UNIVERSITI PUTRA MALAYSIA

MOLECULAR AND PHYSIOLOGICAL RESPONSES OF RECALCITRANT INDICA RICE TO LIGNOSULFONATES DURING CALLUS REGENERATION

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FBSB 2018 31

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By

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

April 2018

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

MOLECULAR AND PHYSIOLOGICAL RESPONSES OF RECALCITRANT INDICA RICE TO LIGNOSULFONATES DURING CALLUS REGENERATION

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April 2018

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Lignosulfonate (LS) is commonly used as an enhancer to promote plant growth. The recalcitrant *Oryza sativa* indica cv. MR219 rice is an important local high yield rice cultivar that is widely cultivated in Malaysia. However, low callus regeneration rate of MR219 hinders further exploitation in cultivar improvement. Hence, LS was introduced in the culture medium in effort to enhance in vitro cultivation of MR219. To date, the effects of LS on regeneration of MR219 has not been reported. Therefore, this study was undertaken to evaluate the effects of LS on callus proliferation, shoot induction and shoot growth of MR219. The MR219 calli were proliferated on MS supplemented with different types (aNaLS and aCaLS) and concentrations (50, 100, 150, 200 mg/L) of LS. The optimum callus proliferation rate (88%) was obtained in week 3 on MS supplemented with 100 mg/L aCaLS in the presence of plant hormone. However, both LSs did not enhance the shoot induction efficiency whereby 50% of the shoot induced was albino in MS fortified with 100 mg/L CaLS. In shoot growth study, shoot apices were cultured in MS supplemented with different types (aNaLS and aCaLS) and concentrations (100, 200, 300 and 400 mg/L) of LS. The optimum shoot growth was observed in MS supplemented with 300 mg/L aNaLS that is taller by 26% of control height. To understand the growth promoting effects of LS, aCaLS treated callus was used as a study model. Results showed that aCaLS increased callus proliferation rate by 67% and adventitious root formation by 62% in MS without hormone. Hence, it was shown that the LS effect was found to be independent of hormone. Under scanning electron microscopy, adventitious roots were seen protruding out from aCaLS-treated calli. Further expression analysis of adventitious root-related genes (*OsWOX11*, *OsAUX1* and *OsIAA23*) on treated calli, *OsWOX11* expression recorded 1.7-fold expression increment, implying a positive role of aCaLS in adventitious root development. In addition, aCaLS-treated calli recorded 1.2-fold higher endogenous indole-3-acetic acid (IAA) content and increment of nutrient ions (Na, K, Ca, Mg, Fe, Mn, Zn and Cu) uptake. Consistently, expression analysis of auxin-related genes (*OsASA1*, *OsTAA1* and *OsYUC1*) and nutrient uptake-related genes (*OsAKT1*, *OsHAK5*, *OsCBL*, *OsCIPK23* and *OsCamk1*) also showed a similar increment trend. The Ca

increment was observed throughout four weeks but the major increment of K was only detected starting from week two. The observed rise of Ca following the enhancement of endogenous K content suggested the possible cross-talk between these ions uptake. The LC-MS/MS analysis suggested that there was an increased in carbon and nitrogen metabolisms in aCaLS treated callus. Taken together, the presence of aCaLS improved MR219 callus proliferation, up-regulated endogenous auxin synthesis, nutrients uptake and carbon-nitrogen metabolisms that ultimately contributed to calli growth enhancement. The findings of this study would be useful on improving the *in vitro* cultivation of the recalcitrant rice cultivars.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PERUBAHAN MOLEKUL AND FISIOLOGI DALAM BERAS INDICA REKALSITRAN TERHADAP LIGNOSULFONATES SEMASA REGENERASI KALUS

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Lignosulfonate (LS) pada kebiasannya digunakan sebagai peransang bagi menggalakkan tumbesaran tumbuhan. Rekalsitran *Oryza sativa* indika cv. MR219 merupakan kultivar beras hasil tinggi tempatan yang banyak ditanam di Malaysia. Walau bagaimanapun, kadar regenerasi kalus yang rendah MR219 telah menghalang eksploitasi dalam pembaikan kultivar tersebut. Oleh itu, LS diperkenalkan dalam medium untuk memperbaiki pertumbuhan MR219 *in vitro*. Sehingga kini, kesan LS pada pertumbuhan semula beras indica rekalsitran belum dilaporkan. Oleh itu, kajian ini dijalankan untuk menilai kesan LS pada proliferasi kalus, induksi pertumbuhan pucuk MR219. Kalus MR219 dikultur di dalam MS yang ditambah dengan LS (aNaLS and aCaLS) pada kepekatan (50, 100, 150, 200 mg/L) yang berbeza-beza. Menurut pemerhatian kami, MS yang ditambah dengan 100 mg/L aCaLS menunjukkan kadar proliferasi yang paling optimum (88%) dalam medium yang mengandungi hormon. Walau bagaimanapun, kedua-dua LS tidak merangsang kadar pengaruhan pucuk dimana 50% tunas yang diinduksi dalam MS yang ditambah dengan 100 mg/L aCaLS adalah albino. Dalam kajian pertumbuhan pucuk, pucuk apeks dikultur dalam MS yang ditambah dengan LS (aNaLS dan aCaLS) pada kepekatan (100, 200, 300 dan 400 mg/L) yang berbeza-beza. Pertumbuhan pucuk optimum diperhatikan pada MS yang ditambah dengan 300 mg/L aNaLS, yang mana pertumbuan pucuk dicatatkan 26% lebih tinggi berbanding dengan kawalan. Untuk memahami kesan LS terhadap kadar pertumbuhan, kalus yang dirawat dengan aCaLS digunakan sebagai model kajian. Hasil kajian menunjukkan bahawa aCaLS meningkatkan kadar proliferasi kalus sebanyak 67% dan pembentukan akar adventif sebanyak 62% dalam media bebas hormon. LS didapati dapat memberi kesan dalam situasi bebas hormon. Di bawah pemeriksaan mikroskopi elektron, akar-akar adventif dapat dilihat menonjol keluar dari kalus yang dirawat dengan aCaLS. Kajian lebih lanjut melalui analisis gen ekspresi yang berkaitan dengan akar adventif (*OsWOX11*, *OsAUX1* dan *OsIAA23*) dijalankan pada kalus yang dirawat dengan aCaLS. Menurut analisis gen ekspresi, gen *OsWOX11* mencatatkan kenaikan 1.7 kali ganda, mengimplikasikan peranan positif aCaLS sebagai perangsang pertumbuhan akar

adventif. Tambahan pula, kalus yang dirawat aCaLS mencatatkan kandungan sebanyak 1.2 kali ganda auksin dalaman yang lebih tinggi dan peningkatan pengambilan ion nutrient telah direkodkan (Na, K, Ca, Mg, Fe, Mn, Zn dan Cu). Analisis ekspresi gen yang berkaitan dengan auksin (*OsASA1*, *OsTAA1* dan *OsYUC1*) dan gen yang berkaitan dengan pengambilan nutrien (*OsAKT1*, *OsHAK5*, *OsCBL*, *OsCIPK23* dan *OsCamk1*) juga turut menunjukkan corak peningkatan yang sama. Peningkatan Ca^{2+} telah dikesan sepanjang empat minggu tetapi kenaikan K^{+} hanya dapat dikesan bermula dari minggu kedua. Peningkatan Ca^{2+} yang diikuti dengan peningkatan kandungan K^{+} mencadangkan kemungkinan pengambilan ion-ion ini berhubung antara satu sama lain. Analisis LC-MS/MS menunjukkan peningkatan metabolisma karbon dan nitrogen dalam kalus yang dirawat aCaLS. Secara kesuluruhannya, aCaLS telah berjaya meningkatkan prolifearsi kalus MR219, penghasilan auksin dalaman, pengambilan nutrien dan metabolisma karbon-nitrogen yang akhirnya menyumbang pada penambahbaikkan pertumbuhan kalus. Penemuan kajian ini berguna untuk memperbaiki pertumbuhan kultivar beras rekalsitran *in vitro*.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to everyone who has helped me throughout the project. The completion of this study could not have been possible without the expertise and guidance of the supervisory committees: Dr. Lai, Dr. Janna and Dr. Wee. I would like to thank my family, friends and labmates for their help and moral support.

Last but not least, I would also like to acknowledge the UPM Graduate Research Fellowship and UPM Putra Grant (GP-IPS/2016/9506100) for supporting and funding the research.

Thank you from the bottom of my humble heart.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
aCaLS	Analytical grade calcium lignosulfonate
aNaLS	Analytical grade sodium lignosulfonate
ABC	Ammonium bicarbonate
ANOVA	Analysis of variance
ATP	Adenine triphosphate
BSA	Bovine serum albumin
DW	Dry weight
FW	Fresh weight
IAA	Indole-3-acetic acid
KED	Kinetic energy discrimination
LC	Liquid chromatography
LS	Lignosulfonate
NAA	1-Naphthaleneacetic acid
PMSF	Phenylmethylsulfonyl fluoride
PCR	Polymerase chain reaction
RT-PCR	Real-Time Reverse Transcription PCR
SD	Standard deviation

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CHAPTER 1

INTRODUCTION

Rice is one of the major staple food worldwide with Asia being the top producer and consumer (Gumma *et al*., 2011). In Malaysia, rice production was recorded at 3 million tons in 2016 (FAO, 2017). However, the current production is unable to sustain the rising local domestic consumption demands in Malaysia. Annual rice yield losses due to climate change as well as pest and disease outbreak worsens food security (Rajamoorthy and Munusamy, 2015). In order to fulfill the local rice demand, Malaysia is still largely dependent on imported rice. Thailand and Vietnam are the major countries that controlled more than 70 percent of rice imported into Malaysia. In 2016, around 675,000 tons of rice valued at 277 million US dollars was imported from these two countries (Abdul, 2017). Hence, improving rice yield and quality are seen as the most effective way in increasing the local rice production in order to reduce the high importing cost.

The *Oryza sativa* indica cv. MR219 is an important local rice cultivar that is widely cultivated in Malaysia. This cultivar is well known for its high yield, short maturation period and resistance to blast and bacterial leaf blight characteristics (FFTC, 2002). However, the recalcitrant characteristic of MR219 rice hinders genetic study on this cultivar. Low regeneration efficiency, long regeneration duration and low transformation rate of recalcitrant rice have been the major obstacles that trouble the plant biotechnologist (Raghavendra *et al*, 2010; Sah *et al*, 2014; Mishra and Rao, 2016). Hence, improving the *in vitro* plant cultivation medium in the critical callus regeneration stages (callus proliferation and shoot induction) is essential, before any traits enhancement modifications are performed in the recalcitrant indica rice cultivar.

Lignosulfonate (LS) is a low-cost by-product from sulfite pulping process in wooding industries that are already being commercialized as binding agent and dispersal for several industrial purposes (Yang *et al*. 2007; Almas *et al*. 2014). In agriculture, LS is widely used as an important component in fertilizer mainly because it is cost-effective and able to chelate different macro- and micronutrient (Carrasco *et al*. 2012). It is used as stimulant in plant growth, plant development and as well as in enhancing *in vitro* rooting and shoot development of ornamental plants (Telysheva *et al*, 1992, 1997; Van der Krieken *et al,* 2004; Rodríguez-Lucena *et al*, 2009; Ertani *et al*, 2011).

To date, a few hypotheses had been suggested on the enhancing effects of LS in plant growth and development. Nevertheless, the molecular mechanisms underlying these growth enhancing responses induced by LS remained largely unknown. In general applications, the incorporation of LS in planting field would allow soil amendment by reducing the soil pH and increased soil organic matters as a form of disease control strategy for soil borne disease through the enrichment of beneficial microbiota (Lazarovits, 2001; Lazarovits *et al*., 2001; Abbasi *et al*, 2002; Almas *et al*, 2014). Besides, researchers also suggested that LS may play role in regulating endogenous auxin concentration (Hausman *et al*, 1995; Gaspar *et al*, 1996); as auxin protector (Soteras, 1994); increasing tissue sensitivity to auxin (Telysheva et al. 1992, 1997); and as mineral-balancer where LS facilitates the transfer of macro- and micronutrients into the plant cell compartments (Yamashita and Thomas, 1996; Cieschi *et al*, 2016; Carrasco *et al*, 2012).

These interesting enhancing effects of LS have made it a potential enhancing supplement for micropropagation of various type of plant without causing any negative side-effects. To achieve a better usage of the LS components as plant media supplement, understanding the mode of activity of LS is necessary. Hence, we hypothesized that LS could improve the *in vitro* cultivation of MR219 through regulation of endogenous auxin and nutrient uptake in MR219. To address this problem, the present study was undertaken to improve the *in vitro* cultivation medium of MR219 rice through the supplementation of growth enhancer namely lignosulfonate (LS). Taken together, this study was aimed to investigate the effects of LS on callus regeneration of MR219 rice *in vitro* at three different stages which were callus proliferation, shoot induction and shoot growth. Furthermore, the present experimental study also aimed to further determine and elucidate the mode of action of LS in MR219 metabolism, in an effort of verifying potential hypothesized mechanisms that lead to plant growth enhancement phenomenon.

Therefore, the objectives of this study were:

1. To study the effects of LS on callus proliferation, shoot induction and shoot growth of recalcitrant MR219.
2. To study the molecular and physiological responses of recalcitrant MR219 to LS.

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