COMPARISON OF TWO SELECTIVE MEDIA FOR THE ISOLATION OF *Agrobacterium tumefaciens* FROM THE SOIL

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By

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ABSTRACT

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OH PEI MIN

Agrobacterium tumefaciens is a soil-borne bacterium that causes the crown gall disease, which is the formation of crown gall tumors on a wide range of plants including dicots and some monocots. Most of the genes that are required for the infection process are found on Ti-plasmid (tumor-inducing plasmid). *Agrobacterium spp*. have been genetically modified and widely used as a tool to genetically transform numerous eukaryotes, particularly plants. However, many plants remain recalcitrant to genetic transformation by *Agrobacterium spp*., resulting in low transformation frequency. This seriously impeded the progress of plant biotechnology, particularly crop improvement. Previous studies have shown that the genetic background of the *Agrobacterium* strain can greatly influence transformation efficiency. Malaysia is rich in biodiversity. This prompted us to isolate *A. tumefaciens* from the local soil. The objective of this study is to isolate new *A. tumefaciens* strains for improving plant transformation. In this study, a total of 229 colonies of *A. tumefaciens* strains were isolated from the soil samples of seven sites in Kampar using two selective media, 1A and D1 media. 1A medium was found to be twice more effective than D1 medium in the isolation of *A. tumefaciens* strains. Bacterial isolates obtained from the preliminary screening

were further tested on King's B, EMB and Hofer's Alkaline media, resulting in the elimination of 64.5% of undesired species. The remaining 79 isolates were further analyzed based on morphology, phenotypic and biochemical characteristics of *A. tumefaciens.* Eventually, six positive isolates were obtained and they were further confirmed by polymerase chain reaction (PCR) using specific primers (UF and B1R) that anneal to the 23S rRNA gene sequences of *A. tumefaciens.* The isolated strains were also tested for resistance to 8 different antibiotics to determine suitable selectable markers for genetic manipulation. Pathogenicity test for the isolates on carrot (*Daucus carota*) was performed and one isolate, S1D1 was found to be able to induce tumor formation on its host plant after 35 days of incubation. Agroinfiltration was carried out on leaves of tobacco (*Nicotiana benthamiana*) using indigenous virulent S1D1, but no outgrowth symptoms were observed on the infected leaves. Isolation and characterization of the Ti-plasmid that may be carried by the isolates remain to be performed. The Ti-plasmid may carry useful virulence genes that may enhance the plant transformation efficiency.

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- Appreciation also goes to postgraduate students that helped me a lot by knowledge sharing and enlightening guidance regarding my project.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

(OH PEI MIN)

APPROVAL SHEET

This project report entitled "**COMPARISON OF TWO SELECTIVE MEDIA FOR THE ISOLATION OF *Agrobacterium tumefaciens* FROM THE SOIL**" was prepared by OH PEI MIN and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) Biotechnology at Universiti Tunku Abdul Rahman.

Approved by:

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PERMISSION SHEET

It is hereby certified that **OH PEI MIN** (ID No. **10ADB01432**) has completed this final year project entitled "**COMPARISON OF TWO SELECTIVE MEDIA FOR THE ISOLATION OF *Agrobacterium tumefaciens* FROM THE SOIL**" under the supervision of Dr. Wong Hann Ling from the Department of Biological Science, Faculty of Science.

I hereby give permission to my supervisor to write and prepare manuscripts of these research findings for publishing in any form, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisor. I also understand that University will upload the softcopy of my thesis in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

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(OH PEI MIN)

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LIST OF ABBREVIATIONS

bp	base pair
B1R	Biovar-1 Reverse
CFU	Colony Forming Unit
CI	Chloroform-Isoamyl alcohol
Cm	Chloramphenicol
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EMB	Eosin Methylene Blue
EtBr	Ethidium bromide
HA	Hofer's Alkaline
h	hour
GFP	Green Florescence Protein
IAA	indole-3-acetic acid
kb	kilobase
LB	Luria-Bertani
MES	2-(N-morpholino)ethanesulfonic acid
$MgCl_2$	Magnesium chloride
min	minute
NAA	α-napthaleneacetic acid
OD	Optical density

PCI	Phenol-Chloroform-Isoamyl alcohol
PCR	Polymerase Chain Reaction
psi	Pound-force per Square Inch
rDNA	ribosomal DNA
Rif	Rifampicin
Rpm	Revolution per minute
RT	Room temperature
sdH_2O	terile distilled water
SDS	Sodium dedocyl sulfate
spp	species
T-DNA	Transfer-DNA
Ti	Tumor-inducing
UF	Universal Forward
UV	Ultraviolet
Vir	Virulence
YM	Yeast Mannitol

CHAPTER 1

INTRODUCTION

Malaysia is well known as a rich heritage of diverse plant species that could be a potential source of virulent *Agrobacterium tumefaciens* strains. Crown gall is a type of neoplastic disease that causes tumor formation in plant and had been generally found to be induced by *Agrobacterium spp.* (Moore et al., 1988). The incited disorganized tissues are common over 600 types of plants and imposed significant detrimental effects on these host plant, generating major economic losses (Hartman, 2007). Besides, ecology of *Agrobacterium spp.* in non-agricultural environments such as abandoned fields or undisturbed playgrounds have received too little attention. This implies an urgent need for rapid and reliable methods to diagnose *Agrobacterium spp.* in natural environment.

Population of *A. tumefaciens* is mainly distributed on and around root surfaces known as rhizosphere (Collins, 2001). It's the vector that carrying the true causative agent of crown galls: Ti-plasmids (COST 873, 2011). Ti-plasmid is a large and transferable extrachromosomal DNA element that confers tumorigenic ability to *A. tumefaciens*. Ti-plasmid composed of T-DNA fragment which carried both oncogenes that perturb normal cell division and a set of virulence genes that

essential to promote the transfer of T-DNA from bacterium to susceptible plant cells (Nautiyal and Dion, 1990).

The discovery of T-DNA region and the virulence (*vir*) genes region can be split into two plasmids had lead to the construction of T-DNA binary system (Lan-Ying Lee, 2008). T-DNA binary system was created from naturally occurring Ti-plasmid found in *A. tumefaciens* to consist of two artificial vectors. For the wide-host-range plasmid, its oncogenes was removed and been substituted with gene of interest (Patentlens.net, 2014). On the hand, the helper plasmid has its entire T-DNA region deleted but with *vir* genes region remained intact (Patentlens.net, 2014). These two plasmid often used together to produce genetically modified plants because their cooperation offers higher plasmid stability even after a long co-cultivation of *A. tumefaciens* with the target host plant cells (Murai, 2013).

Strawberry production become problematic due to frost damage, the freezing temperature can severely damage strawberry fruits (Khammuang et al., 2005). Through binary vector system, AFP genes in plasmid pSW1 was further modified in plasmid pBB and subsequently transformed into strawberry plant using *A. tumefaciens* LBA 4404 (Khammuang et al., 2005). This *Agrobacterium*-mediated transformation allowed the transfer and expression of genes encoding antifreeze protein (AFP) from antarctic fish (DeVries et al., 1970) into strawberry plants and thus significantly increased their frost resistance (Khammuang et al., 2005).

The ability of *A. tumefaciens* to integrate the T-DNA fragment into the genome of plant cells revealed its potential to be used as the vector for genetic engineering in plants and make it a great concern to the agriculture (Islam et al., 2010). At present, many plant species remain recalcitrant to genetic modification using *Agrobacterium spp.* yet some researches were successfully in obtaining transgenic plants through *Agrobacterium*-mediated transformation (Chateau et al., 2000).

World population increases, as does our need to discover new food sources to overcome the global issues like starvation and malnutrition of people nowadays. As an example, '*Golden Rice'*, a genetically modified crop with its nutrition values enhanced through *Agrobacterium*-mediated tansformation, providing mans with additional vitamins A for better vision and immunity system (Swaston, 2014). Besides, this well-developed method also allows us to impose genetic protection over plants, altering the responses of crops toward external stresses (Swaston, 2014). For instance, creation of *'Herbicide Tolerant Soybean'* or commonly known as Soybean MON 89788 (Powell et al., 2009). It was developed through *Agrobacterium*-mediated transformation of soybean meristematic tissue using the binary vector, PV-GMGOX20. *Agrobacterium spp*. strain CP4 allowed *cp4 epsps* gene to be expressed, providing plants to be resistant towards action of glyphosate which is the main constituent in agricultural herbicides. These succeeded cases proved that biotechnologies approaches are efficient strategy to raise the valuable characteristic of many plant species.

The isolation of wild type *A. tumefaciens* from local soils has been carried out on semi-selective media, followed by inoculation of isolates in test-plants. This study was conducted for several purposes including:

- To isolate virulent indigenous *A. tumefaciens* strains from local soils
- To compare the selectivity of two selective media in isolation of *A. tumefaciens*
- To confirm the characteristics of newly isolated strains on the basic of a minimal set of biochemical tests
- To determine virulence activity of *A. tumefaciens* on different test-plants
- To study ecology of Ti-plasmid through the isolation and characterization of Ti-plasmid from potential *A. tumefaciens*

CHAPTER 2

LITERATURE REVIEW

2.1 Current taxonomy state of *Agrobacterium spp.*

Previously, agrobacteria were classifed based on their pathogenicity traits where those isolated bacteria which able to produce diorganized tissues on plants were early described as *Bacterium tumefaciens* and *Phytomonas Rhizogenes* (Smith and Townsend, 1907), meanwhile non-pathogenic agrobacteria were termed as *Bacillus radiobacter*. Nevertheless, Ti-plasmid is conjugative and can be easily transferred to plasmid-free agrobacterium (Hooykaas et al., 1977), thence later strains were delineated upon a criterion of genomic concepts, which led to identification of three biovars, *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, and *Agrobacterium vitis* as biovar 1 to biovar 3 respectively (Tzfira and Citovsky, 2006).

Agrobacterium, a new genus proposed by H.J. Conn to collectively include only plant pathogens that closely related to the crown gall organism and other sapprophytic oraganism that might found in similar morphology and physiology (Conn, 1942). Recently, bacteriologists suggested to group *Agrobacterium spp.* and *Rhizobium spp.* together under *Rhizobiaceae* family as both species showed

marked similarities and appeared to be strongly phylogenetically related (Costechareyre et al., 2010)

2.2 *Agrobacterium tumefaciens* as Biovar 1

Biovars are determined by chromosomal instead of the plasmid species embedded within the bacteria, hence most of the time tumorigenic, rhizogenic and non-pathogenic strains can be found within a single biovar (Shams et al., 2012). Biovar 2 and 3 are homogenous taxa while Biovar 1 is heterogenous (COST 873, 2011), gathering ten genomospecies (Popoff et al., 1984), currently called genomovar G1 to G9 (Mougel et al., 2002) and G13 (Portier et al., 2006), thus it should be collectively called *A. tumefaciens* species complex.

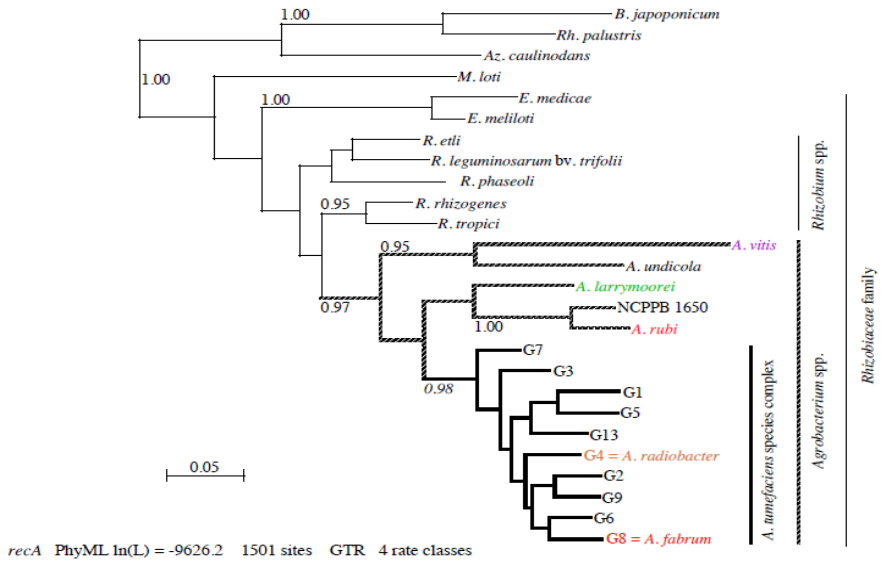


Figure 2.1 Nomenclature of *Agrobacterium spp.* (COST 873, 2011)

2.2.1 *Agrobacterium tumefaciens* as potential plant pathogens

Ti-plasmid containing *A. tumefaciens* can infect 140 genera in over 90 families of plants, most often on stone fruit, brambles, and ornamental plants (Collins, 2001). Crown gall bacteria enter the plant through wounds (Gelvin, 2000). Soon after infection the bacteria induce the plant cells to proliferate forming knobby overgrowths (Gelvin, 2000).

The galls itself also produces low molecular weight compound known as opines which the bacteria use as an energy source (Firmin and Fenwick, 1978). As the galls increase in size, the presence of agrobacteria no longer necessary, therefore galls begin to decay releasing agrobacteria back into the soil (Shams et al., 2012). Agroacteria will remain active in the soil for a minimum of two years after the release and is able to incite crown gall again once this plasmid-free agrobacterium had successfully re-gained a new Ti-plasmid from its neighboring pathogenic-agrobacterium (Shams et al., 2012)

Figure 2.2 Crown gall diseases (Apps.rhs.org.uk, 2014)

2.2.2 Virulence activity of *Agrobacterium spp.*

The virulence of *A. tumefaciens* was contributed by the transduction of T-DNA to plant cells which mediated by the virulence gene located in Ti-plasmid (Gelvin, 2000). Initially, autokinase activity of *vir*A caused a phosphorylation of transcriptional regulator, *vir*G, thence indirectly activated the *vir* gene expression by binding to consensus sequences which located in *vir* operon (Schrammeijer et al., 2003). *vir*D1 and *vir*D2 that induced by *vir*A and *vir*G, were then corporately act as site-specific nuclease which cut T-DNA at its respective borders. T-DNA was linked to *vir*D2 at 5' end and exported to cytoplasm of plant cell via a type IV secretion mechanism (Kohli et al., 2003).

The *vir*B gene is essential to encode proteins that produce a pilus-like structure which act as the bridge connected *A. tumefaciens* and its host. Eventually, the integration of T-DNA into the genome of recipient cell is carried out by *vir*C (Gheysen et al., 1991). Once the section of DNA of host plant that will be replaced is identified, *vir*C cuts into this particular strand of DNA and initiated integration process through illegitimate recombination (Gheysen et al.,1991). Integration of T-DNA can occur randomly at any insertion sites throughout the plant genome (Ishizaki, 2008).

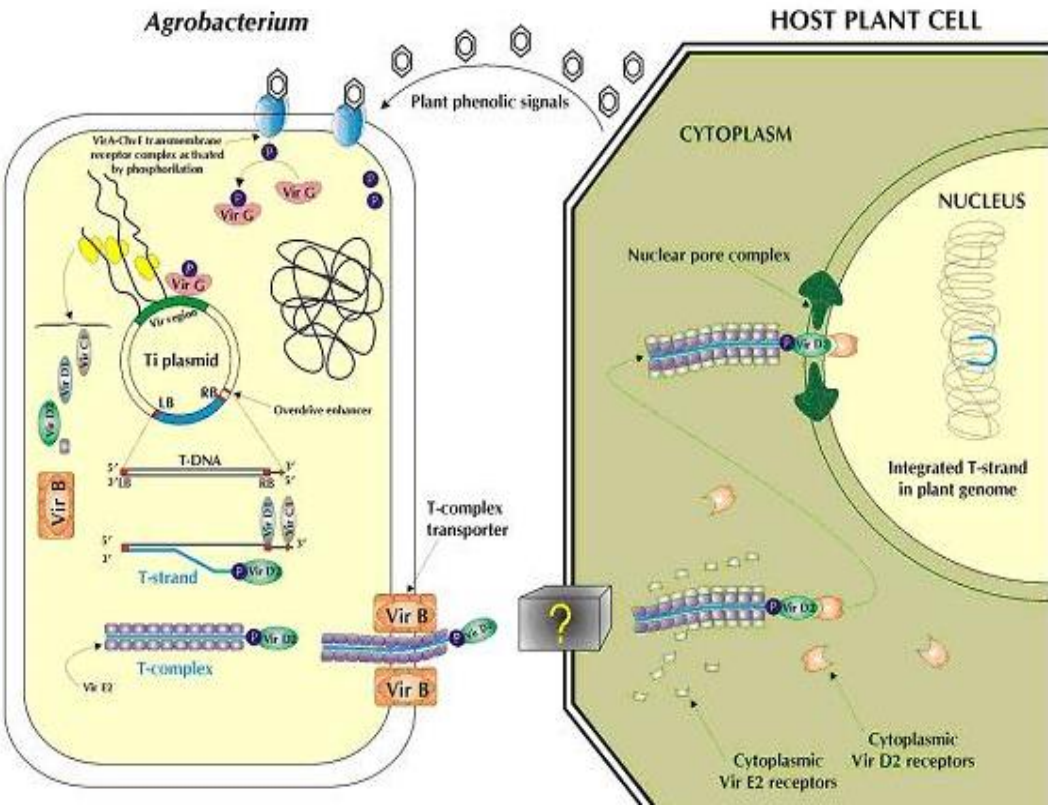


Figure 2.3 Infection Process by *Agrobacterium spp.* on susceptible plant cell (Research.cip.cgiar.org, 2014)

2.3 Isolation of *Agrobacterium tumefaciens* from complex environment

2.3.1 Habitat of *A. tumefaciens*

Distribution of *Agrobacterium spp.* population is depends on soil type and temperature of the particular location. Solarization treatment has significantly reduced *Agrobacterium spp.* population (99%) in Jordan Valley and Uplands (Park et al., 2006). Soil type is one of factor influencing the efficiency of solarization, the silty loam clay soil was less affected by solarization and was

found to be more favorable to the survival of *Agrobacterium spp.* It's recommended to obtain soil samples for analysis at a soil depth of 5 to 10 cm (Bauzar et., 1993) as solarized soil can cause significant reduction on agrobacterial population (Khlaif, 2003).

2.3.2 Opine concept

Agrobacterium spp. catabolizes only opines produced within incited tumor (Firmin and Fenwick, 1978), thence offer a nutritional specificity, providing *Agrobacterium spp*. with a selective growth substrate that favoring its propagation (Tempe et al., 1979). Opine serve as the sole carbon and nitrogen sources of tumor-inducing agrobacteria meanwhile mediated the conjugal transfer of pTi to neighboring non-pathogenic agrobacteria (Moore et al., 1997). However, both *Agrobacterium* and non-agrobacterium microorganisms may inhabit soil or plant roots near a developing tumor, and their colonization could contribute to the population diversity observed (Moore et al., 1997).

2.3.3 Detection through selective media

As proposed by Brisbane and Kerr (1983), biovars-specific media such as 1A medium contain toxic selenite (Sodium Selenite Pentahydrate, Na_2SeO_3) that is useful in isolate and identify agrobacteria. Mougel et al., (2001) concluded that the concentration of toxic selenite must be pre-determined especially when it's

treated on sample that containing low density of *Agrobacterium*. Excess selenite can be toxic and deleterious to *Agrobacterium* meanwhile selenite that is too low in concentration will be insufficient to restraint the growth of competing species (Mougel et al., 2001). Nearly all Agrobacteria are able to tolerate with the toxicity of tellurite metal (200 mg/L), thus it's strongly recommended to supplement tellurite ions (Dipotassium Tellurate (IV) Trihydrate) into selective media to offer a selective advantage over other microbes.

According to Kado and Heskett, (1970), the selectivity of D1 medium is imposed by the alteration on the structure of membrane layer and the cell wall as well. D1 medium was specifically developed for plant-pathogenic bacteria using active constituents such as lithium chloride, which are known to affect the permeability of bacterial membranes to permit the growth of agrobacteria in the meantime, restrict the growth of all other microbes (Kado and Heskett, 1970).

2.3.4 Morphology and phenotypic features of *Agrobacterium tumefaciens*

On solidified agar such as Yeast Mannitol (YM) medium, colonies of agrobacteria are white to cream colored, smooth, convex, glistening circular with entire edges and mucoid (Campillo et al., 2012). When *Agrobacterium spp.* is allowed to growth on carbohydrate containing media, considerable extracellular polysaccharide slime layers are often secreted, leave colonies a voluminous and slimy appearance (Murugesan, 2010).

2.3.5 Characterization of *Agrobacterium tumefaciens* by minimal biochemical tests

A. tumefaciens is a Gram-negative bacterium and positive for motility, lactose, and mannitol utilization test (Putnam, 2006). They can be recognized based on their ability to produce catalase, to consume citrate as their sole carbon and energy source, and most importantly show pigmentation on medium amended with tellurite ions. Moreover, *Agrobacterium spp.* can be easily distinguished from other microbes by 3-ketolactose test, since only *Agrobacterium spp.*, or specically *A. tumefaciens* biovar 1 able to produce intense yellow precipitate of cuprous oxide surrounded the growth on lactose agar (Gaur et al., 1973).

The *A. tumefaciens* have fast acting urease (COST 873, 2011), will induce colorimetric change in urease solution, where pink coloration will be observed after incubation (Shams et al., 2012). For oxidase test, modified by Gaby and Hadley, (1957), suggested that colonies should be grown on media without excess sugar in order to avoid inhibition of oxidase activity.

2.4 Molecular Identification of *Agrobacterium tumefaciens*

2.4.1 Multiplex PCR-based analysis of *Agrobacterium spp.*

Classification of taxa within the genus *Agrobacterium* via the analysis of phenotypic traits corresponds to the biochemical tests is laborious, and the results

are often difficult to interpret. Thence, development of a rapid PCR-based system is exigent. Five primers were designed on the basic of 23S rDNA nucleotide sequence for the fast identification of *Agrobacterium spp.* through a multiplex PCR system (Pulawska et al., 2006). Other than UF primer (Universal Forward), remaining four biovar-specific primers were each complementary to DNA sequence of different species of Agrobacterium (Pulawska et al., 2006). B1R for biovar 1 strains, B2R for biovar 2 strains, AvR for *A. vitis*, and ArR for *A. rubi* (Pulawska et al., 2006).

Conventional PCR process using different combinations of primers together with multiplex PCR process that using 5 primers simultaneously were performed. The analysis of amplified products from both PCR-based systems showed identical expected sizes (Pulawska et al., 2006). The result of this study indicated that the methods presented here are applicable for the classification of *Agrobacterium spp.* into a particular taxon.

2.4.2 Determination of virulence genes region on Ti-plasmid

The genes located on the transmittable Ti-plasmid are useful markers for the detection of *A. tumefaciens.* As proposed by Kawaguchi et al., (2005) the presence of Ti-plasmid within agrobacteria can be detected through the combination of VCF3 (5'-GGC GGGCGYGCYGAAAGRAARACYT-3') and VCR3 (5'- CGAGATTGCGTGCTTGTA GA-3') primers. These primers were

designed on *virC1-C2* genes with an annealing temperature of 57^{o}C. Alternately, cooperation of F14-*vir* (5'-GAACGTGTTTCAACGGT -TCA-3' and F749-*vir* (5'-GCTAGCTTGGAAGAT CGCC-3') primers, which work at an annealing temperature of 57^{o}C, are designed to detect the type of residents plasmids associated in the host agrobacterium (Nesme et al., 1989).

2.4.3 Determination of oncogenes through semi-nested PCR method

Once the presence of plasmid species is identified, semi-nested PCR can be carried out for the rapid determination of tumorigenic agrobacteria in soil. *tms2* gene within the T-DNA fragment was the target for tms2F1 (5'-TTTCAGCTGCTAGGGCCACATCAG-3'), tms2R2 (5'-TCGCCATGGAAACG -CCGGAGTAGG -3') and tms2B (5'-GGAGCACTGCCGGGTGCCTCGGGA-3') primers. The coupled of tms2F1 and tms2R2 bind to the complementary *tms2* gene sequence and resulted in a 617 bp product during the first round of semi-nested PCR. Subsequently, a 458 bp product is generated from the amplification via tms2F1 and tms2B primers.

2.5 *Agrobacterium tumefaciens* as the vector for genetic engineering

Transformation process can be done with electroporation, which is a highly efficient mechanism for the introduction of foreign genes into *Agrobacterium spp*. by creating aqueous pores and causing the plasma membrane become permeable

for the entrance of extrachromosomal DNA molecules. Transformation efficiency of large plasmid, for example Ti-plasmid (>200 kb) can tremendously decline (Wise and Liu et al., 2006). Hence, parameters such as electric field strength and duration of pulse are constantly regulated to ensure high transformation efficiency (Wise and Liu et al., 2006).

At present, *Agrobacterium*-mediated transformation is not practical in pteridophytes, bryophytes and algae. (Hohe and Reski, 2005). However, it has been widely applied to other species, ranging from yeast, to fungi, to human cells, demonstrating the diversity of insertion application (Kunik et al., 2001). Recent years, *Agrobacetrium spp.* becomes an important topic of medical research as it has been suggested to be involved in human diseases (Chalandon, Roscoe and Nantel, 2000)

2.5.1 *Agrobacterium*-mediated transformation in animal cells

The study of Kunik et al, (2001) demonstrated *Agrobacterium tumefaciens*-strain C58C1 harboring Ti-plasmid pGV3850 successfully transformed human cells (HeLa cells) genetically under laboratory conditions by a mechanism similar to that which it uses for the transformation of plants cells. *Agrobacterium*-mediated transformation of mammalian cells can happen at 37^{o}C with relatively high transformation efficiency as long as the components necessary for transformation machinery are all well expressed and induced during the pre-growth of *Agrobacterium* in the presence of *vir*-inducer acetosyringone (Kunik et al., 2001).

2.5.2 Factors influencing *Agrobacterium*-mediated genetic transformation

2.5.2.1 Role of phytohormone

Phytohormone pre-treatment could overcome recalcitrance by increasing the transformation rate (Chateau et al., 2000). The low competence for *Agrobacterium*-mediated transformation of a known recalcitrant genotype, *Arabidopsis thaliana*, is overcome by 4 days pre-culture with phytohormones (Chateau et al., 2000). The transformation frequencies showed 30-60% increment after petioles of Arabidopsis were pre-cultivated with phytohormones such as α-napthaleneacetic acid (NAA) or indole-3-acetic acid (IAA) to induce cell division and to overcome its low sensitivity towards *Agrobacterium* (Chateau et al., 2000).

2.5.2.2 Role of Inducer

Acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone) is the phenolic compound that been widely used as a potent inducer of *vir* genes regulation and expression (Gelvin, 2000). The optimum concentration of acetosyringone must be pre-determined as excess acetosyringone are detrimental to the growth of sample. In the case of haploid liverwort *Marchantia polymorpha* L., transformation efficiency of immature thalli which co-cultivated with *Agrobacterium* cell suspension, in the presence of different concentration of acetosyringone was examined (Ishizaki et al., 2008). Transformation efficiency was dramatically reduced when concentration of acetosyringone exceed 1 mM meanwhile maximum tansformation efficiency was achieved at 100 μM (Ishizaki et al., 2008).

2.5.2.3 Duration of co-cultivation period

Co-cultivation process is an essential step where T-DNA is transferred and integrated into host's genome. The most optimum duration for co-cultivation was determined by comparing the transformation efficiency during distinct periods of co-cultivation (Ishizaki et al., 2008). Amount of transformants obtained increased dramatically from 12 h to 24 h of co-cultivation. Transformation efficiency increased steadily when co-cultivation period was pro-long from 24 h up to 72 h.

2.5.2.4 Over-expression of certain genes within host plants

Components in certain plant species actually involved in *Agrobacterium*-mediated transformation and might aid in bacterial attachment, T-DNA transfer, nuclear targeting or even help in T-DNA integration (Gelvin, 2003). Over-expression of Arabidopsis nuclear protein VIP1 allowing more rapid T-DNA nuclear targeting, thence increased the plant susceptibility to *Agrobacterium* infection (Tzfira et al., 2002). Study showed that mutation of the histone H2A-1 gene (HTA1) resulted in lower transformation efficiency as this gene is crucial for T-DNA integration process. However, the reason of over-expression of HTA1 genes in affecting the transformation process by restoring the susceptibility of plant cells towards *Agrobacterium* remains unknown (Gelvin, 2003).

2.5.2.5 Choice of tissue for infection process

According to Ishizaki (2008), compared with young or mature *M. polymorpha* thalli, immature thalli developed from spores showed higher rate of cell division, thus demonstrated a more stable integration and expression of transgenes in the sample. The developmental stage was crucial for transformation efficiency, suggesting that different cell type might have different competencies for *Agrobacterium* infection

2.6 Pathogeniticity test

Acetosyringone is a phenolic compound originating from the wounded plant cell (Gelvin 2000). Regarding to its impacts on the T-DNA delivery process, concentration of acetosyringone being supplied into *Agrobacterium* culture must be optimized (Wydro, 2006). According to the protocol suggested by Voinnet et al., (2003), bacterial suspension appended with 10 mM MES buffer, pH 5.7 and 150 µM acetosyringone allowed infection process to happen within 2 to 3 h.

2.6.1 Agroinfiltration

Prior to infiltration, 5- to 8- weeks-old tobacco plant (*Nicotiana benthamiana*) that grown under standard greenhouse condition were used. The pre-cultured bacterial suspension was injected into abaxial air spaces of two-top leaves of each host plant via 2 ml syringe without a needle attached. The infiltrated leaves were

observed for bulge at 4 days after agroinfiltration (Wydro, 2006). However when infection was performed through incisions on aerial part or the stem of the plant, autonoumous tumor growth was expected (Campillo et al., 2012). Physiological functions of plants will be affected due to the enlargement of tumors, thus leaving the plant to display reduced growth and may become severely stunted (Campillo et al., 2012).

2.6.2 Carrot disc assay

Acoording to Moore et al., (1988), carrots (*Daucas Carota*) that were collected from fresh market must be surface-sterilized followed by rinsing thrice with double distilled water. Carrot disc can be 0.5 cm–thick, lined with moist filter paper and overlaid with appropriate amount of inoculum. Petri dish must be properly sealed and checked periodically to ensure there is sufficient water to maintain moist environment for the infection process. Every disc can be examined for tumor-like callus formation after from 3 to 7 weeks of incubation (Islam et al., 2010).

2.7 Ti-plasmid

2.7.1 Isolation of Ti-plasmid

A. tumefaciens can easily lose its Ti-plasmid to their surrounding when they were incubated above 28^{o}C. Ti-plasmids are circular extrachromosomal DNA elements

that can have sizes far beyond 200 kb. Basically, they been delineated into different categories according to the type of opines that encoded by the genes found on the non-transferable region of Ti-plasmid. Their special features make them a great natural genetic engineer, but at the same time also revealed that extra cares are needed during the isolation process of these large plasmids.

Extraction of Ti-plasmid can be laborious as *A. tumefaciens* is insensitive to lysozyme-EDTA detergent lysis procedure. It's always time consuming for the agrobacteria cells to lyse, thence additional phenol-chloroform extraction step is necessary to enable the dispensable Ti-plasmid to be released from the host bacterium (Wise et al., 2006). Enhancements such as pro-long treatment with proteolytic enzyme and grow *A. tumefaciens* in the presence of Carbenicilin might resulted in Ti-plasmid of greater yield and purity.

2. 7.2 Characterization of plasmid phenotype

Ti-plasmid been mainly studied and characterized through the presence of oncogenes and *vir* genes, also the genes that responsible for opine synthesis and catabolism, but recently a more reliable way has been established. Agrocin 84 is a type of antibiotic produced by biocontrol agent *Agrobacterium* strain K84O. Researchers suggested purified plasmid could be further classified based on their sensitivity toward agrocin 84 as genes for agrocin sensitivity are located at nopaline Ti-plasmid, thus this test is reliable as a putative test for pathogeniticity.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and Equipments

Reagents and equipments were provided by the Department of Biological Science, Perak campus of University Tunku Abdul Rahman. Details of materials and equipments (refer to Appendices A).

3.2 General Methodology

Semi-selective media, differential media, antibiotic-containing media, stock solutions, and buffer were all prepared according to the instructions (refer to Appendices B). Media that required additional chemicals components were pre-mixed and added with 15 g/L of agar powder before sterilization. Molten agar was poured into petri dishes and allowed to solidify in the laminar flow. Reagents and media were stored in 4°C refrigerator, whereas antibiotic stock solutions and buffers were kept in -20°C freezer and room temperature respectively. Sterilization of media, glassware, microcentrifuge tubes and pipette tips were accomplished through autoclave at 121°C at 15 psi for 15 min. Heat-liable components were all filtered-sterilized before used.

3.3 Soil sampling (Serial Dilution)

Seven samples were collected from different regions of Kampar, Perak during October to November 2013. All the samples were processed within 24 h. A sterilized soil auger was used to obtain moist soil samples from plant rhizosphere, at 8-10 cm beneath the surface soil. Stones and leaves were avoided. Fresh samples were kept in sterilized containers and tightly sealed with parafilm. An amount 2.0 g of sample of bulk soil were macerated in 20.0 g of sterile distilled water (sdH_2O) and vortexed at high speed (18000 - 22000 rpm) for 30 min serial dilution ranged from 10^{-1} to 10^{-5} was performed. Subsequently 100 µL of diluted slurry was then plated on the two semi-selective media, 1A and D1 media. Colonies were examined and enumerated 48 h post plating. Selected isolates were then transferred to Yeast Mannitol (YM) Medium and well separated on grid.

Table 3.1 Sample sites

Date	Location	Sample Site
25 / 10 / 2013	Kampar Old Town Playground	**S1**
25 / 10 / 2013	Kampar New Town Playground	**S2**
26 / 10 / 2013	Football Field	**S3**
26 / 10 / 2013	Household	**S4**
24 / 11/ 2013	C4 Land	**S5**
26 / 11 / 2013	Brangkai Waterfall	**S6**
26 / 11 / 2013	Dragon Path	**S7**

3.4 Preliminary Screening

From the YM medium, replica plating was performed on King's B, Eosin Methylene Blue (EMB), Hofer's Alkaline (HA) media using toothpicks spot technique. Wood-made toothpicks were autoclaved at 121°C at 15 psi for 15 min. The plates were incubated for 24 h at 28^{o}C. EMB and HA media were directly examined for positive reaction. Purple-black colonies and well pronounced growth of isolates were expected from EMB and HA media respectively. On the other hand, King's B medium was irradiated with long UV light to screen for fluorescent-compound- producing bacteria that indicate *Pseudomonas spp*. Isolates that signified positive reaction from these three differential media were proceeded to biochemical test for presumptive identification of *Agrobacterium.*

3.5 Biochemical Tests

3.5.1 Gram Staining

With sterilized toothpicks, fresh colony was placed on clean slide and thoroughly mixed with a small drop of sdH_2O. Once a thin film was formed, the slide was held high above Bunsen burner for heat fixing process. Firstly, the smear was flooded with crystal violet dye solution for 1 minute, and then rinsed with sdH_2O to remove excess dye. Next, the smear was covered with Iodine solution for 1 minute followed by washing with sdH_2O. The decolorization process was carried out by dripping 95% ethanol solution on the smear for 10 second. Lastly, the smear was rinsed with sdH_2O after counterstained with Safranin dye solution for 1

minute. The slide was allowed to dry. The color and morphology of cells were viewed under 100x magnifications by using a light microscope with the aid of immersion oil.

3.5.2 Oxidase Test

Isolates were streaked on YM medium and incubated for 24 h at 28^{o}C, Isolates were allowed to grow into visible mass and subsequently flooded with few drops of oxidase reagents, Gaby-Hadley reagent A and B. Gaby-Hadly reagent A was made up of 1% of 1g alpha-Naphtol, and 95% of 100ml ethanol whereas Gaby-Hadly reagent B contained only N,N-Dimethy-p-phenylediamine (oxalate). The mixture of oxidase reagent was permitted to flow over growth and the formation deep indigo color was immediately observed after 2-3 minutes.

3.5.3 Urease Test

A volume of 5ml of Stuart's Urea broth was transferred to 30 ml universal bottle. Heavy inoculants were inoculated into the broth using sterilized toothpicks. All the universal bottles were fixed on orbital shaker for 24 h at 37^{o}C. Colorimetric change of broth, from yellow to fuschia color was observed by pipetting 200 µl of the broth into the round-bottom-96-well plate.

3.5.4 Potassium Tellurite Test

A volume of 100 µL of potassium tellurite solution was pipette to YM medium and allowed to dry in laminar flow. Isolates were inoculated on the medium and incubated at 28^{o}C. The growth of isolates as well as the formation of black precipitation was examined after 2 days.

3.5.5 3-ketolactose Test

Isolates were streaked on lactose agar and incubated for 2 days at 28^{o}C. Visible growths were fully covered with Benedict's reagent. Formation of yellow precipitation around the growth of isolates was observed after 2 h.

3.5.6 Catalase Production Test

Fresh isolate was transferred to a clean slide using sterilized toothpicks, and thoroughly mixed with a small drop of sdH_2O. Next, a drop of 3% hydrogen peroxide (H_2O_2) was added on the smear. The smear was immediately covered with cover slip and bubbles formation was observed.

3.5.7 Citrate Utilization Test

Simmons Citrate Agar was allowed to harden at an angle. Isolates were streaked on the surface of slant agar in a zigzag manner by using a sterilized inoculating loop. Colorimetric change of slant agar, from green to blue color was observed.

3.6 Molecular Identification

3.6.1 Colony PCR

Colony PCR is crucial to validate isolates as *Agrobacterium tumefaciens.* Reaction mixtures were prepared according to Table 3.2 meanwhile Polymerase Chain Reaction (PCR) was performed using parameters listed in Table 3.3. For *n* reactions, a mix of *n* + 1 reactions was prepared in a 1.5 mL microcentrifuge tube. Sterilized toothpicks were softly touched on colony to transfer only a very small amount of template into the reaction mixture. The amplified product was then electrophoresed on a 2% agarose gel at 80 V for 45 min. As listed in Table 3.4 Universal Forward Primer (UF) and Biovar-1 Reverse Primer (B1R) were used as they specifically anneal to the 23S rRNA gene sequence of *A.tumefaciens* (Campillo et al., 2012).

Table 3.3 Reaction mixture of Colony PCR

Chemicals	Amount (µL)
10x Taq Buffer	1.0
2.5 mM dNTPs	0.6
50 mM $MgCl_2$	0.3
UF primer	0.4
B1R primer	0.4
Taq Polymerase	0.2
dH_2O	7.1
Total	10.0

Table 3.4 Parameters for Colony PCR

	Temperature (^{o}C)	Time (s)	
	94	300	
Denaturation	94	30	35 Cycles
Annealing	57	30	
Elongation	72	60	
	10	∞	

Table 3.4 Primer sequence used in Colony PCR

Primer	Primer Sequence (5'-3')	Length (base)
UF	**GTA AGA AGC GAA CGC AGG GAA CT**	23
B1R	**GAC AAT GAC TGT TCT ACG CGT AA**	23

3.6.2 Genomic PCR

3.6.2.1 Genomic extraction using Fast Boil technique

Isolates were inoculated into 2X YT broth using sterilize toothpicks and incubated on orbital shaker for 24 h at 28^{o}C. A volume of 1.5 mL of cell suspensions was transferred into microcentrifuge tubes and cells were harvested by centrifugation at 12,000 rpm for 5 min. Pellet obtained was resuspended with 500 µL of sdH_2O. Then, cell suspension was boiled using heat blocks at 95^{o}C for 10 min. The supernatant was now containing the desired genomic DNA of isolates, thereby was collected through centrifugation at 12,000 rpm for 5 min. The Optical Density (OD) reading of each sample at 260 nm was measured and recorded.

3.6.2.2 Genomic PCR

The extracted genomic DNA was added into PCR reaction mixture as the DNA template for amplification using UF and B1R primers as well (Table 3.4). The reaction mixture and parameters of PCR process were stated in Table 3.2 and Table 3.3 respectively. The amplified fragments were analyzed on a 2% w/v agarose gel stained with ethidium bromide (EtBr).

3.7 Pathogenicity Test

3.7.1 Preparation of cell suspension

Isolates were inoculated into 2X YT broth and cultured on orbital shaker at 28^{o}C for 24 h. Cell suspension was harvested by centrifugation at 14000 rpm for 15 min. Pellet obtained was resuspended with 5 ml of A1 buffer. Centrifugation and resuspension steps were repeated to rinse the cells. Cell suspension was added with 150 µg/mL of Acetosyringone and kept in dark for 2 h at room temperature (RT).

3.7.2 Carrot Disc Bioassay

Carrot (*Daucus carota*) was purchased from local market in Kampar old town. Carrot samples were cleaned with running tap water and vigorous rinsing was repeated for 3 times. Carrot samples were then further sterilized using commercial 10% of household bleach for 10 minute. In laminar flow, on a sterilized aluminum foil, carrot samples were chopped into discs of uniform height and width. Each disc was properly labeled and placed in the alcohol sterilized plastic container. A piece of autoclaved cotton was immersed with sdH_2O and used as the base for the disc. Next, each carrot disc was overlaid with 100 µl of dense bacterial cell suspension and incubated in growth chamber at 28^{o}C. Each container was tightly sealed with parafilm to keep the internal environment moist. Every disc was checked for the development of undifferentiated cells 6 weeks post inoculation.

3.7.3 Agroinfiltration

The leaves of a 5 weeks-old tobacco plant (*Nicotiana benthamiana*) were selected for the infiltration process and young leaves were preferable. A single piece of leave was infiltrated with four components at different spot, other than the potential isolate, Green Fluorescence Protein (GFP), *Agrobacterium tumefaciens* C58 (positive control), A1 buffer without inoculum (negative control) were also included. The infiltration was done by using syringe without needle. The samples were injected to approximately 4 cm^2 at the abaxial site of leaves. Fingers were placed at the opposite surface of injection spot in order to exert some pressure that enhances the efficiency of infiltration.

3.8 Plasmid Extraction

3.8.1 Antibiotic Resistance Tests

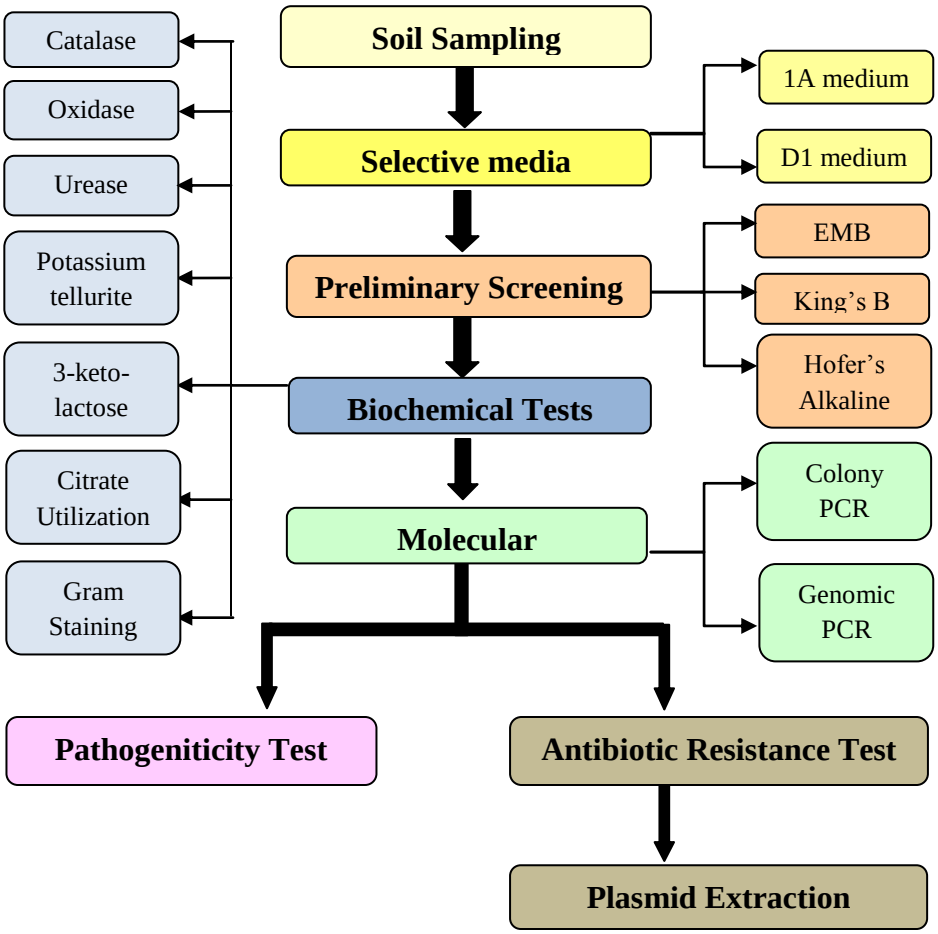
Antibiotics involved are: 100 µg/mL carbenicilin, 100 µg/mL streptomycin, 100 µg/mL gentamycin, 50 µg/mL kanamycin, 50 µg/mL rifampicin, 20 µg/mL hygromycin, 14 µg/mL chloramphenicol (Cm), and 5 µg/mL tetracycline. Luria-Bertani (LB) agar was added with appropriate amount of antibiotics to form antibiotic plates of respective concentration. Isolates were streaked on antibiotic-containing plates using sterilized toothpicks and incubated for 2 days at 28^{o}C. The strength of antibiotic resistance was demonstrated by the growth of each isolate on these antibiotic-containing plates.

3.8.2 Isolation of Ti-plasmid

Firstly, isolates were inoculated into falcon tube containing 20 ml LB broth using sterilized toothpicks and incubated for 8 h at 28^{o}C. Next, the cell suspension was transferred into a conical flask containing 80 mL LB broth and cultured for 16 h at 28^{o}C. A volume of 100 µL of 14 µg/mL Cm was added into the broth in the ratio of 1 mL: 1 µL. The overnight cell suspension was harvested by centrifugation at 6000 rpm for 10 min at RT. The pellet obtained was resuspended in 2.4 mL of 25% sucrose in 50 mM Tris-HCl (pH 8.0). Cells were lysed through the addition of 0.4 mL of lysozyme (10 mg/mL) in 0.25 M Tris-HCl (pH 8.0). The mixture was chilled on ice for 5 min followed by adding of 1 mL of 0.25 M EDTA (pH 8.0). After incubated for 5 min at RT, 1 mL of 20% SDS in TE buffer was added to complete the lysis process. Mixing of 0.3 mL of 3M NaOH with the mixture was carried out gently for 3 min. Subsequently, the mixture was thoroughly mixed after the addition of 2.4 mL of 2M Tris-HCl (pH 8.0), 1 µl of 20% SDS in TE buffer as well as 2.4 mL of ice cold 5M NaCl. After incubated for 20 min at RT, the flocculent precipitate was pelleted by centrifugation at 14000 rpm, 4^{o}C for 30 min. The supernatant was collected and subjected to phenol-chloroform extraction process. Equal volume of Phenol-Chloroform-Isoamyl alcohol (PCI) solution and 0.9 mL of 5M NaCl were added to the supernatant, then centrifugated at 10000 g, 4^{o}C for 10 min, allowed the mixture to separate into 3 layers where only the uppermost aqueous layer was collected into a new falcon tube. This step was repeated until the interface (white precipitate) at the middle layer disappeared. Subsquently, equal volume of

Chloroform: Isoamyl alcohol (CI) solution and 2 volume of ice-cold Isopropanol were added to the collected supernatant. The mixture was then incubated overnight at -20^{o}C. The white precipitate was harvested by centrifugation at 10000 g, 4^{o}C for 20 min. The pellet was washed with chilled Isopropanol and centrifuged at 10000 g, 4^{o}C for 20 min. This step was repeated thrice to ensure high DNA yield. Lastly, the pellet was air-dried in laminar flow and resuspended with 10 µL of TE buffer and 10 mg/mL of RNase.

3.9 Experimental Design



Soil samples were serial diluted and then plated on two selective media, 1A and D1 media. Unwanted microorganisms were discarded through preliminary screening. Subsequently eight different types of biochemical tests were performed for the presumptive identification of *A. tumefaciens.* Desire isolates were determined and confirmed as *A. tumefaciens* by using molecular identification techniques, included colony PCR and genomic PCR. Validated A. tumefacines isolates were subjected to pathogeniticity test as well as plasmid extractions.

CHAPTER 4

RESULT

4.1 Soil sampling (Serial Dilution)

Serial dilution range from 10^{-1}-10^{-5} has been performed on each soil sample. Colonies were picked up from 1A and D1 media of 10^{-1} and 10^{-2} dilution respectively. Plates with too numerous (>200) or too few (<10) colonies were all disregarded. The amount of microorganisms that been added into semi-selective media (1A and D1 media) was tabulated in Table 4.1 in the unit of CFU g^{-1}.

Table 4.1 Estimated CFU/g^{-1} of each sample site

Sample Site	1A medium (CFU g^{-1})	D1 medium (CFU g^{-1})
S1	1.9×10^3	3.2×10^3
S2	8.0×10^2	2.4×10^3
S3	1.1×10^3	2.8×10^3
S4	1.3×10^3	1.8×10^3
S5	4.0×10^2	7.0×10^3
S6	9.0×10^2	1.7×10^3
S7	1.7×10^3	2.2×10^3

***Remarks:* Nomenclature of Isolates**

- S1D1 = **S**ample site **1**, **D**1 medium, Colony **1**
- S4A5 = **S**ample site **4**, 1**A** medium, Colony **5**

4.2 Semi-selective Media

Selective compounds tend to slow down the growth of microorganism, thus plates were incubated for 4 days, followed by the examination for growth, and morphological features such as elevation, surface, metal substrate fermentation and chromogenesis. Colonies with different sizes were observed on both semi-selective media. For instance, by comparing A_1 with A_2, and D_1 with D_2 (Figure 4.1a and Figure 4.1b), it should be noted that different *Agrobacterium* strains will grow at different rates, thus discrete bacterial colonies were picked every 24 h.

4.2.1 1A Medium

A total of 81 colonies that selected from 1A medium have the typical domed glistening morphologies of agrobacteria and a characteristic black color with metallic shine due to the incorporation of tellurite (Figure 4.1a). Colonies with similar morphology was found on 1A medium, revealed that the concentration of selenite used is sufficient to restraint the growth of competing microorganism such as fungus. Plates were free from yeast and fungi due to the supplemented antibiotic, 20 mg/mL of cycloheximide.

4.2.2 D1 Medium

Colonies that vary in morphology were observed on D1 medium (Figure 4.1b), indicated the presence of distinct population of microorganism within the soil sample. Dark-brown colored colonies, nucleated colonies, as well as irregular or filamentous shaped colonies were all disregarded. Eventually, a total of 148 colonies that displayed smooth, convex and glistening appearance together with yellowish and circular edges were chosen for subsequent screening process.

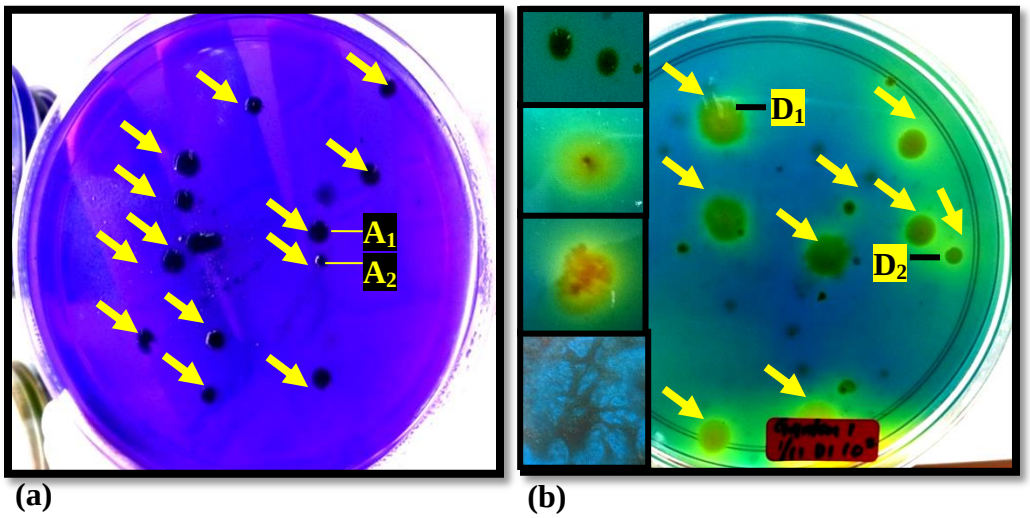


Figure 4.1 Typical agrobacterial colonies on different selective media. a. Plating 10^{-1} dilution slurry on 1A medium. Arrows indicated agrobacterial colonies **b.** Plating 10^{-2} dilution slurry on D1 medium built colonies with different morphology. Arrows indicated agrobacterial colonies.

4.2.3 Contribution of Semi-selective Media

As shown in Figure 4.2, different regions of Kampar contributed different amount of microorganisms. Comparison of the extremes, S1 and S5 revealed that this phenomenon was affected by two factors, the soil type as well as the temperature of the particular sample site. S1 provided the greatest number of isolates, which almost 5 times the amount showed by S5, meanwhile the other regions contributed an average of 34 isolates for further analysis. Furthermore the total amount of colonies selected from D1 medium always higher than those obtained from 1A medium.

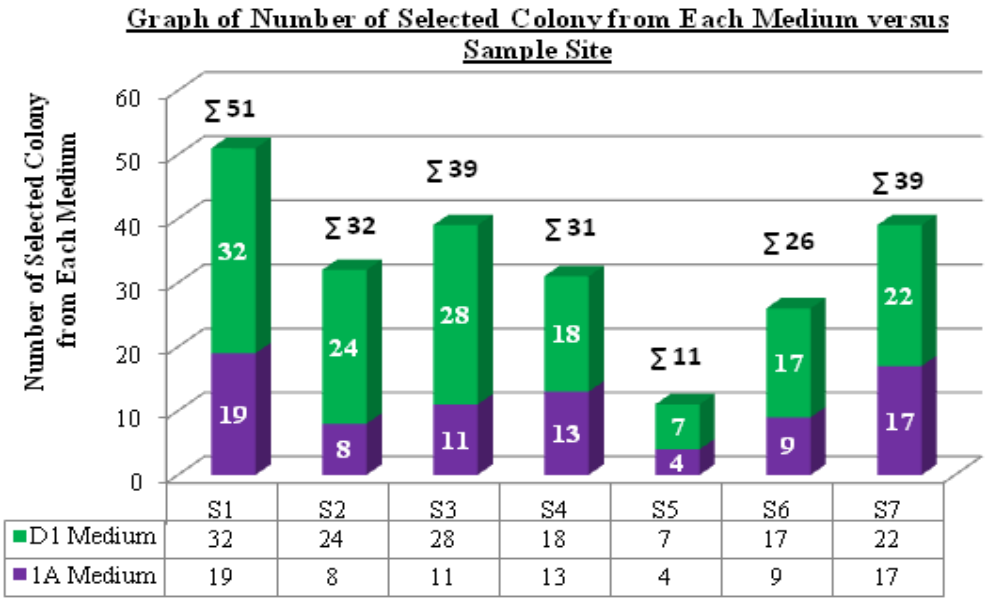


Figure 4.2 Amount of colonies contributed by each selective medium on each sample site. A total of 11, 26, 31, 32, 39, 39, and 51 of colonies were contributed by S5, S6, S4, S2, S3, S7, and S1 respectively in an increasing manner. The total amount of selected colony obtained from S2, S4, S5, and S6 were relatively low compared to S1, S3, and S7.

4.3 Yeast Mannitol Medium

According to the initial observations, a total of 229 isolated colonies (35% from 1A medium; 65% from D1 medium) were tentatively identified as *Agrobacterium* strains. All the selected colonies were then streaked on Yeast Mannitol (YM) Medium where the purity of selected colonies was assured by using toothpicks spot technique as shown in Figure 4.3. Pure *Agrobacterial* cultures were maintained in YM Medium and the plate was use as the master plates throughout the screening process. Based on the observation, selected colonies were typically translucent, domed, and mucoid. On each grid, the bacterial isolates ware allowed to grow and multiply in the form of colonial masses instead of single distinct colony due to the presence of slime layer.

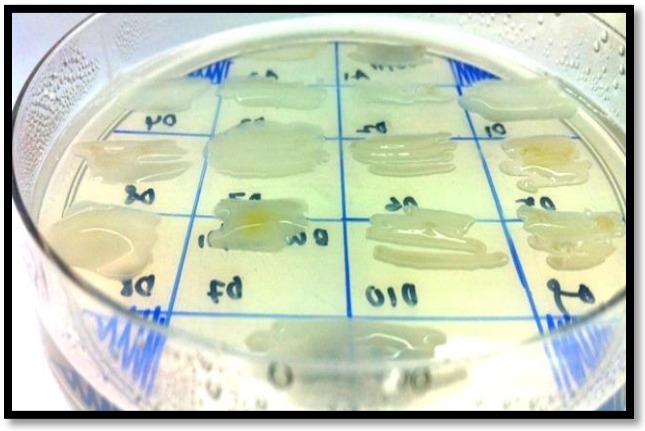
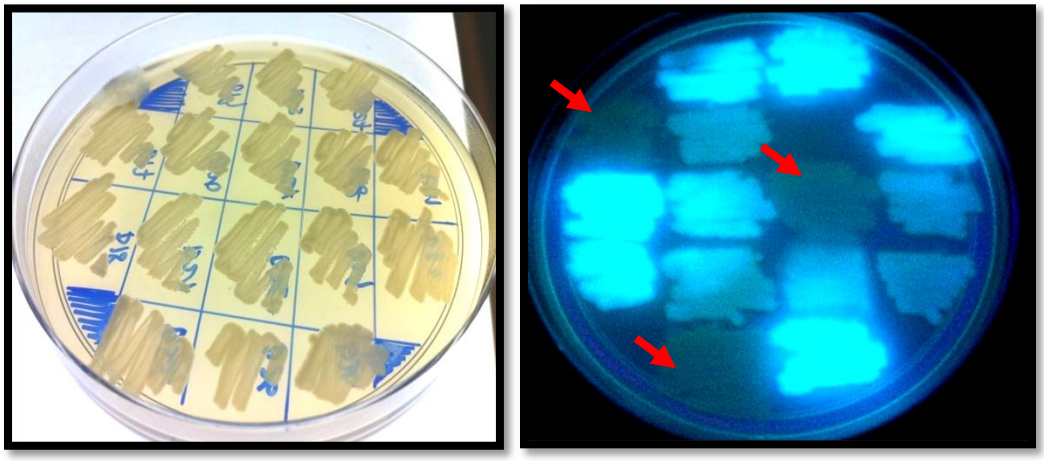


Figure 4.3 Yeast Mannitol medium. Each grid contains single putative agrobacterial isolate.

4.4 Preliminary Screening

4.4.1 King's B Medium

King's B medium allowed the determination and elimination of *Pseudomonas spp.* When growths of bacterial isolates on King's B medium became visible, the plates were exposed to UV light at 365 nm. Approximately 130 isolates were *Pseudomonas aeruginosa* that eventually appeared as colonies with yellow-green fluorescent pigments (Figure 4.4b). Positive results were examined when colonies show no bright green color under dark light (Figure 4.4b). A total of 99 colonies that remained non-fluorescent were tentatively grouped under *Agrobacterium spp.*



(a) **(b)**

Figure 4.4 Typical agrobacterial colonies on King's B a. Before exposure to UV light, isolates exhibit similar morphology on King's B medium. **b.** Arrows indicated positive reactions, putative agrobacterial colonies that remained non-fluorescent under UV light.

4.4.2 Eosin Methylene Blue Medium

Approximately 78% of isolates successfully built dark-purple-colored and irregularly-shaped colonies on Eosin Methylene Blue (EMB) medium due to their ability to ferment lactose. Purple-black undulate colonies that were slightly raised in C_3, C_5, C_7-C_{10}, C_{12}, and C_{15} (Figure 4.5a) were positive for the presence of *Agrobacterium spp*. In contrary with this, large and colorless colonies in C_1 and C_2 are avoided as they are more likely to be lactose-negative species such as *Proteus, Salmonella* and *Shigella.* Meanwhile, colony in C_6 was eliminated as *Pseudomonas spp.* due to its irregular shape and colorless appearance. *Escherichia coli* were easily eliminated as this particular species tends to reflect a distinctive metallic green sheen on its colony (Figure 4.5c). In addition, the selective agents in EMB medium slightly inhibited the growth of Gram-positive bacteria (Figure 4.5c), thus facilitated the removal of undesired species.

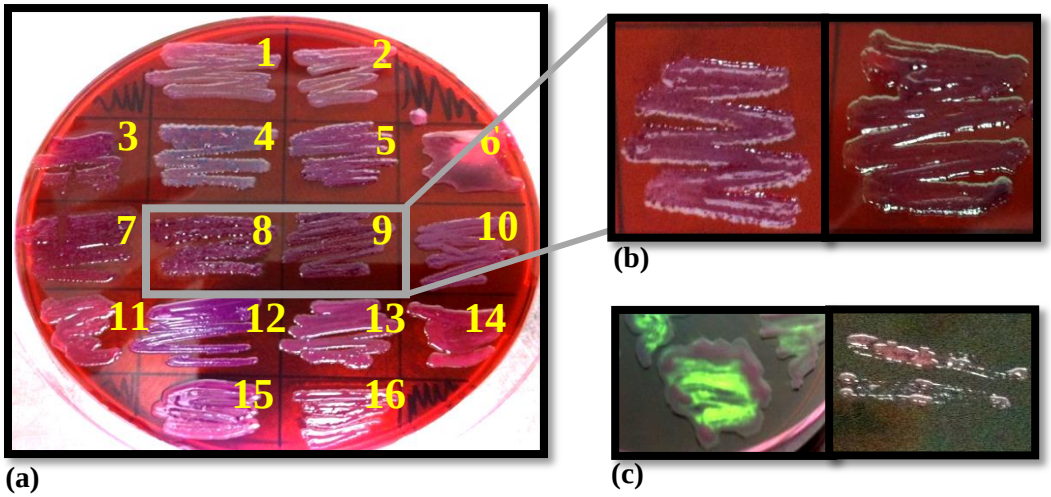


Figure 4.5 Typical agrobacterial colonies on EMB medium. a. Positive reaction-Purple-black colonies on C_3, C_5, C_7-C_{10}, C_{12}, and C_{15}. (eg. C_3 = Colony on grid 3) **b.** Higher resolution of positive colonies. **c.** Negative reaction-Distinctive metallic green sheen reflected by *E. coli* species (left) and Gram-positive colony that displayed inhibited growth (right).

4.4.3 Hofer's Alkaline Medium

Hofer's Alkaline (HA) Medium promotes the growth of *Agrobacterium spp.* while inhibiting *Rhizobium spp.* from soil sample. *Agrobacterial* isolates were expected with well pronounced growth on HA medium (Figure 4.6). An amount 205 isolates showed luxuriant growth on this medium associated with typical circular glistening morphologies of *Agrobacteria*. In Figure 4.6, 'empty' grid represented *Rhizobium spp.* that failed to re-grow on medium with alkaline pH.

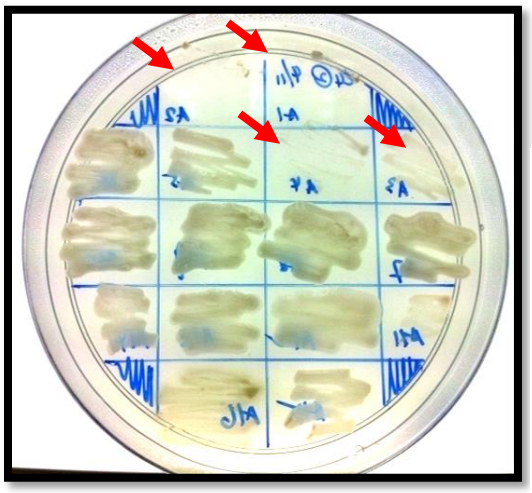


Figure 4.6 Typical agrobacterial colony on Hofer's Alkaline medium. Putative agrobacteria isolates showed well pronounced growth. Arrows indicated negative reactions, 'empty' grids with no visible growth.

4.4.4 Efficiency of differential media in elimination of unwanted species

First round of screening process successfully reduced 65.5% (150 isolates) of unwanted species. According to Figure 4.7, King's B medium showed the greatest contibution with the removal of 56.8% of non-agrobacterial colonies, followed by 21.8% and 10.5% by EMB and HA media respectively. These datas stated that

Agrobacterial strains are more likely to be naturally occurring with *Pseudomonas spp.* instead of *Rhizobium spp.*

Table 4.2 Number of positive isolates on each type of test medium

Sample Site	Initial Number of Isolates	King's B Medium	EMB Medium	Hofer's Alkaline Medium	Total Positive Isolates
S1	51	21	43	47	18
S2	32	17	24	28	13
S3	39	12	33	35	11
S4	31	11	29	28	9
S5	11	7	10	11	7
S6	26	8	15	23	4
S7	39	23	25	33	17
				Total	**∑ 79**

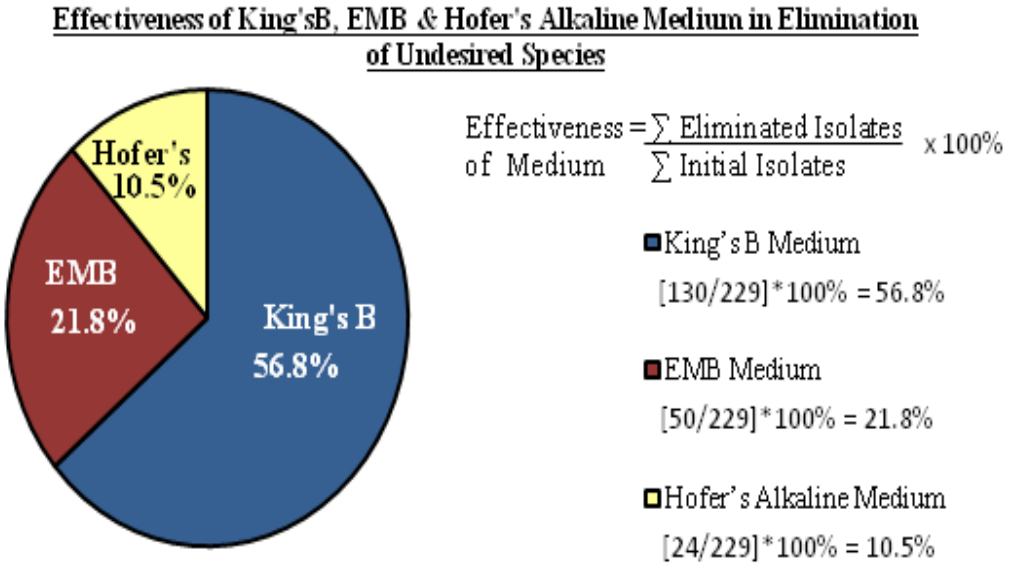


Figure 4.7 Effectiveness of King's B, EMB and Hofer's Alkaline media elimination of undesired species.

4.5 Biochemical Tests

At this stage, 79 isolates (27 isolates from 1A medium, 52 isolates from D1 medium) that successfully sustained from previous screening were believed to exhibit similar features with each other, thus they need to be further characterized through biochemical tests. Eight types of biochemical test have been performed and each of them was associated with vary degrees of efficiency in elimination of non-agrobacterial isolates (Table 4.3). Figure 4.8 illustrated that the greatest reduction was illustrated by 3-ketolactose test (92%), followed by Urease test (72%) and Potassium tellurite test (52%).

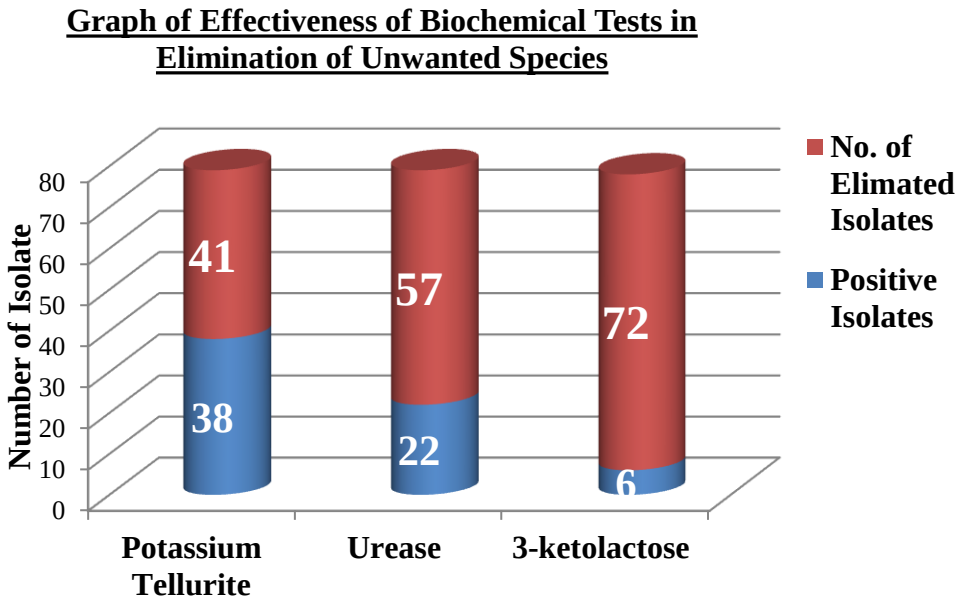
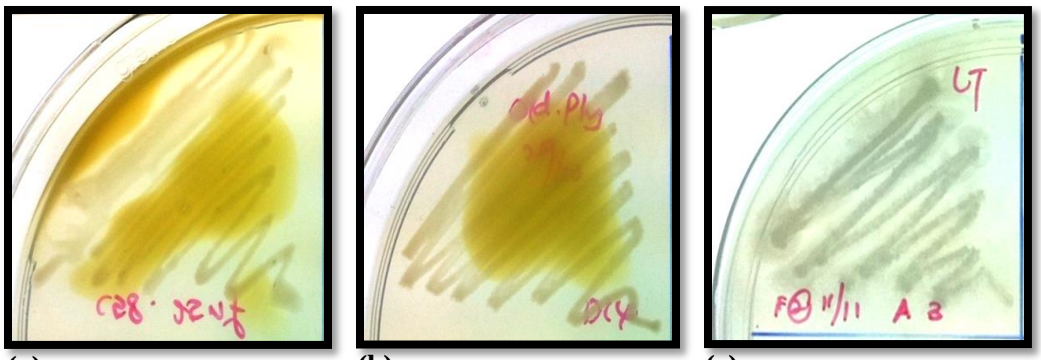


Figure 4.8 Effectiveness of biochemical tests in elimination of unwanted species.

4.5.1 3-ketolactose Test

Bernaerts and De Ley. (1969), stated *A. tumefaciens* is able to convert lactose to 3-ketolactose enzymatically. On lactose agar, fresh growth was flooded with Benedict's reagent for 2 h and then the presence of *A. tumefaciens* was confirmed through the establishment of yellow deposit around the colony resulted from the precipitation of cuprous oxide, CuO_2. Approximately 92% of isolates gave negative reactions due to the absence of yellow precipitation. Other than the standard sample (Figure 4.9a), there are only 6 isolates (8%) demonstrated a positive reaction, which included S1D1 (Figure 4.9b), S1A7, S1A8, S4A5, S5D9 and S7A2.



(a) **(b)** **(c)**

Figure 4.9 3-ketolactose test. **a.** *Agrobacterium* C58 strains as the positive control **b.** Positive reaction was evident by intense yellow precipitation formed by S1D1. **c.** Negative reaction was evident by the absence of yellow deposit.

4.5.2 Urease Test

Stuart's urea broth was formulated to contain phenol red as the pH indicator (Acidic: yellow color, Alkaline: fuchsia color). Urease test is necessary in order to determine the ability of isolates to secrete urease, an enzyme that catalyzes the hydrolysis of urea compounds in the broth. *A. tumefaciens* has fast-acting urease which can degrade urea rapidly, indirectly increased the pH value to alkaline level, and thus induce a colorimetric change of the broth. This phenomenon was observed in 22 isolates, where originally yellow color broth changed to orange-red or fuchsia color (Figure 4.10) within the first 48 h of incubation, and thereby bespeaks a positive reaction. Negative reaction was indicated when urease-negative bacteria grew and increased the turbidity of the broth meanwhile remained identical as the uninoculated broth (negative control).

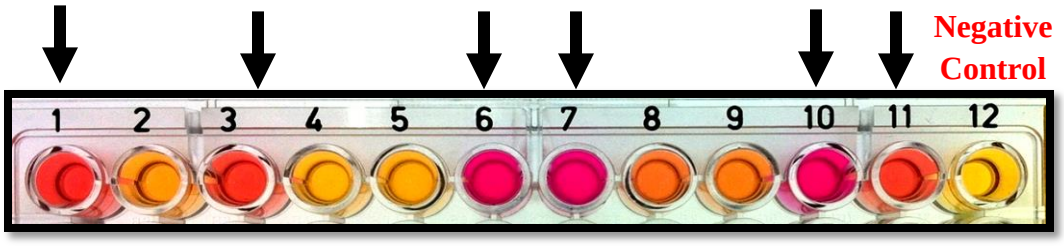
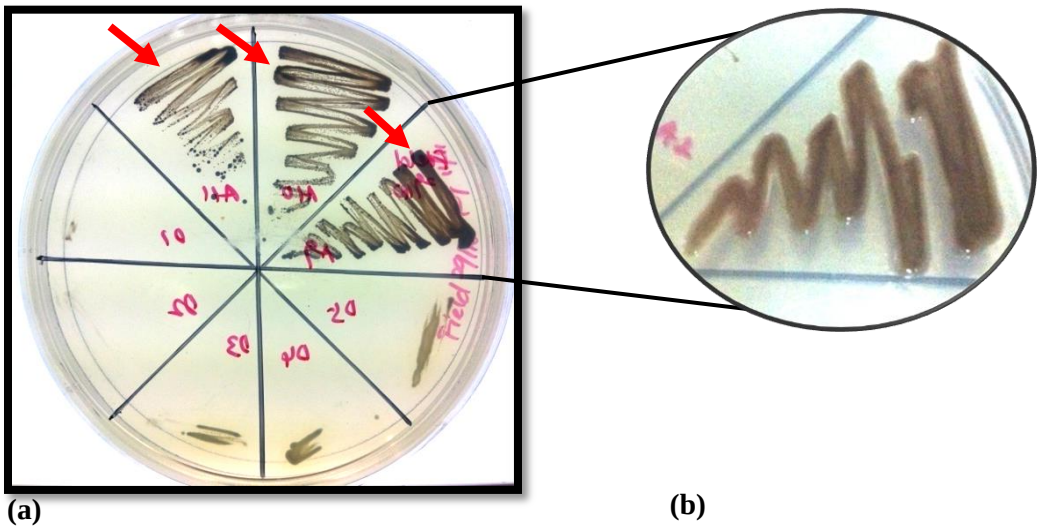


Figure 4.10 Urease test. Inoculated 96 Well Plate was observed for colorimetric changes. Arrows indicated positive reactions, included wells labeled with number 1, 3, 6, 7, 10 and 11. Well labeled number 12 is the negative control.

4. 5.3 Potassium Tellurite Test

Potassium tellurite test is a rapid method of testing the ability of isolates to initiate the reduction of potassium tellurite ions. *A. tumefaciens* are known to be resistant to the toxicity of tellurite ions. Positive reactions were evident by the formation of black deposit in the colonies (Figure 4.11). A total of 38 isolates grew abundantly with shiny, opaque and convex appearance on YMM amended with 8 mg/mL potassium tellurite solution. The presence of tellurite compounds resulted in 52% of the total isolates unable to thrive on the YM medium, thus signified the negative reactions.

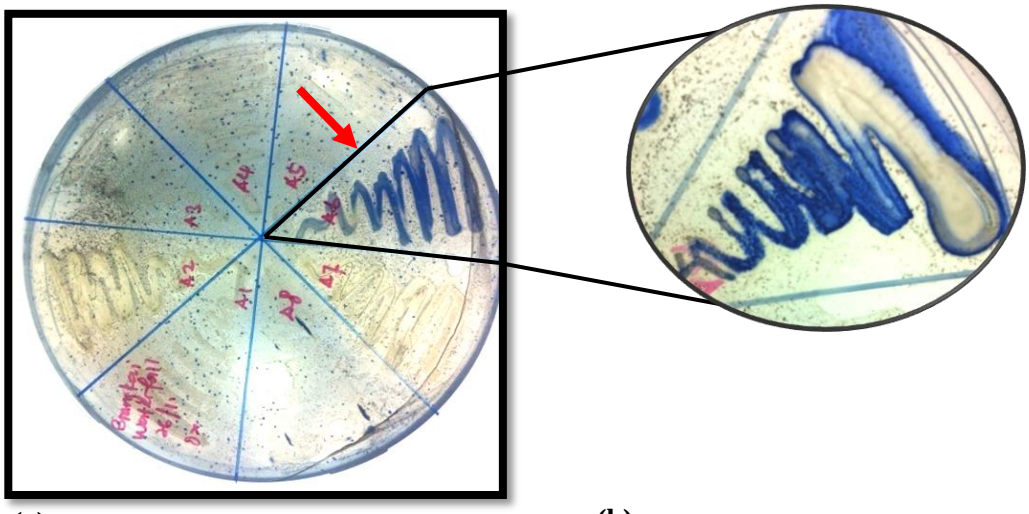


(a) **(b)**

Figure 4.11 Potassium tellurite test a. Arrows indicated positive reaction. **b.** Close-up showed black deposit and shiny appearance of positive isolate.

4.5.4 Oxidase Test

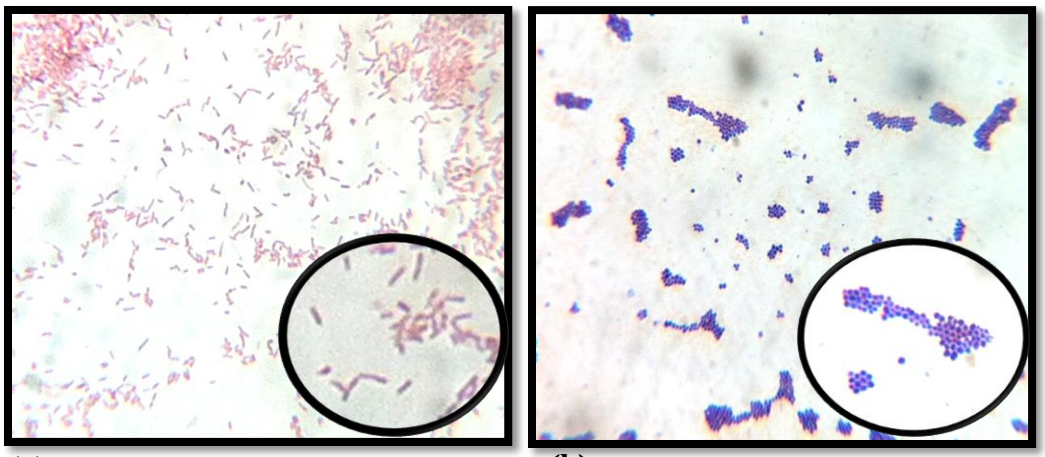
Oxidase test is a biochemical reaction that assays for the presence of cytochrome c oxidase, an essential enzyme of the electron transport chain. By using direct plate method, blackish purple oxidase reagents (Gaby-Hadley reagent A & B) that containing chromogenic reducing agent were permitted to flow over fresh growth on YMM. A total of 40 isolates were successfully produced cytochrome c oxidase to induce the oxidation of oxidase reagents, and thus signifies the positive reactions by forming readily visible deep indigo blue color in and around the border of colonies (Figure 4.12). Oxidase-negative isolates made up 49% of the total isolates, where oxidase reagents remained reduced and colonies retained their original color.



(a) **(b)**

Figure 4.12 Oxidase test a. Arrows indicated positive reaction. **b.** Close-up showed indigo blue color was observed in and around positive isolate.

4.5.5 Gram Staining



(a) **(b)**

Figure 4.13 Image of bacterial cells under 100x magnifications after gram staining was performed. **a.** Positive reaction- Pink colored and rod shaped Gram-negative bacteria **b.** Negative reaction- Cocci and purple-blue colored Gram-positive bacteria.

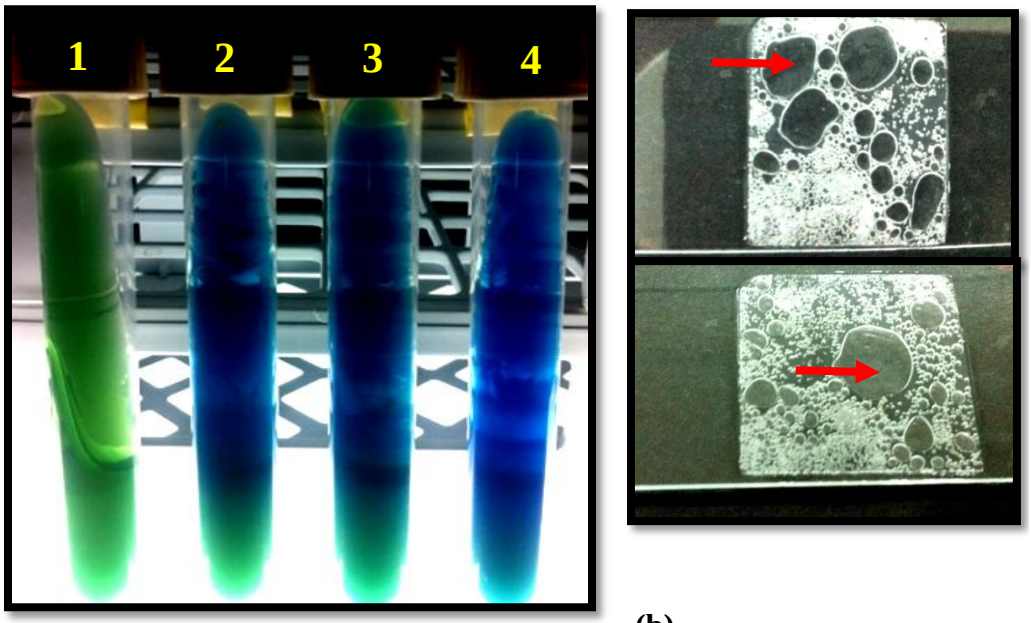
Gram staining was performed to differntiate Gram-positive and Gram-negative bacteria in simultaneously study about the cellular morphology of isolates. A total of 45 isolates signified positive result and appeaered as rod-shaped cells that retained the pink color of Safranin dye (Figure 4.13a), thereby were then classified as Gram-negative bacteria. As shown in Figure 4.13b, clusters of blue cocci cells, which usually the characteristics of *Staphylococcus spp.* were considered as Gram-positive bacteria and eliminated. Coccobacillus bacteria that stained pinkly, the traits that usually found in *Pseudomonas spp.* also been excluded from subsequent screening process.

4.5.6 Citrate Utilization Test

Citrate Utilization test was performed to check for the ability of bacteria to use citrate as its sole carbon source and energy for growth. For citrate positive bacteria, bacteria grew abundantly on Simmons Citrate agar slant. The by-products of citrate catabolism induced bromothymol blue in the medium changed from originally forest green color (pH <7.0) to intense blue color (pH > 7.6). Positive reactions were demonstrated by S1D1, S1A7, S1A8, S4A5, S5D9 and S7A2 (Figure 4.14a). For citrate negative bacteria, they grow weakly on the slant and tend to be virtually indistinguishable from an uninoculated slant as they did not cause any color changes of the medium (Figure 4.14a).

4.5.7 Catalase Production Test

Catalase test is essential for the determination of catalase, an enzyme that affords protection for bacteria to against the toxic by-products of oxygen metabolism. Through slide method, immediate effervescence beneath the cover slip was observed from S1D1, S1A7, S1A8, S4A5, S5D9 and S7A2. These isolates successfully generated copious bubbles upon the addition of 3% hydrogen peroxide (H_2O_2) on the fresh growth (Figure 4.14b). No obvious bubbles are liberated by the smear of colony, indicated the absence of catalase enzyme, and thereby signified a negative reaction.



(a) **(b)**

Figure 4.14 Citrate utilization, and catalase production test. a. Tube 1= Negative Control, Tube 2= Positive Control, Tube 3= S1A7 and Tube 4= S1A8. Positive reactions for citrate utilization test were demonstrated by colorimetric changes in Tube 2, 3 and 4. **b.** Positive reaction was evident by the formation of copious bubbles as indicated by the arrow.

Table 4.3 Number of positive reactions resulted from each biochemical test

Biochemical Tests	Sample Site						
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**
Initial No. of Isolates	18	13	11	9	7	4	17
1. Gram Staining	12	4	6	4	6	4	9
2. Oxidase Test	9	5	5	5	6	3	7
3. Urease Test	7	2	3	3	2	2	3
4. Salt Tolerance Test	5	5	3	3	5	2	5
5. Potassium Tellurite Test	6	7	4	5	3	4	9
6. 3- Ketolactose Test	3	0	0	1	1	0	2
7. Catalase Production Test	3	0	0	1	1	0	1
8. Citrate Utilization Test	3	0	0	1	1	0	1
Total No. of Positive Isolates	3	0	0	1	1	0	1

4.6 Molecular Identification

4.6.1 Colony PCR

The amplified colony PCR products were resolved on 2% agarose gel. As demonstrated in Figure 4.15, Lane 1 is the *Agrobacterium tumefaciens* C58 (positive control) whereas Lane 8 is the negative control. No amplifications were observed in negative controls (Lane 8) containing no inoculum. In the presence of 100 bp DNA ladder as the molecular weight marker, isolates in Lane 2 to Lane 7 exhibited similar bands with the standard sample with a size of 184 bp after the amplification process using UF and B1R primers. They were now confirmed as *A. tumefaciens.*

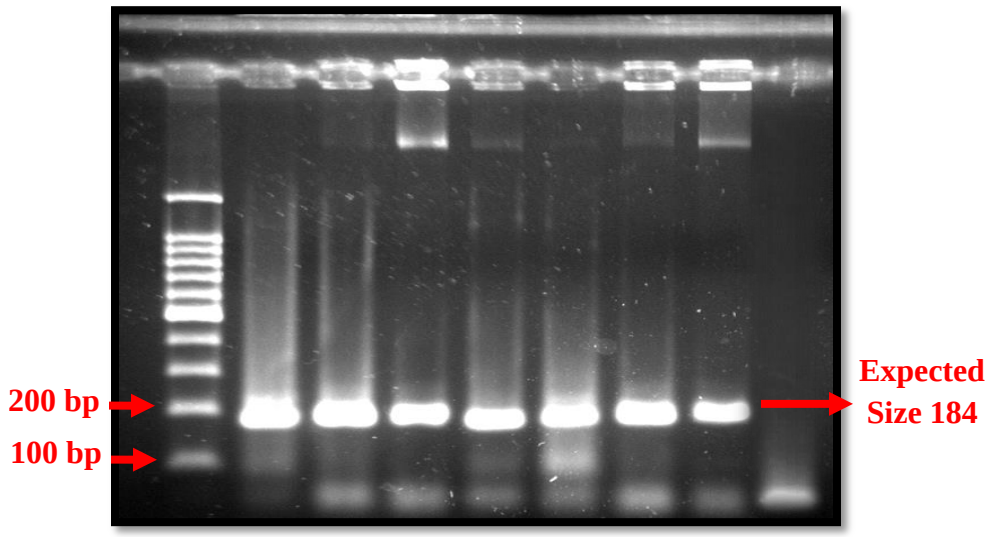


Figure 4.15 Resolution of colony PCR amplified product on 2% agarose gel. Lane 1 = Positive Control, Lane 8 = Negative Control, Lane 2-Lane 7 are S1D1, S1A7, S1A8, S7A2, S4A5 and S5D9 respectively.

4.6.2 Genomic PCR

4.6.2.1 Genomic extraction using Fast Boil Technique

Alternately, the validation of isolates as *A. tumefaciens* also has been performed through genomic PCR method. Genomic DNA of all the isolates was extracted using Fast Boil technique and subsequently subjected to amplification through normal PCR process. According to Table 4.4, the values of $A_{260/280}$ obtained were ranged from 1.82 to 1.91, indicated the high purity of the extracted DNA.

Table 4.4 Optical Density (OD) readings of positive isolates

Sample	Concentration (ng/µL)	Amount (mg)	$A_{260/280}$
Positive Control	207.1	0.1035	1.84
S1D1	541.1	0.2706	1.89
S1A7	452.0	0.2260	1.86
S1A8	597.3	0.2987	1.91
S7A2	818.1	0.4091	1.87
S4A5	722.8	0.3614	1.85
S5D9	355.9	0.1779	1.82

4.6.2.2 Genomic PCR

According to the result obtained (Table 4.4), S7A2 has the highest concentration of DNA (818.1 ng/µL) and was approximately 4 times the amount of DNA extracted from standard sample (207.1 ng/µL). These figures were corresponded with the intensity bands formed on the gel image (Figure 4.16), where S7A2 and S4A5 that associated with higher concentration of DNA eventually resulted in bands of higher intensity. In contrast, amount of DNA extracted from S5D9 was relatively low, thus appeared as faint band. However, the disparity in band intensity can be avoided through proper dilution and standardization in DNA concentration of each isolates prior to gel electrophoresis. The gel images obtained from both molecular identification processes (Figure 4.15 and Figure 4.16) were high in similarity, proved that both techniques were equally reliable and applicable.

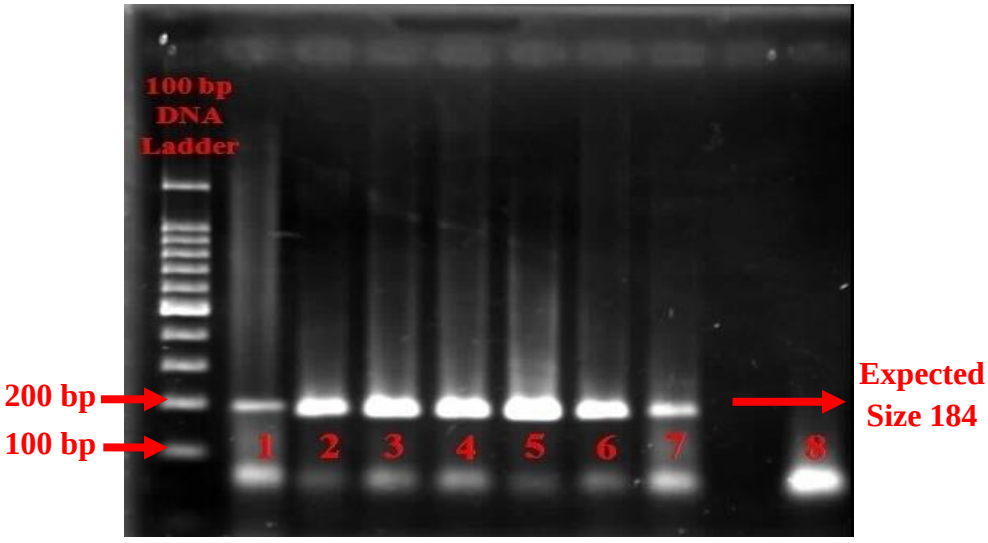


Figure 4.16 Resolution of genomic PCR amplified product on 2% agarose gel. Lane 1 = Positive Control, Lane 8 = Negative Control, Lane 2 to Lane 7 are S1D1, S1A7, S1A8, S7A2, S4A5 and S5D9 respectively.

4.7 Pathogeniticity test

4.7.1 Carrot Disc Assay

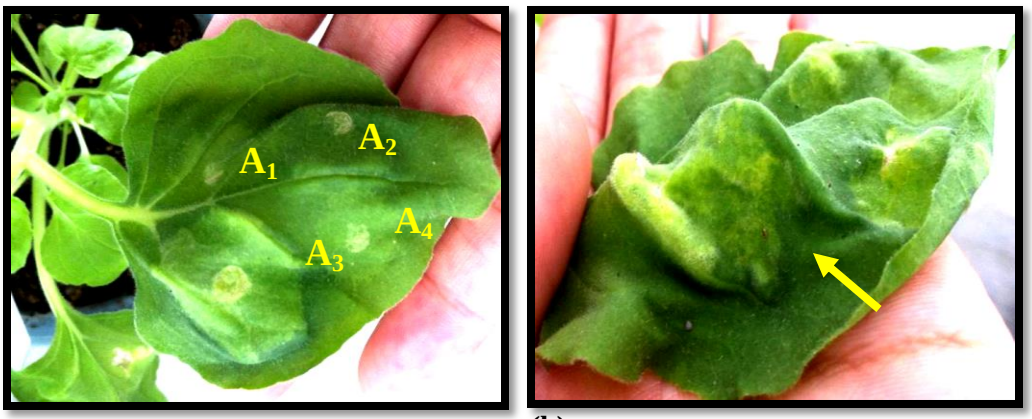
(a)

(b)

Figure 4.17 Young callus formation on *Daucus carota* by S1D1. a. Arrow indicated cream colored young calli on the carrot disc. **b.** Arrow indicated the area where callus tissues slowly exhibited brownish-green color.

After 35 days of incubation, young calli which usually soft, spherical and white to cream color were developed from the meristematic tissues around the central vascular system of the carrot disc (Figure 4.17). The calli formed were solid throughout, and approximately 1.5 cm x 0.5 cm in size. Calli undifferentiated continually and is not protected by an epidermis, leaving the host plant susceptible to secondary infection. When the incubation periods were extended, the aging of callus tissues caused itself began to exhibit brownish-green color. Tumor forming ability in one of the isolate, S1D1 finally confirmed it as indigenous virulent *A. tumefaciens* strains.

4.7.2 Agroinfiltration



(a) (b)

Figure 4.18 Agroinfiltration of *Nicotiana benthamiana.* **a.** Tobacco leave was divided into four injection sites. A_1=S1D1, A_2=GFP, A_3=Positive control (*Agrobacterium tumefaciens* C58), A_4=Negative control (A1 buffer with no inoculum) **b.** Arrow indicated the outward symptom (positive reaction) shown by positive control.

Virulent strain, S1D1 was subsequently agroinfiltrated into the leaves of tobacco plant (*Nicotiana benthamiana*) in order to study its virulence activity in different host plants. After 7 days of incubation, no outward symptom was observed on the site injected with S1D1, negative control and Green Fluorescent Proteins (GFP) (Figure 4.18a). In contrary of this, *Agrobacterium tumefaciens* C58 caused abnormal cell proliferation depending on its original injection site, caused the leaves to deform and eventually resulted in the formation of visible bulge (Figure 4.18b). Meanwhile the absent of obvious bulge on the site injected with S1D1 probably due to the host specificity that associated with this indigenous strain.

4.8 Plasmid Extraction

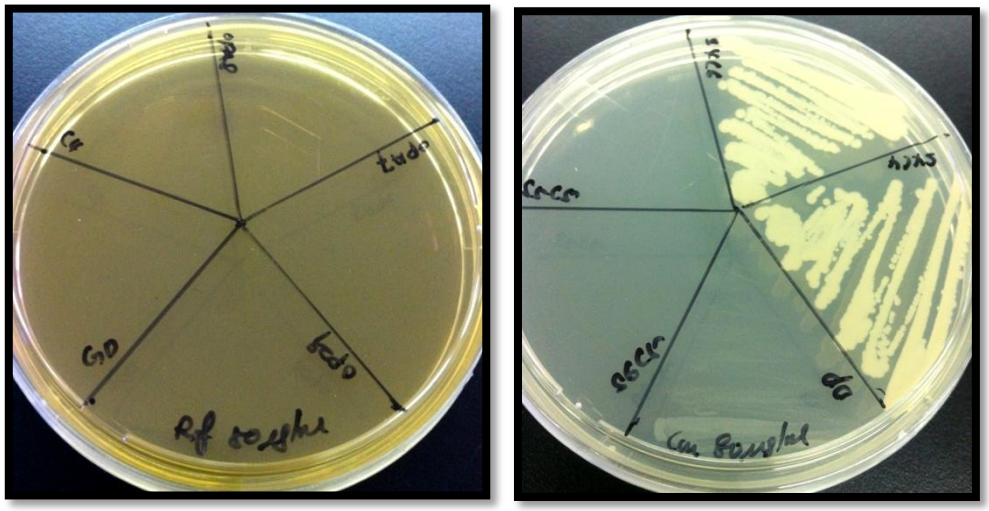
4.8.1 Antibiotic test using 8 types of antibiotics

Positive isolates obtained from this study (S1D1, S1A7, S1A8, S4A5, S5D9, and S7A2) together with four potential strains contributed by previous student (S4C4, S4C6, S5C5 and S6C5) were all treated with different antibiotics in order to detect the presence of antibiotic resistance genes meanwhile to elect a suitable selection marker for the subsequent genetic engineering process. Responses of all the isolates toward these antibiotics were all tabulated in Table 4.5. Two isolates, S4C4 and S4C6 gave anomalous results, where both of them were resistance to all type of antibiotics except 100 µg/mL of Spectinomycin. Besides them, no growth was observed on the medium containing 50 µg/mL of Rifampicin (Rif) from other isolates (Figure 418a). Results showed that most of isolates were resistant to at least four types of antibiotics, indicated the presence of multiple antibiotic resistance genes within these samples. In contrast, S6C5 was susceptible to six types of antibiotics but not for 20 µg/mL of Hygromycin, and 14 µg/mL of Chloramphenicol (Cm).

4.8.2 Antibiotic test using different concentration of Chloramphenicol

Since all the isolates can grow on the medium containing 14 µg/mL of Cm, therefore the antibiotic resistance test was preceded using different concentrations of Cm, ranged from 14 µg/mL to 100 µg/mL (Table 4.6). When the concentration of Cm was elevated to 20 µg/mL, S4A5 turned susceptible and when the

concentration achieved 30 µg/mL, additionally another two isolates (S1A7 and S6C5) failed to multiply on the medium. Analysis showed that 80% of the isolates were susceptible to 60 µg/mL of Cm. Eventually, only S4C4, S4C6 and S1D1 were able to thrive on the medium supplemented with 100 µg/mL of Cm.



(a) **(b)**

Figure 4.19 Antibiotic resistance test. a. Most of the isolates showed inhibited growth on LB medium supplemented with 50 µg/mL of Rif. **b.** Only S4C4 and S4C6 grew abundantly on LB medium supplemented with 80 µg/mL of Cm.

Table 4.5 Antibiotic resistance test of using different type of antibiotics

Positive Isolates	Antibiotics							
	100 µg/mL Carbenicilin	20 µg/mL Hygromycin	50 µg/mL Kanamycin	50 µg/mL Rifampicin	100 µg/mL Spectinomycin	5 µg/mL Tetracycline	100 µg/mL Gentamycin	14 µg/mL Chloramphenicol
S4C4	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**R**
S4C6	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**R**
S5C5	**S**	**R**	**S**	**S**	**S**	**R**	S	**R**
S6C5	**S**	**S**	**R**	**S**	**S**	**S**	S	**R**
S1D1	**R**	**R**	**R**	**S**	**S**	**R**	S	**R**
S1A7	**R**	**R**	**R**	**S**	**R**	**S**	**R**	**R**
S1A8	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**
S4A5	**S**	**R**	**R**	**S**	**R**	**S**	**S**	**R**
S5D9	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**
S7A2	**R**	**R**	**R**	**S**	**R**	**R**	**R**	**R**

Remarks: ***R = Resistance***

S = Susceptible

Table 4.6 Antibiotic resistance test of using different concentration of Chloramphenicol

	Chloramphenicol						
Positive Isolates	**14 µg/mL**	**20 µg/mL**	**30 µg/mL**	**40 µg/mL**	**60 µg/mL**	**80 µg/mL**	**100 µg/mL**
S4C4	**R**	**R**	**R**	**R**	**R**	**R**	**R**
S4C6	**R**	**R**	**R**	**R**	**R**	**R**	**R**
S5C5	**R**	**R**	**R**	**R**	**S**	**S**	**S**
S6C5	**R**	**R**	**S**	**S**	**S**	**S**	**S**
S1D1	**R**	**R**	**R**	**R**	**R**	**R**	**R**
S1A7	**R**	**R**	**S**	**S**	**S**	**S**	**S**
S1A8	**R**	**R**	**R**	**R**	**R**	**S**	**S**
S4A5	**R**	**S**	**S**	**S**	**S**	**S**	**S**
S5D9	**R**	**R**	**R**	**S**	**S**	**S**	**S**
S7A2	**R**	**R**	**R**	**S**	**S**	**S**	**S**

Remarks: ***R = Resistance***

S = Susceptible

4.8.3 Isolation of Ti-plasmid

Virulence gene and T-DNA, two main constituents of Ti-plasmid are believed to have the strength to enhance the plant transformation efficiency. Therefore, to study the structure as well as the characteristics of these components, extraction of Ti-plasmid is imperative. The $A_{260/280}$ reading of extracted plasmid was within the range of 1.8 – 2.0, they are high in purity with no RNA contamination. However, the large disparity among the concentration values of all the isolated strains, indicating that high amount of chromosomal DNA might present in these samples.

Table 4.7 OD readings of extracted plasmid

Positive Isolates	Concentration (ng/µL)	Amount (mg)	$A_{260/280}$
S4C4	3465.9	1.7330	1.90
S4C6	3362.6	1.6813	1.91
S5C5	3456.9	1.7285	1.93
S6C5	3292.0	1.6460	1.97
S1D1	18564.1	9.2820	1.99
S1A7	3275.2	1.6376	1.98
S1A8	2512.2	1.2561	1.96
S4A5	18571.6	9.2858	1.80
S5D9	17246.7	8.6235	1.99
S7A2	15814.9	7.9075	1.95

CHAPTER 5

DISCUSSION

5. 1 Soil Sampling

Seven regions of Kampar were selected for soil sampling. For S1 (Kampar old-town playground), clay soils were collected from the plant rhizosphere beneath 2 m-tall trees meanwhile the sandy soils from S5 (C4 land) were initially placed in a wide-mouth containers that been highly exposed to sunlight during daytime. Loam clay soils in S1 offer higher retention capacity for nutrients and water as well as less affected by sunlight, hence make it a more favorable environment for wide variety of microorganism, including *Agrobacterium spp*. In contrary of this, sandy soils of S5 allowed direct absorption of sunlight, lead to elevation of surrounding temperature and thus resulted in significant reduction of microorganism population. These statements were corresponded with the data shown in Figure 4.2, highest amount of microorganism were successfully isolated from S1 (51 isolates) and lowest contribution by S5 (11 isolates).

Agrobacteria can spread from place to place, via different channels, winds or even water (Tufts.edu, 2014). For S5, soil was transferred from other plantation fields where it might be initially contaminated by the *Agrobacterium spp.* Even though

S5 showed the lowest amount of survivors due to environmental factors, but it actually had contributed a single potential isolate (S5D9) for the study of *Agrobacterium spp*. Except for S5, most of the soil samples were obtained from rhizosphere of trees, thus it's recommended to allow soil suspension to be clarified by filtration using a screen with appropriate size of opening for removal of plant residues (Bouzar et al., 1987). Furthermore, vortexing can be avoided and alternately apply sonication on the slurry obtained (KG, 2014), to promote the dislodgement of microorganism into the surrounding fluid, thereby enhance the efficiency in isolation of *Agrobacterium spp.*

5.2 Semi-selective Media

5.2.1 1A Medium

Microorganisms were recognized based on their ability to degrade the rare sugar alcohol, L-arabitol and to tolerate with the toxicity of selenites and tellurites ions. Selected colonies contain L-arabitol dehydrogenase, an enzyme that act on L-arabitol compounds to acquire carbon and energy source for growth. Competitive bacteria was restricted due to the presence of selenite ions in 1A medium, but as compared with the final result, optimization on the concentration of selenite is imperative and remained to be performed.

Initially a total of 81 colonies displayed typical characteristics of agrobacteria, but ended up only 4.9% of them were identified as *Agrobacterium tumefaciens.* In fact, tellurite compound confers a selective advantage for *Agrobacterium spp.* but it is not sufficiently powerful to discriminate other population of microorganism. This statement was in general agreement with the result by Melodie (2006), indicated not only *Agrobacterium spp.,* but many other species of microbes were resistant to the toxic amount of tellurite as well.

5.2.2 D1 medium

On D1 medium, microorganism was ascertained for their ability to utilize mannitol as the source of carbohydrate. Lithium chloride (LiCl) was added as it strongly inhibits the growth of *Pseudomonas spp.* As a pH indicator, bromothymol blue allows the detection of acidification that occurred in D1 medium in the meantime slightly against the growth of Gram-positive bacteria.

The plate was originally dark green in color (pH 7), medium turned yellowish upon the growth of microorganism, thence created colonies surrounded with yellow zone. Waste compounds (eg. carbon dioxide, CO_2) excreted by bacteria caused the pH value of D1 medium to drop below pH 6, and eventually induced colorimetric changes. This phenomenon explained the patches form of yellow zone that only occurred surrounded the colonies.

5.2.3 Selectivity of 1A and D1 media

In the beginning, a total of 229 isolated colonies (81 from 1A medium; 148 from D1 medium) were tentatively identified as *Agrobacterium* strains. After been through all the identification processes, eventually only six isolates were successfully confirmed as *A. tumefaciens*, including S1D1, S1A7, S1A8, S4A5, S5D9 and S7A2. By relating final results with the initial figures, 5% of the 81 isolates obtained from 1A medium are the desired *A. tumefaciens*. Even though D1 medium contributed a higher proportion (65%) of the total amount of isolates, but at the end only approximately 2% of them was confirmed as *A. tumefaciens*. The percentages showed by both media are relatively low, revealed them as semi-selective media and called for the need of further modifications to enhance the isolation of *Agrobacterium spp.*

Moreover, as shown by the nomenclature of the positive isolates, 4 of them were initially isolated from 1A Medium, and 2 of them from the D1 medium. In another word, 67% of the total positive isolates were actually contributed by 1A medium. Therefore assumption was made where 1A medium confers a higher selectivity (5%) for *Agrobacterium spp.* in compared with that of D1 medium (2%). However the amount of isolates that being processed was too few in amount, thus the result obtained was not significant enough to conclude on the selectivity of 1A and D1 media.

5.3 Yeast Mannitol Medium

Isolates obtained from selective media must not be directly subjected to further analysis since these colonies might contain certain chemical compounds that render the identification impossible to achieve. Therefore, isolates obtained were subsequently re-grow on Yeast Mannitol (YM) medium for purification purpose. However, as recommended by Shams et al., each colony should be purified at least twice to ascertain a pure culture of an *agrobacterial* strain (Shams et al., 2012). For subsequent analysis, pure cultures were taken from the center of isolates to prevent any cross-reactions that might result in false identification.

5.4 Preliminary Screening

5.4.1 King's B Medium

King's B medium is widely used in water analysis for the detection of *Pseudomonas spp*., particularly *Pseudomonas aeruginosa* (King et al., 1954). Peptone was added into King's B medium to promote the production of pyoverdin, enabling them to appear as yellow-green pigments under UV light. This phenomenon won't occur in *Agrobacterium spp.*, and thus facilitates the elimination of *P. aeruginosa.* The efficiency of King's B medium in reduction of 130 non-agrobacterial isolates (Table 4.2) was corresponded with the study of other researchers, where *Pseudomonas spp.* is the soil-habiting bacteria that often found in the habitat similar as *Agrobacterium spp*. (Melodie, 2006).

5.4.2 Eosin Methylene Blue Medium

Eosin Methylene Blue medium is a type of differential microbiological medium made up of two dyes known as Eosin Y and Methylene Blue which combined in the ratio 6 : 1 (microbeonline, 2013). The combination of these stains can slightly retards the growth of Gram-positive bacteria and provides a color indicator in distinguishing between lactose-fermenter and non-lactose-fermenter. A total of 179 isolates (Table 4.2) were purple-black and irregular in shape, thus signified positive reaction for lactose fermentation. These lactose-fermenters produced acids, lowered the pH of surrounding environment, and thence encouraged dyes absorption by the growths.

On the other hand, around 50 isolates (Table 4.2) failed to perform lactose fermentation, instead they elevated pH value of medium through deamination of proteins and limited the dye absorption process, thus was eliminated. Among those discriminated isolates, many of them were colorless non-lactose-fermenter or weak lactose-fermenter with mucoid pink appearance (Figure 4.5a). Methylene Blue can tell whether the growths are alive or not because it has no effect on living cells. C_4 displayed the color of Methylene Blue (Figure 4.5a), indicated C_4 were dead cells that no longer able to keep Methylene Blue molecules from penetrating its cell membrane.

5.4.3 Hofer's Alkaline Medium

Similar with *Agrobacterium spp., Rhizobium spp.* live freely in the soil and the root region of both leguminous and non-leguminous plants (Beijerinck, 2008). Isolates were cultured on Hofer's alkaline medium, in order to eliminate the *Rhizobium spp.* through the strong alkaline environment within the medium. As much as 205 isolates (Table 4.2) have the strength to resist the alkaline pH of medium and grew abundantly, meanwhile only 24 isolates (Table 4.2) that showed no growth on the medium were eliminated.

5.5 Biochemical Tests

5.5.1 3-ketolactose Test

Biochemical test also has been analyzed based on their ability to remove non-agrobacterial isolates. In this case, 3-ketolactose test is the most important differential procedure because among all the strains of *Agrobacterium spp.,* only few of them will ferment carbohydrate such as lactose to signify a positive result (Table 4.3). This test signed the presence of *A. tumefaciens* through the formation of intense yellow precipitate known as cuprous oxide (Figure 4.9). There are 6 isolates that able to produce 3-ketoglycosides enzyme to catalyze the conversion of lactose to 3-ketolactose. Upon catabolism of lactose, isolates were allowed to grow to visible colony (2 to 3 days) since young cultures tend to give weak responses or false positive result. A total of 91% of the total isolates showed no

yellow zones, in fact, means the culture might be something other than *A. tumefaciens,* for instance *E. coli* (Figure 4.8).

5.5.2 Urease Test

Urease is an exoenzyme that can hydrolyze urea to ammonia (NH_3) and carbon dioxide (CO_2). Urease was discovered in many species but only few of them can degrade urea rapidly. One of the recognized 'rapid-urease-positive' microorganisms is *A. tumefaciens.* Stuart' urea broth was formulated to have restrictive amount of nutrients coupled with the use of two pH buffers, in order to prevent common urease-containing microorganism from inducing alkalization in urea broth. Inversely, *A. tumefaciens* that contains fast-acting urease was encouraged to overcome the high buffering capacity of broth, created an alkaline environment and allowed urease to be constitutively expressed thus eventually resulted in color changed from yellow-orange to fuchsia color (Figure 4.10). Results obtained were proven theoretically with relatively low amount of positive results, where only 2-3 isolates was contributed by each sample site. As compared with the final result, noted that some of these positive reactions could be contributed by other rapid-urease-positive microorganisms such as *Proteeae*.

5.5.3 Potassium Tellurite Test

Potassium tellurite test was carried out to determine the ability of isolates to sustain and multiply in the presence of toxic tellurite ions. Potassium tellurite solution was added to YM for the selection of *Agrobacterium* spp. and eventually only 36.7% of non-agrobacterium species were eliminated (Table 4.3). As much as 63.3% of the total isolates displayed positive results as characteristic black color colonies were formed on YM medium (Figure 4.11). These figures were expected as most of the isolates were originally selected from tellurite-containing-1A medium.

5.6 Molecular Identification

The results from biochemical test were quite confirmative. The 6 positive isolates were further validated as *A. tumefaciens* through colony PCR and genomic PCR. Both of these molecular identification processes were performed simultaneously with their effectiveness compared. Identical results were obtained where S1D1, S1A7, S1A8, S7A2, S4A5 and S5D9 displayed bands of expected size (184 bp) on the agarose gel image, in corresponding with the band resulted from the positive control, *Agrobacterium tumefaciens* C58 strain (Figure 4.15 & Figure 4.16). Results obtained indicated that both methods are equally powerful and applicable as the tool to identify the existence of *A. tumefaciens.* Besides, comparison showed that colony PCR was less time-consuming, and a large

amount of colonies can be quickly tested due to the absence of additional genomic extraction and DNA purification steps as shown in genomic PCR.

All PCR reactions were performed with analogous amplification conditions using the combination of UF + B1R primer. The primers were specifically designed on the basic of nucleotide sequence of the 23S rDNA determined for *A. tumefaciens* (Pulwaska et al., 2006). UF is the universal forward primer meanwhile B1R is the reverse primer that complementary to chromosomal DNA of *A. tumefaciens* (Pulwaska et al., 2006). Furthermore, the presence of *A. tumefaciens* in undisturbed, non-agricultural soils (S1, S4, S5 and S7) evidenced by these PCR processes, proved that *A. tumefaciens* is a true inhabitant within the soils of many places in Kampar.

Even though there were 6 potential *A. tumefaciens* been identified, but so far these positive isolates must not be directly dictated as the grown call-inciting strains. This is because only *A. tumefaciens* containing a Ti-plasmid able to induce tumor formation whereas those strains lacking of Ti-plasmid will live as rhizosphere-inhabiting bacteria without causing any disease to their host (Shams et al., 2012). PCR primers that been recently developed to amplify genetic region of Ti-plasmid are essential in order to identify the pathogeniticity of isolated strains. For instance, VCF3 + VCR3 primers been admitted for the determination of virulence genes of Ti-plasmid (Kawaguchi et al., 2005).

Modifications are strongly recommended for the molecular identification techniques mentioned in previous section. Instead of adding colony directly into a PCR mixture, identification of *A. tumefaciens* can actually be done by PCR of a rapid colony lysate (Campillo et al., 2012). Creating colony lysate using sodium hydroxide may avoid the isolates being exposed to contaminations and enable the optimization for the amount of bacteria cells being added for each PCR reaction. Furthermore colony lysate can be stored in -20^{o}C and is re-useable with proper thawing procedure (Campillo et al., 2012). On the other hand, for genomic extraction process, the bacteria suspension can be incubated at 100^{o}C for 45-55 sec and then followed by immediate chilling (DSMZ, 2011). This protocol promotes higher efficiency in denaturation process and is less time consuming (DSMZ, 2011).

5.7 Pathogeniticity Test

5.7.1 Carrot Disc Assay

Callus-like tumor was observed on the surface of *Daucus carota* that been infected with S1D1 (Figure 4.17). The spongy calli were developed on the surface of the carrot disc upon the invasion and incorporation of Transfer-DNA (T-DNA) fragment into the genome of host cell. The succeeded integration allowed the genes that responsible for the production of auxin and cytokinin to be expressed constitutively. Moreover T-DNA fragments were replicated together with the host's genome, thereby perturbed the normal cell division of host plant, and

stimulated the callus formation. The hormones secreted by the virulent cells were uncommon for most of the plants, thus they are unlikely to have regulation pathway to suppress the abnormal level of hormones within their cells. In addition, calli were found continuous with the vascular system of the carrot disc, thence reflected the apparent synthesis site and the flow pathways of the encoded hormones. Over time, the aging of callus tissues caused part of it began to exhibit brownish-green color (Figure 4.17) and eventually the carrot disc dies off as it experienced disrupted flow of water and nutrients.

Furthermore, tumor-forming ability of S1D1 admitted it as an indigenous virulent *A. tumefaciens* strains. The other isolates failed to induce any callus formation on the carrot disc even they been proven as *A. tumrfaciens* wit PCR using 23S rRNA primers. This situation can result from the lost of Ti-plasmids which tend to be dispensable, conjugative and easily spread into their respective surrounding environment (Shams et al., 2012). The ratio of pathogenic to nonpathogenic strains is low, where only 1 out of the 6 positive isolates was associated with the tumorigenic ability. Infrequency of pathogenic strains obtained indicated that the soil seems unlikely to be a significant source for tumorigenic *A. tumefaciens*.

5.7.2 Agroinfiltration

Despite S1D1 failed to induce constitutive cell division on its infected site, but visible bulge was successfully effectuated by the positive control (*Agrobacterium tumefaciens* C58) in Figure 4.18. This observation revealed that the parameters (eg. temperature, humidity) and the growth conditions of the host plant were in acceptable range. In fact, intumesce of leaves was actually manipulated by the host specificity of S1D1. The specific host range permitted S1D1 to induce callus-like tumor on *D. carota* but restricted it to generate any upheaval symptoms on the leaves of *Nicotiana benthamiana*.

5.8 Plasmid Extraction

5.8.1 Antibiotic Resistance Test

Antibiotic resistance test was performed prior to plasmid extraction process. Antibiotic resistance genes reside on the transmissible Ti-plasmid (Tufts.edu, 2014). Therefore, the results obtained from antibiotic resistance test aid in countenance potential isolates as Ti-plasmid-containing *A. tumefaciens*, meanwhile permitted us to study the antibiotic resistance genes confer by each isolates. Isolates demonstrated resistance towards multiple antibiotics (Table 4.5). This phenomenon is possible when originally the isolates obtained multiple resistance traits through the genetic exchange with their neighboring bacterium.

All the isolates were resistant to 14 µg/mL of Cm (Table 4.5), hence Cm was supplemented into the growing medium, to increase the selective pressure that allows *A. tumefaciens* to thrive and the other susceptible species to die off. The growth conditions of isolates in LB medium were normalized but large disparity was observed in the susceptibility level of isolates towards Cm of distinct concentration (14 µg/mL – 100 µg/mL) (Table 4.6). Isolates exhibited vary expression level of antibiotic resistance gene within the medium suggests that the degree of resistance toward Cm was actually strains dependent.

5.8.2 Isolation of Ti-plasmid

Extracted plasmid DNA were analyzed through 0.5% agarose gel, the bands obtained showed that all the extracted plasmid have sizes far beyond 10 kb. Other than the showing the validation in molecular weight of plasmid DNA and the purity of the lysate, the resolved bands also revealed that there's more than one type of plasmid existed in our samples. Modifications and precaution steps were urgently needed in plasmid isolation as contamination of chromosomal DNA was observed in most of the extracted plasmid solutions. Ti-plasmids are very sensitive to mechanical stress, therefore any shearing forces must be avoided as soon as cell lysis occurred.

5.9 Future Study

In this study, conventional microbiological methods were applied for the isolation of *A. tumefaciens* from soil environment. 1A and D1 media are semi-selective media, thus *A. tumefacies* in soil sometimes can be difficult to be distinguishable from other populations of microorganism. Confirmation works using molecular techniques is too laborious and time-consuming especially when we are dealing with a large amount of isolates. Moreover potential *A. tumefaciens* isolates were mainly contributed by sample sites with higher estimated CFU/g^{-1} (Table 4.1). The results obtained were in agreement with the study of Sudaeshana et al., (2006) where *A. tumefaciens* is possible to be detected only when the amount of added bacteria cells was approaching or exceeded 2.0 x 10^3 CFU/mL.

Therefore a development of a culture-independent Real-Time PCR assay that enhances the detection of *A. tumefaciens* in soil had been proposed by researchers (Sudaeshana et al., 2006). These data suggested that a real-time PCR system coupled with direct DNA extraction from soil (Wechter et al., 2003) can actually facilitate the detection and enumeration of *Agrobacterium spp.* in soil. This technique can be performed in 3 to 4 h which will expedite detection of *A. tumefaciens* in soil.

Opine is a low molecular weight compound that can confer a selective advantage on opine-catabolizing cells, for instance tumor-inducing *A. tumefaciens* which can specifically metabolize opines for its growth. (Vaudequin-Dransart et al., 1995).

Therefore other than selenite and tellurite ions, some researchers once isolate *Agrobacterium spp*. using opine-containing medium (Canfield et al., 1989). However, opine was currently unavailable in the market, thereby researchers might need to get it in alternate ways, either by extracting it from natural sources or chemically synthesize an analogous compound of opine. Other than that, 1A and D1 media presented different degree of selectivity, yet both are reliable in isolation of *A. tumefaciens*. As a result, a combination of 1A and D1 media could be fabricated as the best detection tool for *Agrobacterium spp.* from the complex environment.

CHAPTER 6

CONCLUSION

A total of six isolates, including S1D1, S1A7, S1A8, S4A5, S5D9 and S7A2 were successfully be identified as indigenous *Agrobacterium tumefaciens* strains. As proposed by the name of these potential isolates, four of them were contributed by 1A medium, thereby proven that 1A medium was appended with higher selectivity (67%) in isolation of *Agrobacterium spp.* compared to D1 medium (33%). Approximately 97% of the initial amount of isolates was eliminated as non-agrobacterial strains through biochemical tests and molecular identification techniques. The low detection percentage (3%) of *A. tumefaciens* from the complex environement implied the urgent need to develop a highly selective media. *A. tumefaciens* were found to be Gram-negative bacteria, and positive for 3-ketolactose, potassium tellurite, oxidase, and salt tolerance test, in the meantime able to produce catalase and urease enzyme and utilize citrate for growth.

Pathogenicity test for the isolates on carrot (*Daucus carota*) was performed and tumor-inducing ability of S1D1 was authenticated upon the formation of callus-like tumor on the surface of its host plant after 35 days of incubation. Subsequently, agroinfiltration was carried out using the leaves of tobacco tree (*Nicotiana benthamiana*). Indigenous virulent S1D1 was injected and incubated

for 7 days. Unfortunately, no outgrowth symptoms were developed on the infected leaves.

According to the results obtained for antibiotic resistance test, each isolate was confirmed to harbor Ti-plasmid which in turn confers them with multi-antibiotic resistance properties. Ti-plasmid of potential *A. tumefaction* was extracted for the study and understanding of Ti-plasmid ecology. The characterizations of the Ti-plasmid that carried by the isolates remain to be performed.

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APPENDICES A

Table A List of instruments and their manufacturer

Manufacturer	Instruments
AdventurerTM Pro	Electronic Balance
Biometra	PCR Thermocycler
Hettich Zentrifugen	Incubator Shaker
HIRAYAMA	Autoclave Machine
Implen	Nanospectrophotometer
IsocideTM	Laminar Flow Hood
Leica	Light Microscope
Memmert	Incubator, Water Bath, Incubator
Thermo Electron Corporation	Centrifuge Machine, Microcentrifuge, Nanodrop 1000, Electrophoresis, Power Supply
TAKARA BIO INC.	Electrophoresis System
ViPR WMechanical Pipette	Micropipette
UVP	UV Transilluminator

Table B List of materials and their manufacturer

Manufacturer	Materials
BIO BASIC INC.	Ampicillin and Chloramphenicol powder, Ethidium bromide, Tris-HCl, Crystal violet powder, Magnesium Sulphate-Heptahydrate
Biolife	Mannitol
Bendoson	Calcium chloride anhydrous
CALBIOCHEM	PCI (Phenol:Chloroform:Isoamyl alcohol)
Fermentas	100 bp DNA Ladder, DNA loading dye, UF and B1R primer
HIMEDIA	Bromothymol blue
HmbG® Chemical	Absolute Ethanol
iNtRON	dNTP, Magnesium Chloride, $MgCl_2$ Free PCR buffer, *Taq* Polymerase
MERCK	Isopropanol, Gram's Crystal Violet Solution, Luria-Bertani Agar, Luria-Bertani broth, Safranine Solution, L-arabitol, Sodium chloride
Pronadisa	King's B medium, Eosin Methylene Blue medium
QRëC™	Ethylenediaminetetraacetic Acid (EDTA), Glycerol, Dipotassium Hydrogen Phosphate, Monopotassium Phosphate
R & M Chemical	Sodium hydroxide, Gram's Iodine, Phenolphthalein, Sodium selenite pentahydrate, Sodium taurocholate, Ferric chloride anhydrous
Scharlau Microbiology	Yeast Extract
Sigma	Potassium tellurite hydrate
SYSTERM®	Sodium chloride
Vivantis	1 kb DNA Ladder, 100 bp DNA Ladder, Agarose Powder

APPENDICES B

Table C Preparation of buffer and stock solutions

Stock Solutions	Instructions
A1 buffer	An amount of 10.0 mL of 1M MES and 200.0 mL of 50 mM magnesium chloride ($MgCl_2$) was dissolved and topped up to 1 L using sdH_2O.
2% Cycloheximde	An amount of 0.2 g cycloheximide powder was dissolved in sdH_2O and topped up to 10 mL. Stock solution was filter-sterilized with 0.2 µm membrane filter before use.
1% Crystal Violet	An amount of 1.0 g crystal violet powder was dissolved in sdH_2O and topped up to 1.0 mL.
1% Sodium selenite (Na_2SeO_3)	An amount of 0.1 g Na_2SeO_3 powder was dissolved in sdH_2O and topped up to 10 mL. Stock solution was filter-sterilized with 0.2 µm membrane filter before use.
Potassium Tellurite solution	An amount of 1.6g potassium tellurite powder was added into 200 mL of sdH_2O. Stock solution was filter-sterilized with 0.2 µm membrane filter before use.

Table D Preparation of media

Media and broth	Instructions
1A medium	An amount of 3.04 g L-arabitol, 1.04 g dipotassium hydrogen phosphate (K_2HPO_4), 0.54 g monopotassium phosphate (KH_2PO_4) 0.16 g ammonium nitrate, 0.25 g magnesium sulphate-heptahydrate ($MgSO_4.7H_2O$), 0.29 g sodium taurocholate, and 2 mL crystal violet were well mixed and topped up to 1L using sterile deionised water (sdH_2O). Then 20 mL cycloheximide (20 mg/mL), 10 mL sodium selenite pentahydrate and 0.08 g dipotassium tellurate (IV) trihydrate (80 µg/mL) were added into mixture which was sterilized and cooled to around 50^oC
D1 medium	An amount of 15.0 g mannitol, 5.0 g sodium nitrate, 6.0 g lithium chloride, 0.02 g calcium nitrate tetrahydrate, 0.1 g bromothymol blue, 2.0 g K_2HPO_4 and 0.2 g $MgSO_4.7H_2O$, were well mixed and topped up to 1L using sdH_2O. Then 20 mL cycloheximide (20 mg/mL) was supplemented into the mixture which was sterilized and cooled down to around 50^oC.
Yeast Mannitol (YM) Medium	An amount of 10.0 g mannitol, 1.0 g yeast extract, 0.2 g calcium chloride, 0.2 g sodium chloride (NaCl), 0.01 g ferric chloride, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$ were well mixed and topped up to 1L using sdH_2O. Then 20 mL cycloheximide (20 mg/mL) was supplemented into the mixture which was sterilized and cooled down to around 50^oC.
King's B medium	An amount of 38 g of King's B medium powder was added with 10 mL of glycerol and topped up to to 1L using sdH_2O.
Eosin Methylene Blue (EMB) medium	An amount of 32.0 g of EMB medium powder was dissolved in sdH_2O and topped up to 1L.
Hofer's Alkaline (HA) medium	An amount of 10.0 g mannitol, 1.0g yeast extract, 0.016 g phenolphthalein, 0.1 g NaCl, 0.5 g K_2HPO_4 and 0.2 g $MgSO_4$ were well mixed and topped up to 1L using sdH_2O.
Lactose agar	An amount of 10.0 g lactose and 1.0 g yeast extract were well mixed and topped up to 1 L using sdH_2O.

Stuart's urea broth	An amount of 0.1 g yeast extract, 0.01 g phenol red, 9.5 g K_2 HPO_4 and 9.1 g KH_2 PO_4 were well mixed and topped up to 950 mL using sdH_2O. A Fresh urea solution was prepared using 20 g urea powder and topped up to 50 mL. Urea solution was then filter-sterilized and supplemented into the mixture which was sterilized and cooled to around 50^oC.
Carbenicilin-containing media (100 µg/mL)	An amount of 25 µl of carbenicilin stock (200 mg/mL) was added into 50 mL of medium which was cooled to around 60°C, and then well mixed.
Chloramphenicl-containing media (14 µg/mL)	An amount of 23.4 µl of chloramphenicol stock (30 mg/mL) was added into 50 mL of medium which was cooled to around 60°C, and then well mixed.
Hygromycin-containing media (20 µg/mL)	An amount of 20 µl of hygromycin stock (50 mg/mL) was added into 50 mL of medium which was cooled to around 60°C, and then well mixed.
Kanamycin-containing media (50 µg/mL)	An amount of 50 µl of kanamycin stock (50 mg/mL) was added into 50 mL of medium which was cooled to around 60°C, and then well mixed.
Rifampicin-containing media (50 µg/mL)	An amount of 50 µl of rifampicin stock (50 mg/mL) was added into 50 mL of medium which was cooled to around 60°C, and then well mixed.
Streptomycin-containing media (100 µg/mL)	An amount of 100 µl of streptomycin stock (50 mg/mL) was added into 50 mL of medium which was cooled to around 60°C, and then well mixed.
Tetracyclin-containing media (5 µg/mL)	An amount of 25 µl of tetracyclin stock (10 mg/mL) was added into 50 mL of medium which was cooled to around 60°C, and then well mixed.
Gentamycin-containing media (100 µg/mL)	An amount of 50 µl of gentamycin stock (100 mg/mL) was added into 50 mL of medium which was cooled to around 60°C, and then well mixed.
Luria-Bertani (LB) agar	An amount of 37.0 g of LB agar powder was dissolved in sdH_2O and topped up to 1L.
Luria-Bertani (LB) broth	An amount of 25.0 g of LB broth powder was dissolved in sdH_2O and topped up to 1L.