CHAPTER 7

EVALUATION OF THE BIOREMEDIATION POTENTIAL OF THE SINGLE-GENE KNOCKOUT MUTANT STRAINS USING A SIMPLE PHYTOTOXICITY TEST SYSTEM

7.0 Introduction

Main objective for bioremediation process is to transform the noxious compounds to innocuous byproducts or intermediates so that it can be released safely to the environment or can be further treated or degraded by other microbial communities or organisms. Bioremediation process is often monitored by the reduction in concentration of target contaminants. However, this reduction is not always indicating the efficiency of the bioremediation. Incomplete degradation and formation of toxic intermediary metabolites may results in increased toxicity during bioremediation (Philips *et al.* 2000). Chemical analysis does not provide a reliable evaluation of the efficiency of biodegradation because it not considers the effects of the metabolites that may formed during degradation. A combination of chemical analysis and toxicity testing is recommended for monitoring the progress of bioremediation as well as for environmental safety considerations (Molina-Barahona *et al.* 2005). Studies have used individual or combined bioassays to assess the efficiency of bioremediation processes and toxicity potential of the contaminants such as plants, nematodes, earthworm, cladocera and daphnia (Salanito *et al.* 1997; Siddiqui and Adams, 2002; Frische, 2003; Molina-Barahona *et al.* 2005).

In this chapter, phytotoxicity test was selected because vegetation is the dominant biological component of terrestrial ecosystems and may be affected by the toxicity of dibenzofuran component in the environment. Germination and elongation of shoots and roots test are used as indicators of the effects of dibenzofuran toxicity. The phytotoxicity study was carried out with 5µg/mL dibenzofuran and its crude biodegradation byproducts using seeds of monocotyl Sorgho (*Sorghum saccharatum*), dicotyl garden cress (*Lepidium sativum*) and dicotyl mustard (*Sinapis alba*). The test species was recommended in OECD Guideline 208 (OECD, 1984) for the hazard and risk assessment of soil contaminants. The three test species were selected based on their rapid germination and growth of the shoots and roots, which allow completing the assays after only three days of incubation.

7.1 Optimisation of exposure time required for the seeds to be affected by dibenzofuran.

Optimisation of the treatment was conducted using *Lepidium sativum* and *Sorghum saccharatum*. Both seeds were hydrated in water for 60 mins prior to exposure to dibenzofuran for 30mins, 90mins, 3 hours and 24 hours, respectively

For the exposure time of 30 and 90 minutes, seeds of *Lepidium sativum* and *Sorghum saccharatum* were exposed to water, M9 minimal medium and M9 minimal medium supplemented with 5µg/mL dibenzofuran. M9 minimal medium was introduced to seeds in order to determine if the growth of the seeds will be affected by mineral contents in M9 media.

From the observation, no significant difference was obtained between water and M9 minimal media (P_{value} 0.94 > P_{value} 0.05) which indicates that mineral contents in M9 was not affecting the growth of seeds when supplemented with dibenzofuran and any changes are solely affected by the presence of dibenzofuran in M9 minimal media (Figures 7.1 and 7.2). Slight changes were observed in *Sorghum saccharatum* and *Lepidium sativum* that exposed to dibenzofuran for 90 minutes suggesting the effect towards seed is relative to exposure time of the seed to dibenzofuran. Taking that into consideration, the optimisation was continued to a longer exposure time of 3 hours and 24 hours. A longer exposure time will give the chemical a longer contact time with the seeds and allowing greater bioavailability of the test compound. This is important especially to *Lepidium sativum* which formed a protective layer around the seeds upon hydration.

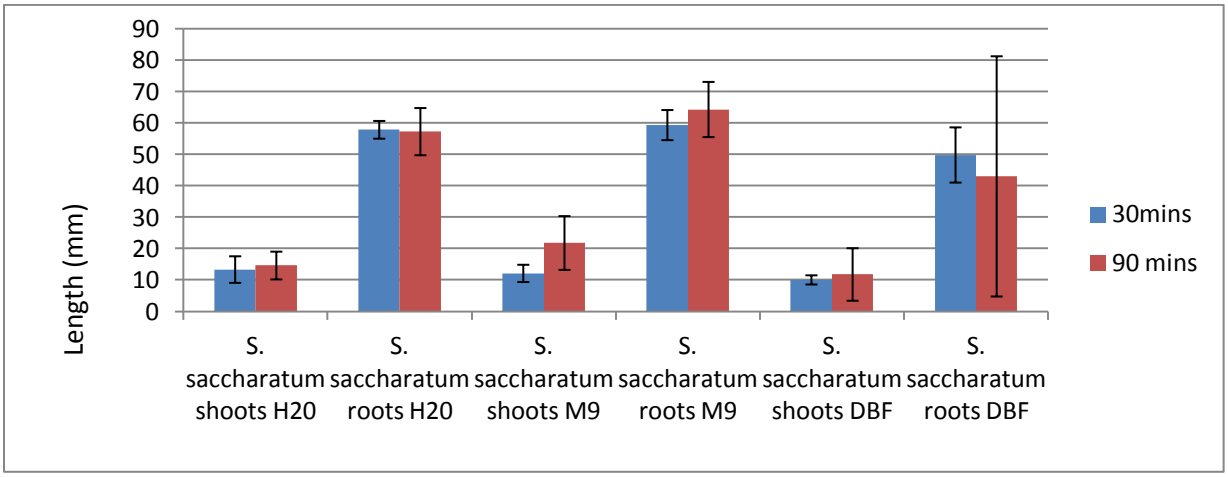

Figure 7.1: Shoots and roots growth of *Sorghum saccharatum* at different exposure time towards water, M9 minimal media and dibenzofuran. Two independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

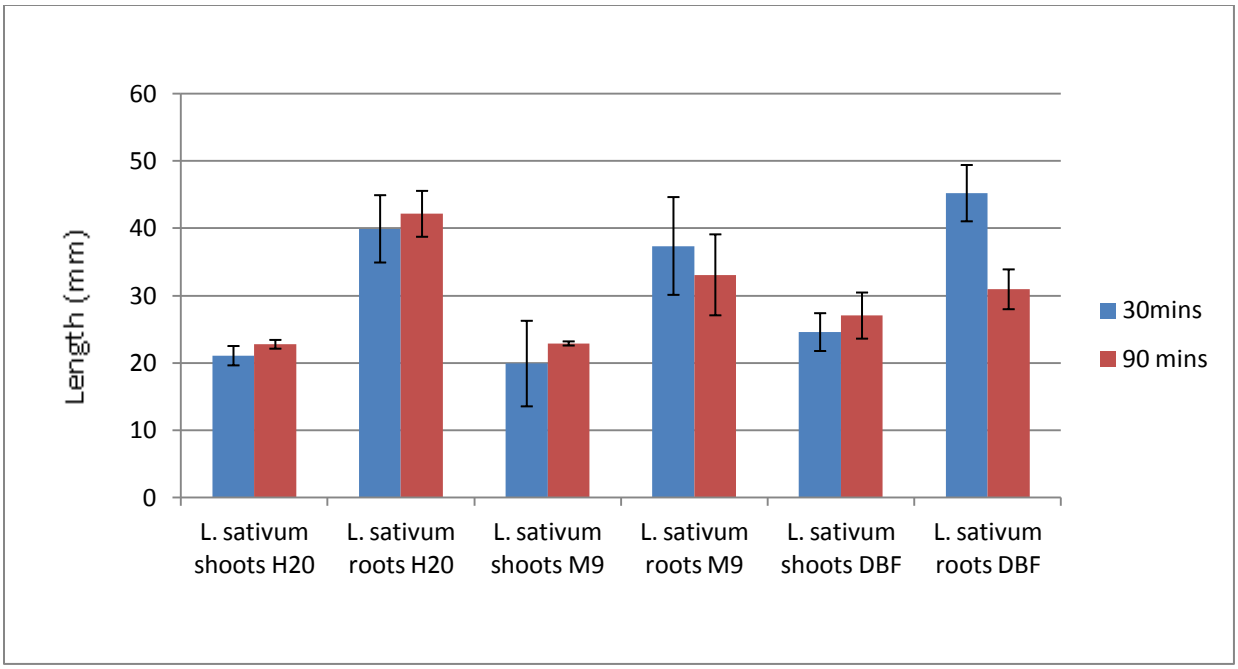

Figure 7.2: Shoots and roots growth of *Lepidium sativum* at different exposure time towards water, M9 minimal media and dibenzofuran. Two independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

However due to limited source of seeds, the optimisation of longer exposure time was conducted only for *Sorghum saccharatum* (Figure 7.3). The results clearly showed that the seeds were affected by longer exposure to dibenzofuran where the growth was reduced. Based on this result, further toxicity study was conducted with the exposure time of 24 hours.

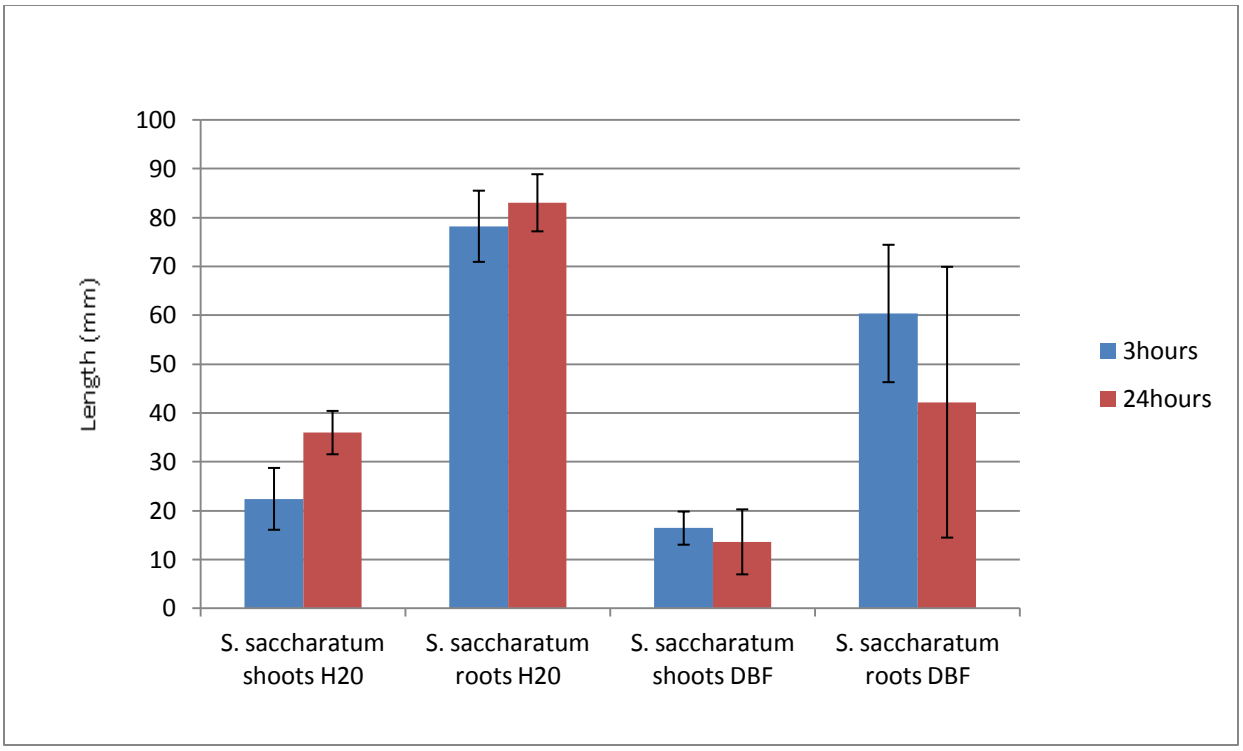

Figure 7.3: Shoots and roots growth of *Lepidium sativum* at different exposure time towards water, M9 minimal media and dibenzofuran. Two independent experiments were performed in triplicates. Error bars represent the standard error of the mean.

7.2 Seed germination percentage of test species towards exposure to dibenzofuran and its crude degradation byproducts.

In this study, seeds of three terrestrial plant species were hydrated with water prior to exposure towards dibenzofurans and crude degradation byproducts. All the seeds were then treated with water as negative control, dibenzofuran and crude byproducts from dibenzofuran degradation by wildtype *Burkholderia xenovorans* LB400, NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant) mutant.

The germination percentage was calculated for each treatment given to the seeds with consideration of the growth of shoots. Overall germination mean percentage based on the sprouting of the shoots was illustrated in Table 7.1. Seeds were considered as germinated with an emergence of at least 1mm protrusion from the seeds after the exposure period. The germination rate of higher than 85% for all three species towards exposure to water indicated that the test system is working well hence any reduction in germination rate or length of shoots and roots might due to the effects of the exposure towards dibenzofuran or crude byproducts from dibenzofuran degradation. High germination rate was observed in *Sorghum saccharatum* when exposed to dibenzofuran and its degradation derivatives suggesting that *Sorghum saccharatum* is more tolerant to dibenzofuran and its degradation derivatives while *Sinapis alba* is the most sensitive towards the influence of the treatment imposed. The germination of *Lepidium sativum* and *Sinapis alba* were reduced when exposed to dibenzofuran and its degradation byproducts when compared to water indicated the effects of the treatment towards their germination. However, germination of *Sorghum saccharatum* when exposed to byproducts from degradation

of dibenzofuran by wildtype *Burkholderia xenovorans* LB400 and NRP2LB (*rpoN2* mutant) were higher (100%) compared to water (90%).

Crude byproduct from dibenzofuran degradation by NRPLB (*rpoN1* mutant) showed low germination in *Lepidium sativum* and *Sorghum saccharatum* at 71% and 83% compared to water at 93% and 90%, respectively. The lowest germination was observed when *Sinapis alba* was exposed to crude byproduct of dibenzofuran degradation by wildtype *Burkholderia xenovorans* LB400 at 57% suggesting the formation of toxic byproduct during the degradation might affect the germination of *Sinapis alba*.

Table 7.1: Seed germination mean percentage of soil plants exposed to different treatments

Treatment	*Lepidium sativum*	*Sinapis alba*	*Sorghum saccharatum*
Water	93%	87%	90%
Dibenzofuran	87%	81%	92%
Degradation byproduct from wildtype *Burkholderia xenovorans* LB400	88%	57%	100%
Degradation byproduct from NRPLB (*rpoN1* mutant)	71%	71%	83%
Degradation byproduct from NRP2LB (*rpoN2* mutant)	86%	86%	100%

7.3 Exposure of *Sinapis alba* towards dibenzofuran and its degradation products

Sinapis alba was observed to be very sensitive towards the exposure to dibenzofuran and its degradation byproducts. Table 7.2 showed the mean of percentage inhibition on shoots and roots of *Sinapis alba*. The effect of each treatment was based on comparison between the treatments and the control (water).

Dibenzofuran showed highest inhibition on the growth of shoot for *Sinapis alba* (51%) compared to other treatments, byproduct from wildtype *Burkholderia xenovorans* LB400 (38.7%), byproduct from NRPLB (*rpoN1* mutant) (41.2%) and byproduct from NRP2LB (*rpoN2* mutant) (19.2%). Crude byproducts from degradation of dibenzofuran by wildtype *Burkholderia xenovorans* LB400 and its single-gene knockout mutants showed reduced inhibition on both shoots and roots of *Sinapis alba*. Even though the inhibition of dibenzofuran on roots was high (60.4%) however, degradation byproduct from NRPLB (*rpoN1* mutant) showed higher inhibition towards the roots of *Sinapis alba* (64.6%) suggested that toxicity of degradation byproduct from NRPLB (*rpoN1* mutant) is higher compared to dibenzofuran might due to incomplete degradation or formation of toxic metabolite. Degradation byproduct from NRP2LB (*rpoN2* mutant) was observed to give less inhibition with 19.2% on shoots and 19.1% on roots. This is lower than shown by degradation byproduct from wildtype *Burkholderia xenovorans* LB400 with 38.7% on shoots and 14.2% on roots. The low inhibition exhibited by degradation byproduct from NRP2LB (*rpoN2* mutant) on shoots and roots might due to the transformation of dibenzofuran to less toxic metabolite.

Table 7.2: Mean percentage of inhibition on shoot and root of *Sinapis alba.*

	Mean percentage of growth inhibition on shoots	Mean percentage of growth inhibition on roots
Dibenzofuran	51.0%	60.4%
Degradation byproduct from wildtype *Burkholderia xenovorans* LB400	38.7%	14.2%
Degradation byproduct from NRPLB (*rpoN1* mutant)	41.2 %	64.6%
Degradation byproduct from NRP2LB (*rpoN2* mutant)	19.2%	19.1%

7.4 Exposure of *Lepidium sativum* towards dibenzofuran and its degradation products

The effect of dibenzofuran and its degradation byproducts from wildtype *Burkholderia xenovorans* LB400, NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant) were also investigated in *Lepidium sativum* (Table 7.3). As observed in *Sinapis alba*, the degradation byproducts showed less inhibition effects on the growth of shoots and roots of *Lepidium sativum*. Dibenzofuran showed 47.8% inhibition on shoots and 63% inhibition on roots compared to control (water). When *Lepidium sativum* was exposed to dibenzofuran degradation byproduct from wildtype *Burkholderia xenovorans* LB400, the root showed growth inhibition of 18.5% but no inhibition was observed on the growth of the shoot. Furthermore the growth of the shoot was 5% better than the control indicated by the negative inhibition value (Table 7.3). This result indicated the reduction of dibenzofuran toxicity in the degradation byproducts towards the seed of *Lepidium sativum*. The crude degradation byproducts of dibenzofuran from NRPLB (*rpoN1* mutant) also showed reduction in growth inhibition compared to dibenzofuran with 15.9% and 31.3% for shoot and root, respectively.

The lowest growth inhibition was found when seeds of *Lepidium sativum* were exposed to degradation byproduct from NRP2LB (*rpoN2* mutant). The growth inhibition of roots exhibited by degradation byproduct from NRP2LB (*rpoN2* mutant) was only 9% compared to control (water) but the growth of shoot was observed 7.9% better than was indicated by negative growth inhibition value. The results suggested that degradation byproducts from wildtype *Burkholderia xenovorans* LB400 and NRP2LB (*rpoN2* mutant) not only less toxic than dibenzofuran but may consist of compound that able to enhance the growth of *Lepidium sativum*.

Table 7.3: Percentage effect on shoots and roots of *Lepidium sativum*

	Mean percentage of growth inhibition on shoots	Mean percentage of growth inhibition on roots
Dibenzofuran	47.8%	63%
Degradation byproduct from wildtype *Burkholderia xenovorans* LB400	-5%	18.5%
Degradation byproduct from NRPLB (*rpoN1* mutant)	15.9%	31.3%
Degradation byproduct from NRP2LB (*rpoN2* mutant)	-7.9%	9%

7.5 Exposure of *Sorghum saccharatum* towards dibenzofuran and its degradation products

The effect of dibenzofuran and its degradation byproducts from wildtype *Burkholderia xenovorans* LB400, NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant) were also investigated in *Sorghum saccharatum* (Table 7.4). *Sorghum saccharatum* showed high tolerance towards dibenzofuran and its degradation byproducts as observed in germination percentage.

Percentage of growth inhibition on shoot and root of *Sorghum saccharatum* were high with with 62.9% and 63.9%, respectively. Moreover, the inhibition in growth of shoot and root of *Sorghum saccharatum* were higher than *Lepidium sativum* and *Sinapis alba*. This observation may be due to the larger area available for dibenzofuran for attachment as seed of *Sorghum saccharatum* is bigger compared to *Lepidium sativum* and *Sinapis alba*.

Generally, the degradation byproducts of dibenzofuran were less toxic for *Sorghum saccharatum* compared to dibenzofuran. The shoots of *Sorghum saccharatum* showed 35.8% inhibition when exposed to degradation byproducts from wildtype *Burkholderia xenovorans* LB400 but exhibited the better growth of roots with inhibition value of -5%. As previously mentioned, the negative value indicated better growth compared to control (water).

The degradation byproduct from NRPLB (*rpoN1* mutant) also showed better growth in both shoots (7.8%) and roots (8%) compared to (control) water. This is different from results obtained in *Lepidium sativum* and *Sinapis alba* where the degradation byproducts from NRPLB (*rpoN1* mutant) inhibit the growth of their shoots and roots. This finding suggested that degradation byproducts from NRPLB (*rpoN*1 mutant) may consist of compound that able to enhance the growth of monocotyl *Sorghum saccharatum* but not dicotyl *Lepidium sativum* and *Sinapis alba*.

Sorghum saccharatum showed negative growth inhibition for both shoots (-51%) and roots (-66%) towards exposure to degradation byproduct from NRP2LB (*rpoN2* mutant) indicating the better growth of shoots and roots of *Sorghum saccharatum* in degradation byproduct from NRP2LB (*rpoN2* mutant) compared to control (water). The growth of more than 50% better than water suggested that the degradation byproduct from NRP2LB (*rpoN2* mutant) may also consist of compound that enhance the growth of *Sorghum saccharatum*.

Table 7.4: Percentage effect on shoots and roots of *Sorghum saccharatum*

	Mean percentage of growth inhibition on shoots	Mean percentage of growth inhibition on roots
Dibenzofuran	62.9%	63.9%
Degradation byproduct from wildtype *Burkholderia xenovorans* LB400	35.8%	-5.0%
Degradation byproduct from NRPLB (*rpoN1* mutant)	-7.8%	-8.0%
Degradation byproduct from NRP2LB (*rpoN2* mutant)	-51.0%	-66.0%

7.6 Discussion

Dibenzofuran is a non-chlorinated member of dioxins and a precursor for polychlorinated dibenzofuran (PCDFs). Even though currently no solid evidence shows the toxicity of non-chlorinated dibenzofuran, the toxicity analysis of dibenzofuran and its derivatives is important. As the precursor for highly toxic chemical, dibenzofuran and its derivatives must be regarded as the same. The degradation might transform the potent chemical to less harmful or vice versa. This study was conducted to determine the toxicity of the dibenzofuran degradation byproducts produced by wildtype *Burkholderia xenovorans* LB400 and its single-gene knockout mutants. The seeds of three terrestrial plant species were used in this study were *Sinapis alba*, *Lepidium sativum* and *Sorghum saccharatum*. The test was initiated by optimisation of exposure time of dibenzofuran and its degradation byproducts towards seeds of *Sorghum saccharatum* and *Lepidium sativum* which represent the monocot and dicot plants, respectively. The results showed that exposure time-dependent reduction of growth inhibition on shoots and roots of the plants. It was observed that 24 hours exposure of dibenzofuran and its derivative was sufficient to induce an observable growth inhibition of both *Sorghum saccharatum* and *Lepidium sativum*. It was also observed that mineral contents in the M9 minimal media used as media in degradation of dibenzofuran did not affect the growth of the seeds compared to water since no significant difference was obtained from ANOVA analysis. Hence, all subsequent experiments adopted water as negative control.

Germination percentage is higher for *Lepidium sativum* compared to *Sinapis alba* and *Sorghum sativum*. This result might due to the water holding capacity of the *Lepidium sativum* seed which form a “gel-like” layer when hydrated with water. Germination of both dicot plants (*Lepidium*

sativum and *Sinapis alba*) were low when exposed to dibenzofuran and its degradation byproducts compared to monocot (*Sorghum saccharatum*), nevertheless the germination of *Lepidium sativum* is higher compare to *Sinapis alba* which may be due to the gel-like layer that might act as protective layer for *Lepidium sativum* from toxic effects of dibenzofuran and its degradation byproducts. However, the germination percentage values obtained showed that the system is working well, as more than 85% germination rates were observed in all three species tested. In general, germination of all three species tested were reduced when exposed to dibenzofuran and its degradation byproducts compared to water indicating the toxicity of dibenzofuran and its degradation byproducts even though that is not the case for *Sorghum saccharatum*. Germination rate showed that *Sorghum saccharatum* was relatively more tolerance towards the exposure of dibenzofuran and its degradation byproducts while *Sinapis alba* is the most sensitive among the tested plants. Dibenzofuran was found toxic to *Sinapis alba* as demonstrated by Sverdrup *et al.* (2003) where it was found as the most toxic compared to other tested PAHs and PACs.

The inhibition of dibenzofuran and its degradation byproducts towards the seeds of *Sinapis alba* was investigated. Generally the toxicity of dibenzofuran was reduced in its degradation byproducts towards the seeds of *Sinapis alba* when degraded by wildtype *Burkholderia xenovorans* LB400, NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant). However the growth inhibition in *Sinapis alba* towards degradation byproducts from NRPLB (*rpoN1* mutant) was found higher compared to degradation byproducts from wildtype *Burkholderia xenovorans* LB400 and NRP2LB (*rpoN2* mutant) in both shoots and roots suggesting the degradation byproducts from NRPLB (*rpoN1* mutant) is more toxic compared to degradation byproducts

from wildtype *Burkholderia xenovorans* LB400 and NRP2LB (*rpoN2* mutant). On the other hands, the degradation byproduct from NRP2LB (*rpoN2* mutant) showed the least effect to *Sinapis alba* seeds. This suggested that dibenzofuran was transformed to less toxic metabolite by NRP2LB (*rpoN2* mutant).

The exposure of *Lepidium sativum* seeds to dibenzofuran and its degradation byproducts was also investigated. The results showed that the growth inhibition by dibenzofuran is higher towards the roots compared to its shoots. In general, all degradation byproducts from wildtype *Burkholderia xenovorans* LB400, NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant) were observed to have lower inhibition compared to dibenzofuran indicated that the toxicity of dibenzofuran was reduced during the degradation. Furthermore, degradation byproduct from wildtype *Burkholderia xenovorans* LB400 and NRP2LB (*rpoN2* mutant) showed better growth on shoots of *Lepidium sativum* compared to control (water) suggesting that the byproducts might consist of the compound that able to enhance the growth of *Lepidium sativum* shoots but it was not able to enhance the growth of the roots.

The exposure of *Sorghum saccharatum* seeds towards dibenzofuran and its degradation byproducts was also investigated. From the results, the degradation byproducts by wildtype *Burkholderia xenovorans* LB400, NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant) were less toxic compared to the original compound (dibenzofuran). This finding suggested that dibenzofuran was transformed by wildtype *Burkholderia xenovorans* LB400 and its single-gene knockout mutants (NRPLB and NRP2LB) to the less toxic compounds. Furthermore, the degradation byproducts from NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant) were able to enhance the growth of shoots and roots of *Sorghum saccharatum*. The better growth of *Sorghum*

saccharatum when exposed to the degradation byproducts from single-gene knockout mutants suggested that the byproducts not only less toxic than the original compound (dibenzofuran) but may also consist of compound that able to enhance the growth of *Sorghum saccharatum*.

The results obtained in this chapter suggested that degradation of dibenzofuran by wildtype *Burkholderia xenovorans* LB400, NRPLB (*rpoN1* mutant) and NRP2LB (*rpoN2* mutant) was successfully reduce the toxicity of dibenzofuran towards the growth of *Sinapis alba*, *Lepidium sativum* and *Sorghum saccharatum*. This results also indicated the potential of utilisation of NRP2LB (*rpoN2* mutant) as bioagent in bioremediation process of dibenzofuran.

7.7 Summary

The phytotoxicity test system that was used in this study is working well as the germination percentage of all three test species were higher than 85% indicating that any germination reduction is due to the effect of dibenzofuran and its degradation byproducts and not because of the seeds. The *Sinapis alba* was observed to be the most sensitive among the test species while *Sorghum saccharatum* was the most tolerant towards the exposure to dibenzofuran and its degradation byproducts. All the degradation byproducts exhibited the reduction of inhibition effect to all three test species compared to the original compound (dibenzofuran) suggesting the successful of biodegradation process by wildtype *Burkholderia xenovorans* LB400 and its single-gene knockout mutants. The NRP2LB (*rpoN2* mutant) might be a potential biodegrader of dibenzofuran as its not only able to transform dibenzofuran to less toxic compound but the degradation byproduct might also consist of compound that able to enhance the growth of test species especially *Sorghum saccharatum*.