UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF IMPROVED PROTOCOLS FOR PLANT REGENERATION AND GENETIC TRANSFORMATION OF RUBBER (Hevea brasiliensis Muell. Arg.)

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By

MD. MAHBUBUR RAHMAN

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

June 2014

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Specially dedicated

to

My respected late father Dr. Md. Monsur Rahman,

My dearest mother Asia Khatun

&

My beloved wife Dr. Waheeda Parvin

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the Degree of Doctor of Philosophy

DEVELOPMENT OF IMPROVED PROTOCOLS FOR PLANT REGENERATION AND GENETIC TRANSFORMATION OF RUBBER (*Hevea brasiliensis* Muell. Arg.)

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Chairman: Professor Maziah Mahmood, PhD
Faculty: Biotechnology and Bio-molecular Sciences

Hevea brasiliensis Muell. Arg. is the major source of commercial natural rubber which accounts for 99% of the world natural rubber production. The narrow genetic base, highly heterozygous nature, the long breeding cycle, low fruit set etc are the major limitations of crop improvement by conventional breeding of rubber tree. The recombinant DNA technology combined with tissue culture technique provides opportunity to introduced novel characters into commercially important crop plants, which cannot be achieved easily by conventional breeding. This study was undertaken to establish embryogenic callus culture and plant regeneration protocol via somatic embryogenesis from potential explants of *H. brasiliensis* (Clone RRIM 901) and evaluate the potential target tissues to design a genetic transformation protocol through *Agrobacterium* mediated transformation. In attempt to establish the embryogenic callus and somatic embryos of *H. brasiliensis*, the potential of zygotic embryo, leaf, cotyledon and root in forming embryogenic callus were examined in the basal MS (Murashige and Skoog) medium supplemented with different auxins and cytokinins at various concentrations. The highest callus formation frequency was observed from zygotic embryo explants, in MS medium containing 2.0 mg/L 2,4- D (90%) followed by NAA **(**50%), picloram (40%) and Dicamba (20%) after 8 weeks of culture. In response to embryogenic callus induction the highest 50% of the zygotic embryo explants produced embryogenic callus. A higher yield 70% of embryogenic callus was obtained when explants were cultured in MS medium containing 2.0 mg/L 2,4- D and 2.0 mg/L KIN (6-furfuralaminopurin) in presence of 0.5 mg/L NAA. Maximum embryos induction frequency (76.66%) were obtained on modified MS medium supplemented with 1.0 mg/L BAP (6- benzylaminopurine), and 2.0 mg/L GA_3 (Gibberellic acid) in presence of 0.1 mg/L NAA after 4 weeks of culture. Frequency of embryo maturation was improved (70%) by inducing amino acid glutamin100 mg/L in the culture medium. Maximum plant conversion (80%) was observed on a medium supplemented with 0.3 mg/L GA_3 and 0.2 mg/L IBA (Indole - 3- butyric acid). The *in vitro* micro-propagation capacity of somatic embryo derive plants in compare to mature grafted mother tree was evaluated. Maximum mean number of shoots (9.33 shoots / explant) formation were observed in MS medium supplemented with 2.0 mg/L BAP alone and 9.66 shoots / explant were produced in a

combine effect of 2.0 mg/L BAP with 0.5 mg/L KIN after 8 weeks of culture. Root formation was observed only the shoot regenerated from the explant of somatic embryo derive plant. The plants were successfully acclimatized in natural conditions. A transformation protocol with LBA 4404 harbouring pCAMBIA1304 was established by evaluating the effect of different parameters on transformation efficiency by the expression of reporter gene *gfp* in rubber callus culture. The maintenance conditions for the embryogenic callus cultures, particularly a high auxin to cytokinin ratio (2.0 mg/L 2,4 D **:** 2.0 mg/L BAP **:** 0.5 mg/L NAA), the age of the culture and the use of a yellow green callus phenotype, were the most important factors for achieving efficient transformation. At the histological level, successful transient expression was related to the number of pro-embryogenic masses present in the embryogenic callus tissue. Transformed callus lines were selected and the stable expression of *gfp* gene detected without antibiotic pressure in rubber callus. In conclusion, the plant regeneration protocol via somatic embryogenesis developed using zygotic embryo explants of *H. brasiliensis* (Clone RRIM 901) has not been reported previously that could be applied to several rubber genotypes for production of large scale planting materials. The present regeneration system also used for developing transgenic callus lines by *Agrobacterium*- mediated gene transfer.

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

PERKEMBANGAN BAGI PENAMBAHBAIKAN PROTOKOL REGENERASI TUMBUHAN DAN TRANSFORMASI GENETIK GETAH (*Hevea brasiliensis* Muell. Arg.)

Oleh

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Hevea brasiliensis Muell. Arg. adalah sumber utama getah asli komersial dan ia mencakupi 99% daripada pengeluaran getah asli di dunia. Asas genetik yang terhad, sifat heterozigot semulajadi yang tinggi, kitaran pembiakan yang panjang, penghasilan set buah yang rendah dan sebagainya adalah batasan-batasan utama dalan penambahbaikan tanaman melalui pembiakan konvensional pokok getah. Teknologi DNA rekombinan digabungkan dengan teknik kultur tisu memberi peluang untuk memperkenalkan ciri-ciri baru ke dalam tanaman komersial yang penting, di mana ia tidak boleh dicapai dengan mudah melalui pembiakan konvensional. Kajian ini telah dijalankan untuk memantapkan protokol bagi kultur kalus embriogenik dan regenerasi melalui pembentukan embrio somatik daripada eksplan-eksplan *H. brasiliensis* yang berpotensi (Klon RRIM 901) dan menilai tisu sasaran yang berpotensi untuk menbentuk satu protokol bagi transformasi genetik melalui *Agrobacterium*. Dalam usaha untuk menghasilkan kalus embriogenik dan embrio somatik *H. brasiliensis*, embrio zigotik, daun, kotiledon dan akar yang berpotensi dalam pembentukan kalus embriogenik telah diuji di dalam media asas MS (Murashige dan Skoog) ditambah dengan auksin dan sitokinin pada kepekatan yang berbeza. Kekerapan pembentukan kalus yang paling tinggi telah diperhatikan daripada eksplan embrio zigotik di dalam media MS yang mengandungi 2.0 mg/L 2,4- D (90%), diikuti oleh NAA **(**50%), picloram (40%) dan Dicamba (20%) selepas 8 minggu dikultur. Hasil bagi induksi kalus embriogenik, sebanyak 50% eksplan embrio zigotik menghasilkan kalus embriogenik. Hasil yang lebih tinggi sebanyak 70% kalus embriogenik diperolehi apabila eksplan-eksplan dikulturkan di dalam media MS yang mengandungi 2.0 mg/L 2,4- D, 2.0 mg/L KIN (6-furfuralaminopurin) dan 0.5 mg/L NAA. Kekerapan induksi embrio maksimum (76.66%) diperolehi di dalam media MS yang diubahsuai, di mana ia ditambah dengan 1.0 mg/L BAP (6- benzylaminopurine), 2.0 mg/L GA_3 (Gibberellic acid) dan 0.1 mg/L NAA selepas 4 minggu dikultur. Kekerapan kematangan embrio telah dipertingkatkan (70%) dengan menambah asid amino, 100 mg/L glutamin ke dalam media kultur. Maksimum petukaran tumbuhan (80%) diperhatikan di dalam media yang ditambah dengan 0.3 mg/L GA_3 dan 0.2 mg/L IBA (Indole - 3- butyric acid). Kapasiti mikropropagasi *in vitro* embrio somatik yang terhasil dari pokok berbanding dengan pokok induk matang yang dicantumkan telah dinilai. Purata

maksimum pembentukan pucuk (9.33 pucuk / eksplan) telah diperhatikan di dalam media MS ditambah dengan 2.0 mg/L BAP, manakala 9.66 pucuk / eksplan telah diperoleh daripada gabungan 2.0 mg/L BAP dengan 0.5 mg/L KIN selepas 8 minggu dikultur. Pertumbuhan akar hanya diperhatikan pada pucuk yang diperolehi daripada somatik embrio. Tumbuhan ini telah berjaya diaklimatisasi dalam keadaan semula jadi Satu protocol transformasi dengan LBA 4404 yang mangandungi pCAMBIA1304 telah dimantapkan dengan menilai parameter yang berbeza terhadap kecekapan transformasi melalui ekspresi gen pelapor *gfp* di dalam kultur kalus getah. Keadaan terbaik untuk penyelerengaraan kultur kalus embriogenik adalah nisbah auksin kepada cytokinin yang tinggi (2.0 mg/L 2,4 D **:** 2.0 mg/L BAP **:** 0.5 mg/L NAA), umur kultur dan penggunaan kalus berfenotip hijau kuning, merupakan faktor-faktor yang paling penting bagi mencapai transformasi yang cekap. Di peringkat histologi, ekspresi transien' yang berjaya adalah bilangan gumpalan pro-embriogenik yang hadir dalam tisu kalus embriogenik. Kalus-kalus yang ditransformasi dipilih dan ekspresi gen *gfp* yang stabil dikesan tanpa tekanan antibiotik dalam kalus getah. Kesimpulannya, protokol bagi regenerasi tumbuhan melalui pembentukan embrio somatik dengan menggunakan eksplan embrio zigotik *H. brasiliensis* (Klon RRIM 901) yang tidak pernah dilaporkan sebelum ini boleh digunakan untuk beberapa genotip getah untuk pengeluaran bahan tanaman secara skala besar. Sistem regenerasi ini juga boleh digunakan untuk menghasilkan kalus transgenik melalui pemindahan gen dengan *Agrobacterium*.

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

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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