In Vitro PRODUCTION AND ASSESSMENT OF SALT TOLERANT LINES OF MALAYSIAN *Indica* RICE (*Oryza sativa* L.) CV. MARDI SIRAJ 297

By

NOORHAZIRA BINTI SIDEK

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

My beloved family – En Sidek bin Idris, Pn. Kamariah Awang, Nurul Izzaty
Sidek, Mohd Khalil Salleh and Imran Harith bin Mohd Khalil.

May this journey humbles me,
and benefit others.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in
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As most of other economically important crops, rice (*Oryza sativa* L.) is sensitive to salinity. Due to emerging salinity impacts on global food security, different strategies have been implemented including the development of salt tolerant varieties to minimize yield loss. Therefore, this study was conducted to produce salt tolerant lines of an importantly grown local rice cultivar, MARDI Siraj 297 through *in vitro* callus selection. The first objective of this study was to optimize the embryogenic callus induction medium. Sterilized MARDI Siraj 297 seeds were inoculated on MS basal medium supplemented with 0 to 3.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0 to 0.5 mg/L kinetin (Kin) for 35 days. The MS medium supplemented with 2.0 mg/L 2,4-D and 0.2 mg/L Kin exhibited the maximum response in all callus growth parameters evaluated. Hence this combination was selected as the optimum medium for embryogenic calli proliferation in the subsequent experiments. The second objective was to produce, screen and select the salt tolerant calli. Embryogenic calli were treated in selection medium containing 0 to 150 mM NaCl for 5 months, followed by screening and selection of salt tolerant variants using morphology and biochemical markers. Normal callus morphology was observed in NaCl concentration up to 75 mM. The biochemical profile of these surviving calli showed that the salt tolerant lines had significantly higher content of proline, total soluble sugar, catalase activity, ascorbate peroxidase activity and K^+/Na^+ ratio compared to the non-tolerant control. Meanwhile, reduction of protein content and elevated MDA production was observed in these salt tolerant calli with increasing salinity level. The third objective involves the salt tolerance enhancement of the selected calli by supplementation of salicylic acid (SA) as phytoprotectant in the growth medium. The addition of 1.0 mM SA reduced the morphological injury while maximized the regeneration frequency and number of shoots as compared to the non-SA-treated calli. In the fourth objective, the regenerated salt tolerant plants (R_0) were acclimatized. The evaluation of growth and agronomic traits found that 6 tolerant lines derived from 25 and 75 mM NaCl were morphologically normal and able to produce seeds of the first generation of salt tolerant lines (R_1) while 2 lines derived from 100 mM NaCl were sterile. In the final objective, the comparison of agronomic traits between the R_1 salt tolerant lines and control plant showed that the salt tolerant lines exhibited significantly improved agronomic traits, lower stress susceptibility index (SSI) and higher stress tolerance index (STI) in different salinity level during germination, vegetative and reproductive stage. In summary, *in vitro* selective salinity pressure in this study has successfully produced the R_0, R_1 and R_2 generation of MARDI Siraj 297 salt tolerant lines. These established salt tolerant lines have potential to be utilized by farmers in salinity affected rice field areas. This study also provides a reliable protocol for the establishment of salt tolerant rice lines through tissue culture selection.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGHASILAN DAN PENILAIAN GALUR TAHAN GARAM BAGI PADI *Indica* MALAYSIA (*Oryza sativa* L.) CV. MARDI SIRAJ 297 SECARA *In Vitro*

Oleh

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Seperti kebanyakan tanaman utama yang lain, padi (*Oryza sativa* L.) adalah sensitif terhadap kemasinan. Berikutan impak kemasinan yang semakin meluas terhadap jaminan makanan global, pelbagai strategi telah dilaksanakan termasuklah pembangunan varieti tahan garam bagi mengurangkan kehilangan hasil. Oleh itu, kajian ini dilaksanakan bertujuan untuk membangun galur padi tahan garam bagi kultivar tempatan yang penting iaitu MARDI Siraj 297 melalui pemilihan kalus. Objektif pertama dalam kajian ini adalah untuk mengoptimumkan medium bagi induksi kalus embriogenik. Biji benih MARDI Siraj 297 diinokulasi ke dalam medium MS yang ditambah dengan 0 hingga 3.5 mg/L 2,4-dikloroasetik asid (2,4-D) dan 0 hingga 0.5 mg/L kinetin (Kin) selama 35 hari. Medium MS yang ditambah dengan 2.0 mg/L 2,4-D dan 0.2 mg/L kinetin menunjukkan kesan maksimum terhadap kesemua parameter pertumbuhan kalus. Oleh itu, kombinasi ini telah dipilih sebagai medium yang optimum untuk proliferasi kalus embriogenik bagi eksperimen seterusnya. Objektif kedua adalah untuk menghasilkan, menyaring dan memilih kalus yang tahan garam. Kalus embriogenik dikultur di dalam medium pemilihan yang mengandungi 0 hingga 150 mM NaCl selama 5 bulan, diikuti dengan saringan dan pemilihan varian tahan garam menggunakan ciri morfologi dan penanda biokimia. Morfologi kalus yang normal dapat diperhatikan di dalam kepekatan NaCl sehingga 75 mM. Profil biokimia bagi kalus *in vitro* yang masih hidup menunjukkan bahawa galur tahan garam tersebut mempunyai kandungan prolina, jumlah gula terlarut, aktiviti katalase, aktiviti askorbat peroksida dan nisbah K^+/Na^+ yang lebih tinggi berbanding kawalan yang tidak toleran. Sementara itu, penurunan kandungan protin dan penghasilan MDA yang tinggi dapat diperhatikan di dalam kalus tahan garam apabila tahap kemasinan meningkat. Objektif ketiga adalah untuk mempertingkatkan toleransi garam pada kalus yang telah dipilih dengan penambahan asid salisilik (SA) sebagai fitopelindung di dalam medium pertumbuhan. Penambahan 1.0 mM SA dapat mengurangkan kecederaan morfologi serta memaksimumkan frekuensi regenerasi dan bilangan pucuk berbanding kalus yang tidak dirawat dengan SA. Di dalam objektif keempat, anak pokok yang diregenerasi dari kalus (R_0) telah diaklimitasi. Penilaian terhadap pertumbuhan dan ciri agronomi bagi anak pokok tahan garam (R_0) mendapati bahawa 6 galur tahan garam yang terhasil dari medium 25 dan 75 mM NaCl adalah subur dan normal dari segi morfologi dan berjaya menghasilkan biji benih tahan garam generasi pertama (R_1) manakala 2 galur dari medium 100 mM adalah mandul. Dalam objektif terakhir, perbandingan ciri agronomi di antara generasi pertama (R_1) galur tahan garam dan pokok kawalan menunjukkan bahawa galur tahan garam mempunyai ciri agronomi yang lebih baik, indeks kerentanan tekanan (SSI) yang rendah serta indeks ketahanan tekanan (STI) yang tinggi sewaktu peringkat percambahan, vegetatif dan pembiakan. Secara ringkasnya, tekanan kemasinan selektif *in vitro* dalam kajian ini telah berjaya menghasilkan galur tahan garam generasi R_0, R_1 dan R_2 bagi MARDi Siraj 297. Galur padi tahan garam yang dihasilkan ini berpotensi untuk digunakan oleh pesawah di sawah padi yang terjejas dengan kemasinan. Kajian ini juga menyediakan protokol bagi penghasilan galur padi tahan garam melalui kaedah pemilihan kultur tisu.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
APX	ascorbate peroxidase
CAT	catalase
CIM	callus induction medium
CRD	complete randomized design
dH_20	Distilled water
DMRT	Duncan's Multiple Range Test
dSm^{-1}	deciSiemens per meter
EC	electrical conductivity (unit = $dS\ m^{-1}$)
g	relative centrifugal force
ha	hectare
H_2O_2	hydrogen peroxide
MDA	malondialdehyde
mM	millimolar
MS	Murashige & Skoog
N	normality (unit for solution concentration)
NaCl	sodium chloride
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NAD(P)H	nicotinamide adenine dinucleotide phosphate hydrogen
PGR	plant growth regulator
RCBD	randomized complete block design
ROS	reactive oxygen species
R_0	regenerated salt tolerant plantlets (from callus)
R_1	first generation of salt tolerant lines

SA	salicylic acid
TSS	total soluble sugars
v/v	volume/ volume
w/v	weight/ volume
ε	absorption coefficient

CHAPTER 1

INTRODUCTION

1.1 Background of study

Rice (*Oryza sativa* L.) is the world's most important food crop that feeds about 4 billion people around the globe with an estimation of 25% increase in demand between 2010 to 2030 (IRRI, 2019). However, rice production in most regions has progressively been affected by various abiotic stress such as drought, salinity and flood, which are expected to worsen due to climate change (Mandal et al., 2018; Pareek et al., 2020; Dar et al., 2021). In Malaysia, the impact of climate change such as temperature increase and soil degradation has imposed severe threats to rice production (Firdaus et al., 2020), whereby salinity became one of the main contributors for soil degradation and productivity loss in cultivable lands (Machado & Serralheiro, 2017; Raoufi et al., 2021).

Salinity induced various major responses in rice plants in terms of morphology, physiology, biochemical and agronomic attributes (Riaz et al., 2019; Irakoze et al., 2020; Razzaq et al., 2020; Dramalis et al., 2021). Dissolved salt in soil water exerts two phases of growth response in plants, as revealed by Munns & Tester (2008). The first phase is known as osmotic effect whereby it reduces plants ability for water uptake, followed by ionic effect that interferes with transpiration and causes cell injury in the transpiring leaves (Lefevre et al., 2001; Munns, 2005). These two events hamper cellular metabolisms, accelerate senescence, interrupt source-sink relationship and finally impair plant growth and development (Rahman et al., 2017). In rice, increased severity of salinity effect occurs during seedling and early vegetative phase (Amirjani, 2010; Krishnamurthy et al., 2016) and later during reproductive stage (Reddy et al., 2017; Sen et al., 2017).

In order to survive, plants are endowed with various mechanisms to surge their tolerance during this stressful condition (Borsani et al., 2003), which includes the activation of antioxidant enzymes (Kim et al., 2018) and enhanced accumulation of compatible solutes such as amino acids and soluble sugars for osmotic adjustment (Chen & Murata, 2002). Previous studies by Zeng & Shannon (2000) and Reddy et al. (2017) demonstrated that certain physiological and morphological parameters serve as reliable indicators for salinity tolerance evaluation in rice. Thus, assessing the cumulative effect of these morpho-physiological traits can help to build a comprehensive protocol for salinity tolerant selection and elicit the underlying mechanisms involved in order to develop salt tolerant varieties (Kakar et al., 2019)

1.2 Problem statement

Similar to the majority of crop species, rice, including the Malaysian varieties are categorized as a glycophyte or salt sensitive plant (Green et al., 2017). The MARDI Siraj 297 which is the majorly grown local variety, was also recognized as susceptible to salinity level as low as 4 dSm^{-1} (Sazali et al., 2021). Therefore, salinity could pose significant threat to the country's rice supply, since 47.9% of the paddy fields are cultivated with this variety (Rahim et al., 2021). In Malaysia, sea water intrusion into the paddy fields during high tide phenomenon had been reported in northern states of Malaysia (Perak, Kedah and Perlis), with an estimated destruction of 35 hectares of productive rice cultivation areas, including the area where MARDI Siraj 297 is cultivated (Rahman et al., 2021). Due to increased frequency of such event, it was expected that salinity occurrence will continue to worsen and cause a huge loss in this highly demanded variety (Aling, 2020).

In addition, studies on climate change impacts in Malaysia demonstrated that few factors contributing to salinity such as temperature increase, precipitation variability, intensified use of nitrogen fertilizers and rising seawater level also posed severe threats to paddy productivity in the main rice granary areas of Peninsular Malaysia including MADA (Kedah), IADA (Pulau Pinang), KADA (Kelantan), Perak and Selangor (Herman et al., 2015; Sazali et al., 2021). These factors are inevitably contribute to the increased salinity level in agricultural lands including rice field especially in the coastal line areas and therefore compromising the nation's food security (Jamaluddin et al., 2018).

Due to the emerging impacts of salinity towards plant productivity in Malaysia and worldwide (Pareek et al., 2020; Rahman et al., 2021), different strategies have been implemented to mitigate this problem including the development of salt tolerant cultivars (Mandal et al., 2018) and application of various phytoprotectant such as salicylic acid (Jini & Joseph, 2017; Rahman et al., 2017). Salicylic acid is regarded as a key phytohormone involved in plant systematic acquired resistance (Kim et al., 2018), with ability to regulate many physiological and biochemical responses including modulation of other endogenous hormones and antioxidant activities (Janda et al., 2006; Yusuf et al., 2013).

Research on salt tolerant rice cultivars have been conducted through various approaches including conventional breeding, transgenic manipulation and *in vitro* induced mutation. However, each of them imposed its own limitations (Reddy et al., 2017; Chen et al., 2021). Conventional breeding which depends on wild germplasms for salt tolerant traits mostly caused reduction in agronomic characters such as light-sensitivity, decreased yield and poor grain quality, making it difficult for breeders to introduce them into domesticated varieties (Das et al., 2015). In molecular breeding, limited progress in the research was mainly due to limited genetic resources with sufficient tolerance level and lack of reliable salinity tolerance genes with huge effects (Chen et al., 2021). Despite numerous salinity-responsive genes have been identified in rice, none of them have been successfully incorporated into commercial germplasm so far (Kotula et al., 2020; Liu et al., 2020). Therefore, tissue culture selection was opted in this study due to its feasibilities such as faster development compared to conventional breeding, high frequency of trait changes, possibility of obtaining novel variants and allowing the use of large population of cells for selection purpose (Deepthi, 2018).

1.3 Significance of study

To date, Malaysia has yet to develop its own salt tolerant rice cultivar. Although few studies have successfully regenerate *in vitro* salt tolerant rice (Kalhori et al., 2017; Atabaki et al., 2018), the performance of these lines in salinity at different growth stages has not been evaluated. At present, rice farmers in stress-prone areas depend on cultivating local low yielding traditional cultivars and landraces (Dar et al., 2021) while some others left their paddy fields uncultivated during stressful seasons (Ismail et al., 2013), thereby causing significant loss in the rice supply. Hence, this study aims to develop salt tolerant rice lines that could be used by farmers in salt affected lands in order to ensure sustainable rice production for the future.

1.4 Objectives of study

Previous findings showed that *in vitro* selection is likely to have a significant role in the recovery of stable somaclonal variants with improved stress tolerance. This study was therefore looked at the feasibility of producing salt tolerant rice lines of MARDI Siraj 297 through *in vitro* selection and evaluates the role of salicylic acid in enhancing salt tolerance ability of these lines. Hence, this study was conducted to achieve the following objectives:

1. To optimize embryogenic callus induction from MARDI Siraj 297 seeds.
2. To produce, screen and select the salt tolerant callus of MARDI Siraj 297 using morphological and biochemical markers.
3. To enhance salt tolerance traits of MARDI Siraj 297 callus by supplementation of salicylic acid as phytoprotectant.
4. To acclimatize the regenerants (R_0) salt tolerant lines of MARDI Siraj 297.
5. To compare the growth and agronomic traits between first generation (R_1) salt tolerant lines and control plant of MARDI Siraj 297 at different growth stages.

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