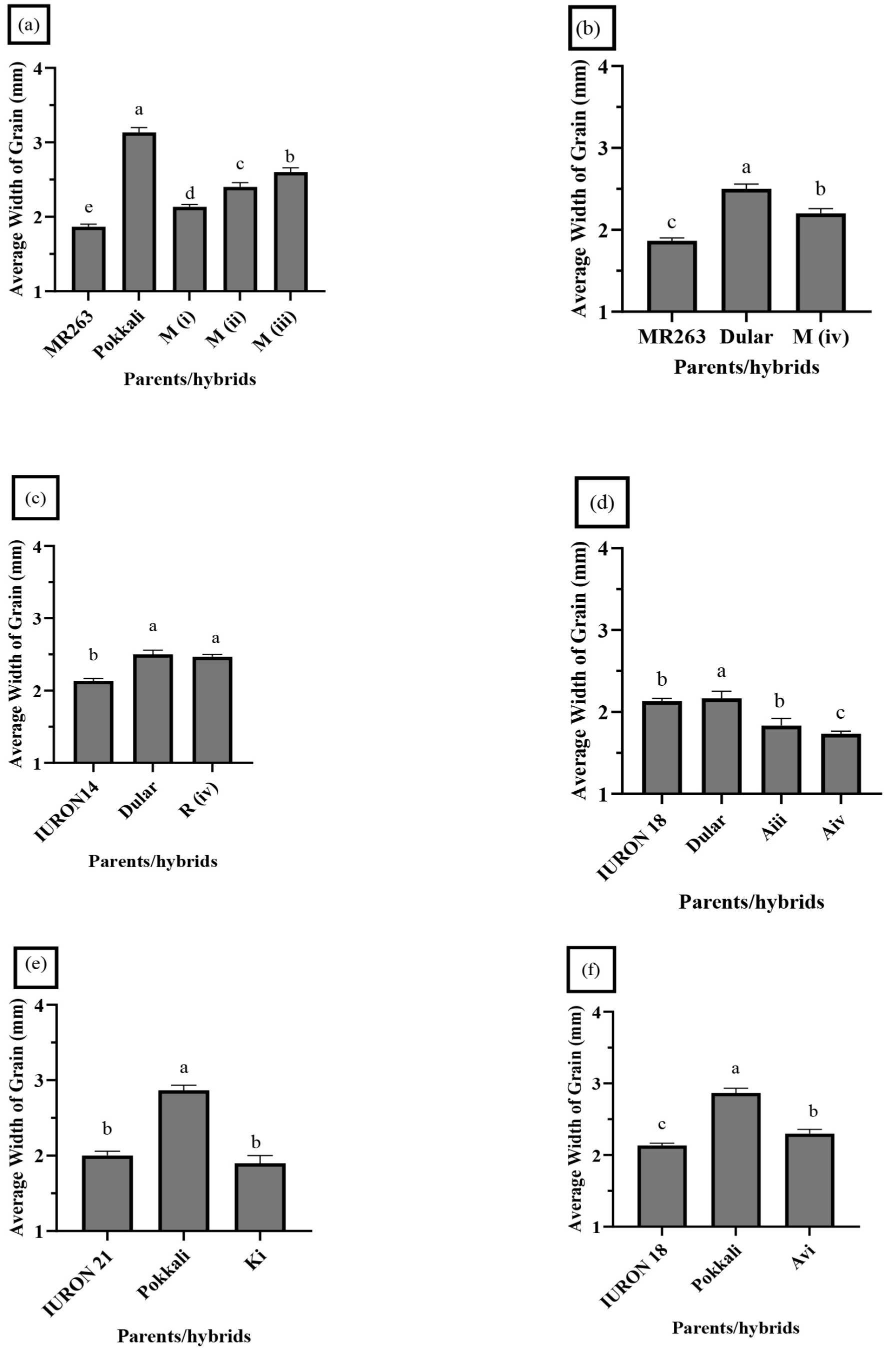


Figure 5: Comparison between potential $F_1$ hybrids of (a) MR263 x Pokkali, (b) MR263 x Dular, (c) IURON14 x Dular, (d) IURON 18 x Dular, (e) IURON 21 x Pokkali, (f) IURON 18 x Pokkali and its parents in width of grain. Different letters indicate significance at ($p < 0.05$). The values are means and the error bars are the standard error (replicates, n=3). M(i, ii, iii) and A (iii, iv) represent $F_1$ hybrids obtained from different individual female plant

## Conclusion

The high vigour of hybrids compared with their parents in terms of stomata opening at midday, grain characteristics and other plant morphological characteristics that differed significantly between the hybrids and parental lines support previous evidences that these hybrids may inherit the drought (Dular) or salinity (Pokkali) tolerance of their parents. This has been observed in hybrids M(i-iii) and M(iv), which have shown significantly less stomata opening at midday, with a high grain width compared with the female plant (MR263) and a high grain length compared with the male (Pokkali). Also, the potential $F_1$ hybrids have been continued for embryo rescue for double haploid production to speed up the breeding program and they are pure enough for the seed certification process after reaching $F_4$ (filial 4$^{th}$). Therefore, it eliminates genetic variations that remain within a breeding line through self-fertilization. However, further experiments should be carried out under two contrasting moisture regimes to identify the successful transfer of desirable traits from the male parent.

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*Abbreviations:* Cv, cultivar; IRRI, International Research Rice Institute; CIAT, International Center for Tropical Agriculture.

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**Appendix**

**Appendix A**

Stem colour of (a) MR263 which is green, (b) Dular which is red and (c) Hybrid ($F_1$ of MR263 X Dular) which is also red.