AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION OF *NICOTIANA BENTHAMIANA* WITH *DEHALOGENASE* GENE RESISTANT TO MONOCHLOROACETIC ACID

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This thesis is dedicated with love and gratitude

To
My Beloved Mother & My Father
(**Allahyarhamah Azizah binti Aziz & Mohamed bin Sayuti**)
Who taught me the first word to speak,
the first alphabet to write, the first step to take and have raised me to be the person I am today.

To
My lovely husband:
Khairul Amin bin Othman

My lovely children:
Elisya Ainul Madihah binti Khairul Amin
Umar al Fetih bin Kahirul Amin
Emeer al Faiq bin Khairul Amin
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I couldn't have done this without your love, care & support

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ABSTRACT

Weeds give adverse effects to crops because of the competition to get nutrients, light and moisture. Many farmers used broad-spectrum herbicide such as monocloroacetic acid (MCA) which is effective at killing a wide range of weeds. Unfortunately, broad-spectrum of herbicide can also kill valuable crops and cause significant losses in agricultural productivity. One of the solutions to this problem is by developing herbicide resistant plant using *dehalogenase D* (*dehD)* gene isolated from *Rhizobium sp.* RC1. A *dehD* gene encoding dehalogenase enzyme that has the capability to degrade monochloroacetic acid (MCA) was isolated and characterized from *Rhizobium* sp. RC1. *dehD* gene was used as herbicide resistance gene and selectable marker gene in *Nicotiana benthamiana* plant transformation. The 798 bp *dehD* gene was inserted into pCAMBIA 1305.2 under the control of the Cauliflower Mosaic Virus 35S (CaMV35S) promoter and designated as pCAMdehD, with a total size of 10,592 bp. A few parameters of *Agrobacterium tumefaciens*-mediated transformation were optimized including hygromycin concentration (40 µg/mL of hygromycin), and the MCA toxicity level to *N. benthamiana* at tissue culture (60 µg/L of MCA) and whole plant stage (2.0 g/L of MCA). pCAMdehD was introduced into *N. benthamiana* via *Agrobacterium* mediated transformation method. Based on the screening of the transformants on MS media containing 60 µg/L MCA, the results showed that *N. benthamiana* was successfully transformed with *dehalogenase D* gene with 50 % of transformation efficiency. The integration and expression of *dehD gene* in *N. benthamiana* were confirmed by PCR, Southern Blotting and reverse transcription PCR. Analysis of leaf-painting assay revealed that transgenic *N. benthamiana* (T_1) was resistant to 4.0 g/L MCA compared to 2.0 g/L for non-transformed plants control. The Chi Square analyses of five transgenic plants (T_1), suggested that the *dehD* gene was segregated according to Mendelian 3:1 ratio. These findings showed that transgenic *N. benthamiana* plant resistant to MCA herbicide was successfully produced.

ABSTRAK

Rumpai memberikan kesan buruk kepada tanaman kerana persaingan untuk mendapatkan nutrien, cahaya dan kelembapan. Ramai petani menggunakan herbisid berspektrum luas seperti asid monokloroasetik (MCA) yang efektif membunuh pelbagai jenis rumpai. Walau bagaimanapun, herbisid berspektrum luas juga boleh membunuh tanaman yang berfaedah dan menyebabkan kerugian dalam hasil pertanian. Salah satu penyelesaian kepada masalah ini ialah menghasilkan tumbuhan yang rintang terhadap herbisid menggunakan *dehalogenase D* (*dehD)* gen daripada *Rhizobium sp.* RC1. Gen *dehD* mengkodkan enzim dehalogenase yang berupaya mendegradasi asid monokloroasetik (MCA) telah dipencilkan dan dicirikan daripada *Rhizobium sp.* RC1. Di dalam kajian ini, gen *dehD* digunakan sebagai gen rintang herbisid dan gen penanda pemilihan dalam transformasi pokok *Nicotiana benthamiana*. Gen *dehD* bersaiz 798bp telah dimasukkan ke dalam pCAMBIA 1305.2 di bawah kawalan promoter Virus Mozek Kubis Bunga 35S (CaMV35S) dan dinamakan sebagai pCAMdehD bersaiz 10,592 bp. Pengoptimuman beberapa parameter transformasi berperantarakan *Agrobacterium tumefaciens* telah dijalankan termasuklah kepekatan higromisin (40 µg/mL higromisin), dan tahap ketoksikan MCA kepada *N. benthamiana* pada peringkat kultur tisu (60 µg/L MCA) dan pada peringkat pokok (2.0 g/L MCA). pCAMdehD telah dimasukkan ke dalam *N. benthamiana* menggunakan kaedah transformasi berperantarakan *Agrobacterium*. Berdasarkan kepada saringan transforman di atas MS media yang mengandungi 60 µg/L MCA, menunjukkan *N. benthamiana* telah berjaya ditransformasikan dengan gen *dehD* dengan 50 % kecekapan transformasi. Integrasi dan pengekspresan *dehD* di dalam *N. benthamiana* telah dibuktikan menggunakan kaedah PCR, Southern Blotting dan transkripsi berbalik PCR. Analisis asai 'leaf-painting' menunjukkan *N. benthamiana* (T_1) transgenik rintang kepada 4.0 g/L MCA berbanding 2.0 g/L bagi pokok kawalan tidak tertransformasi. Analisis Chi Square ke atas kelima-lima pokok transgenik (T_1), mencadangkan gen *dehD* telah tersegregasi mengikut nisbah 3:1 seperti dalam Hukum Mendel. Hasil kajian menunjukkan transgenik *N. benthamiana* yang rintang terhadap herbisid MCA telah berjaya dihasilkan.

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2,2-DCP	-	2,2-dichloropropionate
2,4-D	-	2,4-dichlorophenoxyacetic acid
BAP	-	6-benzylaminopurine
BLAST	-	Basic local alignment search tool
C.V	-	Cultivar
CaMV	-	Cauliflower mosaic virus
DehD	-	D-specific dehalogenase
dehD	-	D-specific dehalogenase gene
DIG	-	Digoxigenin
hpt	-	*Hygromycin phosphotransferase* gene
LB	-	Luria Bertani
MCA	-	Monochloroacetic acid
MS	-	Murashige and Skoog
NAA	-	1-naphthaleneacetic acid
NCBI	-	National Centre for Biotechnology Information
Ri	-	Root inducing
USDA	-	United States Department of Agriculture
Var.	-	Variety
vir	-	Virulence gene

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CHAPTER 1

INTRODUCTION

1.1 Background of the study

Weed infestation is a major problem for agricultural activities and these invasive plants directly affect production through competition for nutrients, moisture and light that reduce crop yields to below economic levels, reduce quality of the produce and can render pastures virtually unproductive (Gressel, 2000; Singh and Yadav, 2012). Weed would cost billions in economic losses every year. According to Weed Science Society of Amerrica (2016), United State and Canada loses $43 billion annually in their corn and soybean crops industries. The advent of worldwide industrialization and fast economic development, have boost the cost of farm labor, hence increasing the necessity for cost-effective chemical weed control using herbicides. The increase preference for herbicides for control weed have resulted in worldwide herbicide market which grew by 39% between 2002 and 2011 and it is projected to grow by another 11% by 2016 (Hossain, 2015).

Intensive use of herbicides has been associated with a number of drawbacks such as environmental pollution through surface run-off that leach into deep soil strata and ground water, or adsorption of herbicides in soil (Michaelidou *et al*., 2000), human and animal health issues (Milosevic and Govedarica, 2002) and most troubling is the evolution of herbicide-resistant weeds (Vencill *et al*., 2012). As of the beginning of the year 2012, a total of 372 unique, herbicide-resistant weed biotypes have been confirmed in the top 19 countries with intensive agriculture. The United States, Australia and Canada recorded the highest number of herbicide

resistant weeds of 139, 60 and 52 biotypes, respectively (Vencill *et al*., 2012). Therefore, it is pertinent that alternative methods that would overcome such disadvantages, improve crop yields and productivity would be of significant advantage.

Given the harmful implications of herbicides, development of transgenic crops that are resistant toward specific herbicides using biotechnological method is timely. Herbicides resistance in selected plants involves the addition of a gene coding for an enzyme that detoxifies the herbicide, or encodes for an altered form of an enzyme targeted by the herbicide. In this context, bacterial genes able to degrade toxic compounds are inserted into plants to render new generation of cultivars insensitive to herbicides. Crops displaying resistance to bromoxynil (Taghipour, 2013) and the herbicide Basta and Buster (Zhang *et al*., 2009) following transformation of a synthetic *bxn* and *bar* gene, respectively, were reported.

Current study will focus using the bacterial genes encodes for production of dehalogenases that cleavage the carbon-halogen bond of the active component of herbicides such as the D-enantiomers monochloropropionate (D-2CP) and monochloroacetate (MCA). The dehalogenase D (DehD) previously isolated from *Rhizobium* sp. RC1 (Berry *et al*., 1979) that was shown to act specifically on D-2-chloropropionate (D-2CP) and monochloroacetate (MCA) (Huyop and Sudi, 2011).

Broad-spectrum herbicide such as monocloroacetic acid (MCA) is effective at killing a wide range of weeds. Unfortunately, they also kill valuable crops and cause significant losses in agricultural activity. One of the solutions to this problem is by developing herbicide resistant plant for instance using *dehD* gene from Rhizobial system. An application of herbicide resistant plant technology has been reported on many plants and crops such as tobacco (Cicero *et al*., 2015), rice (Li *et al*., 2016) and canola (Oliver *et al*., 2016). In this study we successfully produced plant transformation vector for development of herbicide MCA resistant tobacco cultivar *Nicotiana benthamiana* using the *dehD* gene as herbicide resistance gene and in the same time as a selectable marker gene. The transgenic *N. benthamiana* cultivars

resistant towards the herbicide MCA were obtained and its efficacy in resisting herbicide effects was then evaluated.

From our country perspective, "Plant Biotechnology" has been identified as one of the technologies to accelerate Malaysia's transformation into a highly industrialized country by the year 2020. It has received strong government support and commitment with significant funding for R&D, infrastructure, and human resource development for instance in Kuala Lumpure there was 3rd Plant Genomics Congress on 11-12 April 2016, attended by many researcheres all over the country co-hosted by Malaysian Biotechnology Corporation. In Europe, they have started to plan development new crops and cultivars to secure the competitiveness of agriculture. In Strasburg, “The European Plant Science Organisation (EPSO), the European Commission, and the European Association for Bioindustries (EuropaBio) presented the plan entitled “Plants for the Future: A European Vision for Plant Genomics and Biotechnology, for 2025”. The document recommends using genetic engineering to achieve some of its goals. Therefore, since Malaysia is still at infant stage since there is no commercialization as yet, at least at fundamental level we will prove that the technology is there to be realized in the near future.

1.2 Problem Statement

The presence of weeds in the farm can adversely affect crop production in a number of ways. Losses may be occurring through the increasing of harvest costs. The greatest cause of economic loss is a reduction in crop yield due to weed rivaly with the crop for available light, nutrients and moisture. Some weeds release toxins that inhibit crop growth, and others may harbor insects, diseases or nematodes that attack crops. Weeds often interfere with harvesting operations, and at times contamination with weed seeds or other plant parts may render a crop unfit for market. Besides that, framers also spend a lot on weed control activities, labour charges and use of herbicides. Profitable crop production depends on effective weed control.

Many farmers used broad spectrum herbicide such as monochloroacetic acid (MCA) which is cost effective and efficient at killings a wide range of weeds. MCA is a phytotoxic chemical that used as broad spectrum of herbicide against broad leaf weeds, grasses and woody plants (Munn *et al*., 2005). The high concentration of MCA may kill desired plant that lack of herbicide reistance. Unfortunately, MCA and the others broad-spectrum of herbicide can also kill valuable crops and also causing significant losses in agricultural productivity because the herbicides cannot differentiate between plants that are crops and plants that are weeds.

Effords should be made to study the production of herbicide-resistant plants that resistant to broad-spectrum herbicides as one of the solution to this problem. Herbicide resistance gene for a wide range of herbicides have been recognised, isolated, characterised and transferred into a wide range of plants leading to rapid progress in the development of herbicide resistance transgenic plants. The *dehalogenase D* gene (*dehD*) encoding dehalogenase enzyme from *Rhizobium* sp. was found to act on monchloroacetic acid (MCA) by cleaves the carbon halogen bond of MCA.

Transferring herbicide resistance genes into agronomically plants is a useful strategy for controlling weeds and increasing agricultural production. However, before transfer the technology into agronomically important plant, the technology must be tested on model plant plant. In this study, *Nicotiana benthamiana* was used as model plant in this study because this plant can genetically transform and regenerate with good efficiency (Martin *et al*., 2009).

Therefore, current study is to develop a plasmid containing herbicide resistance gene like *dehD* (previously isolated from *Rhizobium sp*.) and transfer them into a *N. benthamiana* plant via *Agrobacterium tumefaciens*. In addition to the function of *dehD* gene as herbicide resistance gene, it also has potential to be used as selebtable marker gene. Transferring herbicide resistance genes such as *dehD* into model plant is a useful strategy for controlling weeds and unwanted plants whereby in the future can be applied for agronomically important plants.

1.3 Objectives

The objectives of this study are as follows:

1. To construct recombinant plasmid pCAMdehD that contains CAMV35S promoter, *dehalogenase D* (*dehD*) gene, NOS terminator and transform the recombinant plasmid into *Agrobacterium tumefaciens*.
2. To transform *Nicotiana benthamiana* plant tissue with *dehD* gene by using *Agrobacterium*-mediated transformation, and the use of *dehD* gene as selectable marger gene.
3. To analyse the integration and expression of the *dehD* gene in transformed *N. benthamiana* plants and verify the T_1 progenies inheritance pattern by segregation analysis.

1.4 Scope of Study

In order to achieve the objectives of this study, six basic research outlined have been proposed:

1. Study the *dehalogenase D* encoding DNA fragment in order to develop a recombinant plasmid construct, pCAMdehD and plant selectable marker based on detoxification of herbicide MCA.
2. Perform preliminary test of the MCA toxicity against untransformed *N. benthamiana* at tissue culture and whole plant level.
3. Transformation of binary plant transformation vector pCAMdehD into *N. benthamiana* plant and the use of *dehD* gene as selectable marker gene in screening stage.
4. Analyses of integration and expression of *dehD* gene into plant genome, by using molecular analyses such as PCR, reverse transcriptase PCR and Southern Blotting analysis.
5. Transgenic plants were analyzed against MCA by leaf painting analysis and chlorophyll content analysis.

6. Segregation analysis using Chi-square analysis was performed to check Mendelian inheritance pattern of *dehD* gene in T_1 generation.

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