Herbicidal activity of marine sponge associated actinobacteria against herbicide-resistant ryegrass (*Lolium rigidum*) on greenhouse trial

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DECLARATION

I certify that this thesis does not contain material which has been accepted for the award of any degree or diploma; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis.

Pamela Joyce Gorriceta
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Abbreviations

°C	degree Celsius
µl	microlitre
µm	micrometer
g	grams
HPLC	high performance liquid chromatography
L	litre
mls	millilitre
	millimeter
PDA	potato dextrose agar
RO water	reverse osmosis water
S.M.	sulfometuron methyl
TLC	thin layer chromatography

Abstract

The Australian agriculture sector experience a widespread problem caused by weeds resulting in lower quality and quantity of crops. The annual loss reported by the GRDC, Grains and Development Corporation is set at $3.27 million. *Lolium rigidum* known as annual ryegrass is one of the most common weed species that cause record loss on Australian crops. It is resistant to commercial herbicides with 11 modes of action in the market. This species can also withstand different weather conditions to allow for growth year wide. Discovery of novel herbicides from biological sources are making rounds in the research community.

Marine sponges and their symbiotic microbiota, important contributors of the novel bioactive compounds, are taken our greatly consideration to be employed for potential herbicidal compounds discovery. Therefore, this study aims to 1) to validate the herbicidal activity of the extracts of the selected 12 candidate actinobacteria strains; 2) to induce the secondary metabolites from 15 newly isolated actinobacteria strains using two different cultivation media; 3) to investigate and compare the herbicidal activity of the metabolites produced by all the 27 strains under different cultivation conditions; 4) to screen the strains and their metabolites fractions with herbicidal activity.

Two rounds of activity investigations were conducted: (1) 12 actinobacteria strains were selected for the first round to validate the performance of the selected strains that showed potential herbicidal activity in a previous study; (2) fifteen newly isolated sponge associated actinobacteria were included to screen the strains with potential herbicidal activity. In the validation round, to produce the compounds from the previously used actinobacteria, methanol extracts of inoculated plates using M4 and ISP2 media was employed and the dried methanol extracts were used for

germination and greenhouse assay test. In the screening round, liquid media (M4 and ISP2) cultures were employed to evaluate the best inoculation time for an improved compounds production. The germination test from the validation round did not produce significant results in inhibiting the growth of the annual ryegrass, thus no further germination test was employed. Thin layer chromatography (TLC) was additionally applied for 15 strains showing different performances in seed germination test. High performance liquid chromatography (HPLC) analysis on the active extracts from ISP2 showed different isolation times produce different compounds.

As a result, *in vitro* germination test showed that one of the 27 tested strains had positive effect to inhibit the annual ryegrass seeds. However, the results of its greenhouse trail did not show significant inhibition against the annual ryegrass plant. Moreover, the analysis of the dry weight of the tested annual ryegrass plants also consistently reflected the greenhouse test results.

The best inoculation time is between 5 – 9 days using ISP2 liquid media. This is shown by the results from the TLC and confirmation of compound detection using HPLC.

Table of Contents

List of Tables and Figures

Tables

Figures

CHAPTER 1: INTRODUCTION

1. Introduction

1.1 Australia's problem with weeds

Weeds are plants that usually grow where they are not wanted and are not planted on purpose (Ligenfelter, n.d.). In Australia, weeds can spread at a very rapid pace and can become difficult to manage and control (Weed Management Society of South Australia, 2018, Heap, 2018). The Australian agriculture is affected by weeds with reducing the quality and quantity of crops produced, it can also damage farm machinery (Commonwealth of Australia, 2018). The Grains Research and Development Corporation (GRDC) reported about $3.27 billion per annum as the cost for weed management and agricultural losses, expenditure cost of $2,573 million and total yield revenue loss of AUD$ 745 million (Invasive Plants and Animals Committee, 2016). Expenditure and losses of Australian grain growers per hectare is AUD 146, as these growers spend AUD 13 for both herbicide and non-herbicide weed control. Loss in grain production amounting to 2.76 million tonnes are due to emergence of weeds (Llewellyn RS et al., 2016).

Weeds can affect the economy, the environment and the health of the population. Weeds compete with the nutrients, natural sunlight and space of the naturally occurring species of the environment. Natural substances from weeds can inhibit crop growth and they can also host attacking pathogens (Mark Schonbeck, 2013). Weeds can also affect the human population by causing health problems such as dermatitis, allergies and respiratory problems. Weeds that grow in water can become dangerous to the health of consumers if they inhabit the source of the water supplies. Some weeds can be poisonous which can be very dangerous to human and animals. (Commonwealth of Australia, 2018, Invasive Plants and Animals Committee, 2016).

1.2 Annual ryegrass

Lolium rigidum more commonly known as annual ryegrass, belongs to the Poaceae family (Figure 1). It is one of the most common weeds that causes much damage to the annual winter agriculture crops in Southern Australia. This grass species can live from winter to spring season. Mature annual ryegrass weeds can grow up to 900mm tall (Government of Western Australia, 2017). Regarded to be one of the most costly weeds during winter cropping has a very high capacity of seed production. One annual ryegrass plant can produce as much as 45,000 seeds per square metre. This grass species can also host the bacteria *Clavibacter* spp., the causative agent of annual ryegrass toxicity (Murrumbidgee Catchment Management Authority, 2008)

A B

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Figure 1.2.1. Annual ryegrass photos in the field. A. Glyphosate resistant annual ryegrass in wheat fields, b. Ryegrass growing in the plant row in the Trifluralin treatments. (A. Storrie, n.d., Barry Haskins and Angus MacLennan, 2014)

Annual ryegrass is a big problem for agriculture and for weed control as it has become resistant to selective and non-selective herbicides. Among the ryegrass population only 2% can be controlled by a herbicide and the remaining 98% have developed resistance to more than one mode of action in herbicides. The total cost for herbicide resistance for annual ryegrass is higher than the sum of all the other forms of resistance from other weeds (Llewellyn RS et al., 2016).

In the state of South Australia and Victoria there is an increase of resistance of annual ryegrass. In South Australia this weed has developed to multiple resistance to almost 10 herbicide modes of

action, refer to Table 1.1 in the study of Mechelle Owen et al (2013). In 2001, ryegrass has developed resistance to the popular herbicide glyphosate (John Matthews, n.d.).

1.3. Weed control methods

1.3.1 Chemical

1.3.1.1. Action mode of chemical herbicides

The process applies the chemicals as herbicides to that are protect the crops by infestation of weeds which is convenient, effective and economical. The weeds are either dropping their leaves or drying out (Queensland Government, Dwight Lingenfelter and Nathan L. Hartwig, 2018). The chemical herbicides are divided into selective and non-selective groups. The selective group of herbicides kills or damage the specific target weed without harming other plants. The non-selective group of herbicides kills all plants in the area sprayed by the chemical (The Pennsylvania State University, 2018).

The efficiency of the herbicides depends on its mode of action that is determined by the process of how the chemical is applied and has detrimental effect on an essential plant function (Table 1). The mode of action can be a physical or biochemical process by which the plant growth is inhibited leading to killing of weeds. Herbicides applied to soil before germination of the weed seeds are regarded as pre-emergent. These herbicides can result in the disruption of the germination of the plant seedling. Herbicides applied after the seeds germinate or sprayed to the leaves and soil are defined as post-emergent herbicides (Mandy Tu et al., 2001).

Table 1.3.1.1. Action mode of chemical herbicides based on the chemical family in different herbicide groups (CropLife Australia, 2018)

HERBICIDE GROUP	MODE OF ACTION	CHEMICAL FAMILY
Group A	Inhibitors of acetyl co-enzyme A carboxylase (ACCase) (Inhibitors of fat synthesis/ACCase inhibitors)	Arxloxyphenoxypropionates, Cyclohexanedione, Phenylpyrazoline
Group B	Inhibitors of acetolactate synthase (ALS)/ acetohydroxyacid synthase (AHAS)	Sulfonylurea, Imidazolinone, Triazolopyrimidine, Pyrimidinylthiobenzoate
Group C	Inhibitors of photosynthesis at photosystem II (PSII inhibitors)	Triazine, Triazinone, Uracil, Pyridazinone, Phenylcarbamate, Urea, Amide, Nitrile,
Group D	Inhibitors of microtubule assembly	Benzothiadiazinone, Dinitroaniline, Pyridine, Benzamide, Benzoic acid
Group E	Inhibitors of mitosis/ microtubule polymerisation	Carbamates
Group F	Bleachers: Inhibitors of carotenoid biosynthesis at the phytoene desaturase step (PDS inhibitors)	Norflurazon, Diflufenican, Picolinafen
Group G	Inhibitors of protoporphyrinogen oxidase (PPOs)	Diphenyl ether, Phenylpyrazole, N-phenylphthalimide, Oxadiazole, Triazolinone, Pyrimidindione
Group H	Inhibition of 4hydroxyphenyl-pyruvatedioxygenase (4-HPPD)	Isoxazoles, Pyrazoles, Triketone
Group I	Disruptors of plant cell growth (Synthetic Auxins)	Arylpicolinate, Benzoic Acids, Phenoxycarboxylic acids, Pyridine carboxylic acids, Quinolone carboxylic acids
Group J	Inhibition of lipid synthesis – not ACCase inhibition	Thiocarbamate, Phosphorodithioate, Benzofuran, Chloro-carbonic-acid
Group K	Inhibitors of cell division/ Inhibitors of very long chain fatty acids (VLCFA)	Chloroacetamide, Acetamide, Isoxazoline

Group L	Inhibition of photosynthesis at photosystem I via electron diversion (PSI inhibitors)	Bipyridyls
Group M	Inhibitors of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase	Glysines
Group N	Inhibitors of glutamine synthesase	Phosphinic acids
Group O	Inhibitors of cell wall (cellulose) synthesis	Alkylazine, Benzamides, Nitrites
Group P	Inhibitors of auxin transport	Naptalam
Group Q	Inhibitors of carotenoid biosynthesis unknown target	Isoxazolidinones, Triazoles
Group R	Inhibitors of DHP (dihydropteroate) synthase	Carbamates
Group Z	Herbicides with unknown and probably diverse sites of action	Arylaminopropionic acid, Pyrazolium, Organoarsenicals

1.3.1.2. Herbicide resistance of weeds

There is a big problem worldwide in the increase of the emergence of herbicide resistance that poses a big threat to the world's food sources (Matzrafi et al., 2014). In particular, weeds that mutate into herbicide resistant species can produce weed populations that are no longer controlled even after the exposure or treatment of an herbicidal agent. This resistance quality of weeds can be natural or produced by genetic modification. In Australia, 36 weed species have been discovered to contain herbicide resistant properties (Grains Research and Development Corporation, 2014). Among of them, 21 species have been reported in South Australia (Table 2). Herbicide resistance of weeds is influenced how often the herbicide which the percentage of weeds that survive the herbicide treatment (CropLife Australia, 2017).

Table1.3.1.2. Herbicide resistant weeds in South Australia (Heap, 2018)

No	Species	Co on Name	Mode of Action
1	*Lolium rigidum*	Ryegrass	ACCase inhibitors (A) ALS inhibitors (B) DOXP inhibitors (F) Microtubule inhibitors (K) Mitosis inhibitors (K) Long chain fatty acid inhibitors (K) Lipid Inhibitors (N) Photosystem II inhibitors (C) PSI Electron Diverter (D) EPSP synthase inhibitors (G)
2	*Avena fatua*	Wild Oat	ACCase inhibitors (A)
3	*Avena sterilis*	Sterile Oat	ACCase inhibitors (A) ALS inhibitors (B)
4	*Sisymbrium orientale*	Oriental Mustard	ALS inhibitors (B) Synthetic Auxins (O)
5	Hordeum murinum ssp. glaucum	Smooth Barley	PSI Electron Diverter (D) EPSP synthase inhibitors (G)
6	*Digitaria sanguinalis*	Large Crabgrass	ACCase inhibitors (A) ALS inhibitors (B)
7	*Lactuca serriola*	Prickly Lettuce	ALS inhibitors (B)
8	*Brassica tournefortii*	African Mustard	ALS inhibitors (B)
9	*Echium plantagineum*	Patersons Curse	ALS inhibitors (B)
10	*Diplotaxis tenuifolia*	Perennial Wallrocket	ALS inhibitors (B)
11	*Pentzia suffruticosa*	Calomba daisy	ALS inhibitors (B)
12	*Mesembryanthemum crystallinum*	Crystalline Iceplant	ALS inhibitors (B)
13	*Sisymbrium orientale*	Oriental Mustard	ALS inhibitors (B) Synthetic Auxins (O)
14	*Raphanus raphanistrum*	Wild Radish	ALS inhibitors (B) Carotenoid biosynthesis inhibitors (F) Synthetic Auxins (O)
15	*Bromus diandrus ssp. rigidus*	Rigid Brome	ACCase inhibitors (A) ALS inhibitors (B) EPSP synthase inhibitors (G)
16	*Conyza bonariensis*	Hairy Fleabane	EPSP synthase inhibitors (G)
17	*Galium tricornutum*	Threehorn Bedstraw	ALS inhibitors (B)
18	*Sonchus oleraceus*	Annual Sowthistle	Synthetic Auxins (O) ALS inhibitors (B)
19	*Chloris virgata*	Feather Fingergrass	EPSP synthase inhibitors (G)
20	*Arctotheca calendula*	Capeweed	Synthetic Auxins (O)

21	*Poa annua*	Annual Bluegrass	EPSP synthase inhibitors (G) Photosystem II inhibitors (C) Unknown (Z) Microtubule inhibitors (K) ALS inhibitors (B)

*Refer Column 4 letters A-Z Mode of Action to Table 1 for Herbicide Group.

1.3.1.3.Negative impact of chemical herbicide

Chemical herbicides have shown to result in negative effects on soil with repeated applications. Glyphosate, the most common form of chemical herbicide, has shown undesirable effects on a variety of beneficial soil microorganisms (BSM) (Druille et al., 2016). The study has provided evidence that chemicals applied to soil negatively affects the production and the microbial diversity of the plant ecosystem (Druille et al., 2016). Chemical weed control can also affect the production of crops by decrease germination and lower number and weight of harvest (Mrs. Joyce Chinyere Best-Ordinioha et al., 2017). Chemicals that accumulate in crops showed symptoms of decrease in chlorophyll content (Jun Zhang et al., 2014).

Chemical herbicides decreased the mycorrhization of grapevines by more than 50% (Zaller et al., 2018). Mycorrhization helps in the improvement of the plant growth and quality. With decrease in mycorrhization there is implication of lower plant survival rate (Alizadeh et al., 2636).

1.3.2 Biological

Weed control methods that introduce microorganisms that can control or kill the weeds is referred to as biological control. This weed control method works well for integrated weed management, but it can be limited as it is a time-consuming method. Many research studies on weed control are ongoing to try to improve the efficacy of the treatment in order to solve the problem (Dwight Lingenfelter and Nathan L. Hartwig, 2018) Compounds from biological sources are used in sustainable agriculture. Studies have been done to develop metabolites

from biological sources that can be easily degraded in the environment (Priyadharsini et al., 2013a) after they act on target organism. Biological herbicides can target single or multiple weeds and are not harmful to crops if it is applied (Bailey, 2014). A study by Singh et.al., 2018 on endophytic actinobacteria for their herbicidal activity have shown promising results that biological sources can be effective weed control agents.

Studies involving the use of plant pathogens as bioherbicides have started since the 1970s (Charudattan and Dinoor, 2000). Plant pathogens are found to show herbicidal activity (Charudattan and Dinoor, 2000). Many metabolites from plant pathogens have properties that inhibit the growth and germination of weeds (Radhakrishnan et al., 2018). The use of plant pathogens as herbicides have showed potential in inhibiting growth and germination of weed seeds (Radhakrishnan et al., 2018). Herbicides from plant pathogens features host-specific property that cannot address mixed weed populations, the prediction for the success of the introduced pathogen is unpredictable. It can be very difficult to stop the damage once the introduced plant pathogen is not effective. In the United States of America the rate of herbicides from plant pathogen-based weed control project is 21% (Yandco-Ables and Charudattan, 2006).

1.4 Marine derived herbicides

1.4.1. Resources for herbicides discovery: from land to ocean

Land-based discovery for new products for biotechnology have been exhausted by researchers. Scientists have now explored the potential of marine microorganisms to source natural products of discovery. The marine environment presents chemicals that are unique only to the sea and not to land creatures (National Research Council, 2007).

The earth's surface is roughly covered with 70% of ocean water. The marine ecosystem offers a different chemical and biological diversity. Different conditions and natural compounds

by marine microorganisms have become the target of researchers in the discovery of products from natural sources (Brinkmann et al., 2017, Sabdono and Radjasa, 2008). Marine microorganisms have species that are only found in the marine ecosystem (Motti et al., 2007a). Different microorganisms from marine origin have shown good potential source for pharmaceutical and novel bioproducts (Lindequist, 2016). Large numbers of microbes that are present in the marine environment can be found in marine organisms as host-associated members. In particular the marine sponge (Phylum Porifera) are made up of diverse microbial populations that make it a great source of novel biotechnological products (Simister et al., 2011).

Microalgae has been reported as a good source of bioactive metabolites, including a number of compounds with direct or indirect biological activity with herbicide potential (Hernández-Carlos and Gamboa-Angulo, 2011). Common marine bacteria Proteobacteria, Planctomycetes and Cyanobacteria have revealed the presence of Phosphonates, a compound that can be found in glyphosate herbicides (Asuncion Martinez et al., 2010). Marine fungi screened and showed to inhibit the C_4 plant enzyme pyruvate phosphate dikinase (PPDK). The enzyme PPDK has been a target for development for new herbicide (Motti et al., 2007b).

1.4.2. Marine sponge derived compounds with herbicidal activity

Marine sponges (Phylum Porifera) are the oldest multicellular animals. To the date, about 9,045 (Van Soest et al., 2018) valid species have been identified and more than half of this number are still unidentified (Gopi et al., 2012). Sponges do not have the ability to move freely and generally lack a physical defense mechanism. Therefore, the production of chemicals are their preferred method of protection for themselves and their environmental space (Beesoo et al., 2017). Their effective water pumping system allows the sponges to interact with different

microorganisms, (Van Soest et al., 2018) and those that endure this process and bypass the sponge immune response remain and live in the sponge.

Sponges are known to be important manufacturers of secondary metabolites (Braekman and Daloze, 2004). Sponge-derived metabolites have shown several biological activities and applications that include enzyme inhibitors, cell division-inhibitors, and cytotoxic/ cardiovascular properties, as well as the antiviral, antifungal, antimicrobial, anti-inflammatory, and anti-tumor activities (Lee et al., 2000). Since early 1950s there have been reports on that the studies of marine sponge isolation and up to this date there have been increasing studies in this area (Lee et al., 2001, Motti et al., 2007a).

1.4.3 Sponge-associated actinobacteria with herbicidal activity

The class Actinobacteria is considered to hold important prokaryotes. Actinobacteria from marine sources were found to be a good source of new bioactive metabolites. (Jiang et al., 2008). Actinobacteria are abundant gram-positive bacteria. They have become one of the best sources for studies that involve discovery of antibiotics that can be useful in treatment of human diseases (Jose and Jha, 2016). Apart from being a good source for the production of antibiotics, actinobacteria have a bigger role in the marine ecosystem (Das et al. 2006). Different types of microorganisms are responsible for degrading and replacement of a number of materials (Jensen et al., 2005a, Lam, 2006).

From the sea, *Actinobacteria* associated with marine sponges were identified with herbicide activity. One novel species, *Streptomyces atlanticus* isolated from *Aplysina fulva* produced butenolide, a lactone compound that showed a potent herbicide property, inhibiting the growth of monocotyledonous annuals (Melo, 2017).

The production of extracellular active compounds like Anisomycin, Bialaphos, Herbicidin A and B play the vital roles in actinobacteria (Singh et al., 2018). For example, one compound that showed herbicidal activity isolated from *Streptomyces* sp. KA1-3 is Propionanilide with other names Propanamide or *N*-phenyl-Propionamide (Figure 2.A). It has showed herbicidal activity against the weed species *Cassia occidentalis* L. and rhizome *Cyperus rotundus*. The herbicide activity showed positive results by inhibiting germination of seeds by 80% (Priyadharsini et al., 2013b). Another compound produced from *Streptomyces viridochromogenes* and *Streptomyces hygroscopicus* is Phosphinothricyl-alanyl-alanine (Pt tripeptide (Ptt), bialaphos) contains glutamine syntheses (Figure 2.B)(Grammel et al., 1998). This compound showed bactericidal, fungicidal, and herbicidal activities (Schwartz et al., 2004).

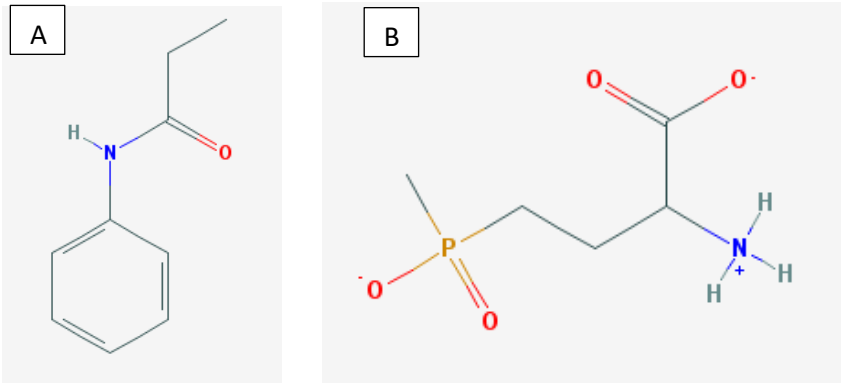

Figure 1.4.3. A) The chemical structure of Propionanilide (https://pubchem.ncbi.nlm.nih.gov/compound/12107) B) The chemical structure of Phosphinothricyl-alanyl-alanine, (https://pubchem.ncbi.nlm.nih.gov/compound/25244585)

1.5. Study plan

This study was designed as a follow-up of a Masters research project in which twelve actinobacteria pure cultures isolated from marine sponges were selected as candidates. They showed a weak herbicide activity against annual ryegrass in previous *in vitro* assay on seed germination test. As reproducibility was an issue, for comparison, an additional 15 newly isolated sponge-associated actinobacteria strains were further analyzed.

Therefore, the aims of this study are:

1) to validate the herbicidal activity of the selected 12 candidate actinobacteria strains;

2) to screen the secondary metabolites from 15 newly isolated actinobacteria strains using two different cultivation media;

3) to investigate and compare the herbicidal activity of the metabolites produced by all the 27 strains under different cultivation conditions;

4) to screen the strains and their metabolites fractions with herbicidal activity.

1.6.Hypothesis

Marine sponge-associated actinobacteria are good sources for herbicides.

1.7.Significance of the study

The significance of studying the potential for actinobacteria from marine sponge will present a great opportunity for the agriculture and herbicide industries. It will benefit mostly the farmers that are affected by the increasing number of weeds that are resistant to co ercially available herbicides. This study is focused to find alternative sources of production of herbicide that can target weeds hat are found to be resistant into multiple mode of action of chemical herbicide.

CHAPTER 2: METHODOLOGY

2.1 Marine actinobacteria strains, positive control and annual ryegrass seeds

The 12 strains (first round) of actinobacteria from marine sponge are provided by Dr. Qi Yang from Flinders University, 15 strains (second round) from Yitayal Anteneh PhD student from Flinders University. Positive control Sulfometuron Methyl (S.M.) and annual ryegrass seeds are provided by Associate Professor Chris Preston from the University of Adelaide.

Selection of the actinobacteria stains for the second round was chosen based on the antimicrobial property shown on Table 2.1.3.

Table 2.1.1. Twelve actinobacteria strains for the first round testing

Sample Code	Appearance on plate	Species
SP-H-1	Light yellow	*Streptomyces* sp.1
SP-H-2	Brown/white	*Streptomyces* sp.2
SP-H-3	Light yellow	*Streptomyces* sp.3
SP-H-4	light yellow/transparent yellow	*Streptomyces* sp.4
SP-H-5	Yellow	*Streptomyces* sp.5
SP-H-6	White	*Mycobacterium* sp.1
SP-H-7	Light yellow/white	*Mycobacterium* sp.2
SP-H-8	Brown/light yellow	*Mycobacterium* sp.3
SP-H-9	Yellow	*Mycobacterium* sp.4
SP-H-10	Orange	*Microbacterium* sp.5
SP-H-11	Orange	*Microbacterium* sp.6
SP-H-12	White/transparent yellow	*Microbacterium* sp.7

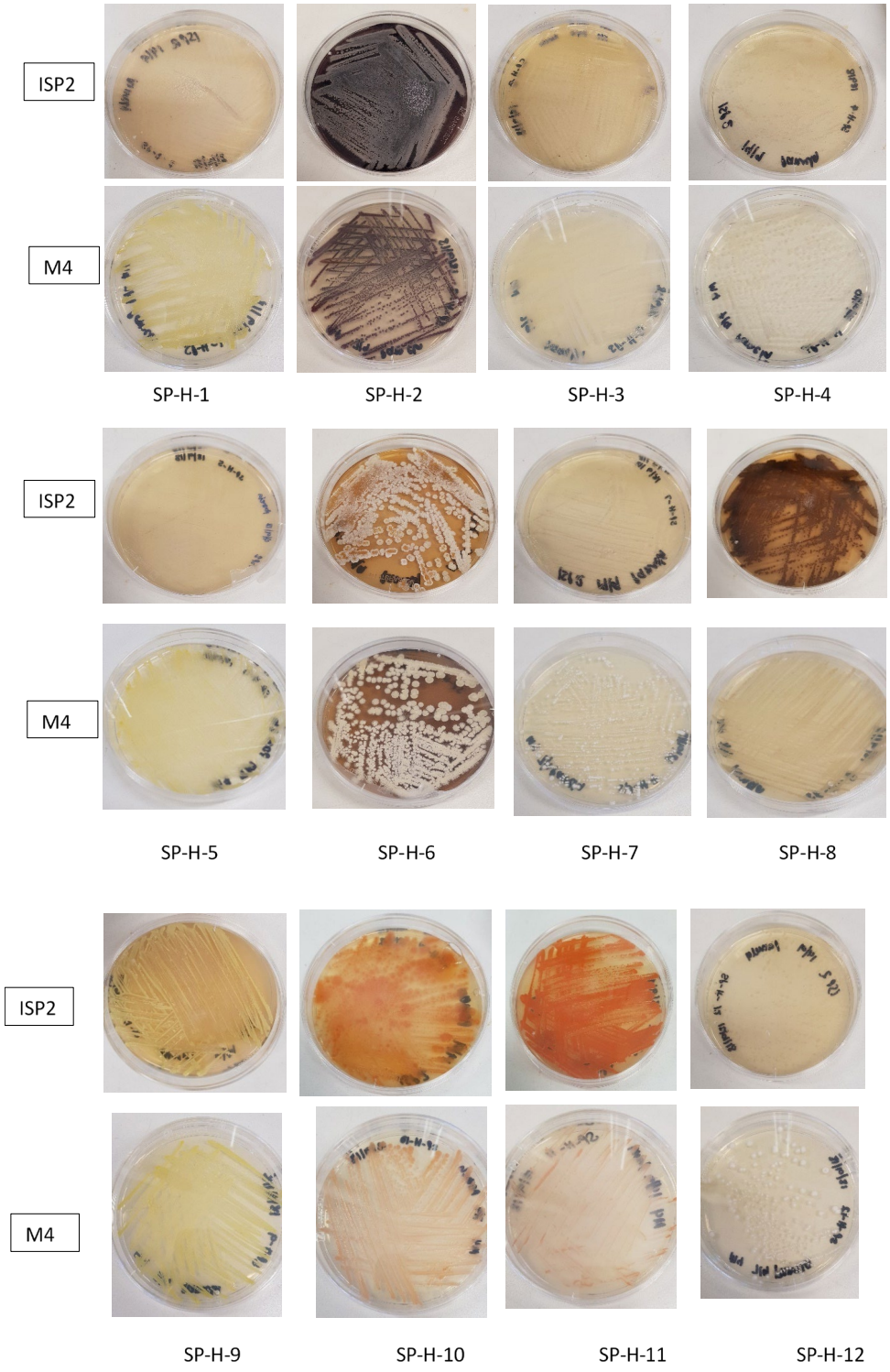

Figure 2.1.1. Morphology of 12 actinobacteria strains for the first round testing

Table 2.1.2. Fifteen strains of actinobacteria for second round of testing

Sample No.	Appearance on Plate
YA-27	White
YA-11	White
YA-20	White
YA-1	White
YA-28	White
RB-128	Dark purple
RB-27	Light yellow
RB-53	Transparent yellow
YA-2	Dark purple
YA-21	White
YA-32	White
YA-12b-152	Dark purple
YA-13	White
YA-19	Brown
RB-76	Transparent yellow

Table 2.1.3. Antimicrobial testing for strains of actinobacteria for second round of testing

Zone diameter in mm

Strains	Genus	S. aureus (SF)	S.aureus (LF)	MRSA (SF)	MRSA (LF)	C. albicans (SF)	C. albicans (LF)	T. rubrum	T. intedigitalis	Sacclophosis sp.	M. gympsum
YA11	Pseudonocardia	12	14	-	-	10	12	+1	-	-	+2
RB53	Streptomyces	13	16	11	15	-	-	-	-	-	-
RB76	Streptomyces	12	16	11	16	-	-	-	-	-	-
YA1	Streptomyces	12	16	12	15	12	14	+3	+3	+3	+3
YA13	Streptomyces	10	15	10	12	10	13	+3	+3	+3	+2
YA2	Streptomyces	12	15	10	13	-	-	-	-	+1	+1
YA20	Streptomyces	10	15	-	10	12	16	+4	+4	+4	+4
YA21	Streptomyces	10	15	11	13	11	14	+3	+3	+3	+2
YA27	Streptomyces	10	12	10	11	12	15	-	-	-	-
YA28	Streptomyces	12	18	-	-	-	-	+4	+4	+4	+4
YA32	Streptomyces	10	16	10	13	10	13	+4	+3	+2	+3
RB27	Streptomyces	15	20	13	18	-	-	-	-	-	-
YA-12b-152	Streptomyces	11	14	-	-	-	-	-	-	-	-
RB128	Streptomyces	-	-	-	-	-	-	-	-	-	-

*Data from Yitayal Anteneh

The strains were isolated from marine sponge and were chosen based on their activity against *Staphylococcus aureus, Methicillin resistant Staphylococcus aureus*, Yeast (*Candida albicans*), and dermatophytic fungi (*Tinea rubrum, Tinea interdigitalis,* Sacclophosis sp*., Microsporum gypsum*). RB128 was chosen because no activity was observed.

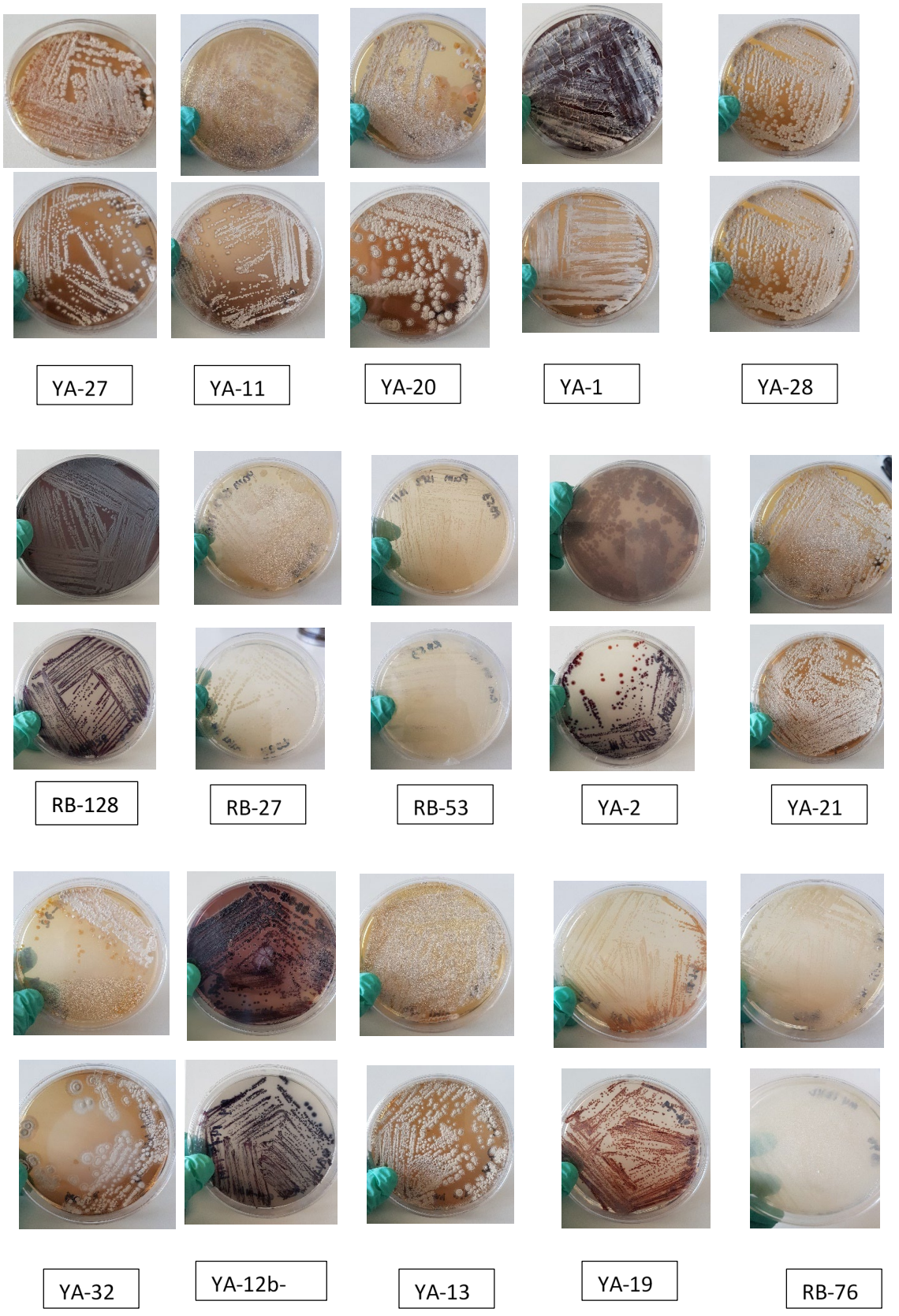

Figure 2.1.2. 15 Morphology of 15 actinobacteria strains for the second round of testing

2.2 Media for actinobacteria growth and inoculation

The culture media used to inoculate the actinobacteria from marine sponge are M4 and ISP2.

Table 2.2.1 M4 medium preparation

Component	Weight (g)
Yeast	1g
Tryptone	5g
$FeSO_4.7H_2O$	0.018g
NaCl	20g
Agar	20g

Weigh dry components then add into 1L RO water. Adjust the pH to 7.6-7.8 and then add the agar into the solution. Autoclave the solution at 120^0C for 1 hour. This solution can now be poured into sterile petri dishes in the laminar flow.

Table 2.2.2. ISP2 medium preparation

Component	Weight (g)
Yeast	4g
Malt Extract	10g
Glucose	4g
Agar	18g

Weigh dry components then add one by one into 1L RO water. Adjust the pH to 7.2-7.4 and then add the agar into the solution. Autoclave the solution at 120^0C for 1 hour. This solution can now be poured into sterile petri dishes in the laminar flow.

2.2.3. Inoculation of actinobacteria

The actinobacteria are inoculated using the streaking method following sterile techniques inside the laminar flow. All strains of the actinobacteria from marine sponge are inoculated in both M4 and ISP2 media with 5 plates for each strain. The plates inoculated with the actinobacteria are stored in the culture room at 27ºC up to 14 days. After good growth of culture was obtained, the agar plates containing actinobacteria strains were extracted with methanol to obtain compounds secreted by actinobacterial culture as well as the compounds contained in the mycelium.

2.2.4. Methanol extraction and freeze drying of actinobacteria from M4 and ISP2 media

After 9 days (M4 plates) and 14 days (ISP2 plates) the actinobacterial strains from marine sponge underwent extraction using methanol. 2 agar plates of each strain on both ISP2 and M4 were used for extraction. The agar culture was cut into cubes and transferred into 250ml baffled flasks, add 60ml of methanol was added. The opening of the flask was covered with alfoil and kept on the shaker for 15 hours at 150rpm. After 15 hours the methanol from the flasks was decanted to 50ml centrifuge tubes without the pieces agar cubes. The 50mls tubes were centrifuged for 10min ate 4000rpm then the supernatant liquid was transferred into the petri dish inside the laminar hood. These petri dish will undergo evaporation for 15-18hrs by leaving them open inside the laminar hood. The resultant methanol-free aqueous extracts from the petri dishes were transferred to Eppendorf tubes and stored in the -80ºC freezer overnight and subjected freeze drying for 3-5 hours. The actinobacterial extracts were weighed and then stored in the refrigerator.

2.3 Seed germination assay of annual ryegrass with actinobacteria from marine sponge

2.3.1. Preparation of positive control Sulfometuron Methyl (S.M.) and actinobacteria extract solution

The positive control for the seed germination test Sulfometuron Methyl (S.M.) 100uM is produced by dissolving 3.6mg in 100ml 70% ethanol. 3 positive control concentrations are used in the in-vitro seed germination 0.1 uM, 10uM, and 100uM.

2.3.2 First round of seed germination of annual ryegrass with actinobacteria from marine sponge

From the first round of actinobacteria strain dissolve 2.16mg of actinobacteria extract in 200uL 70% ethanol. Transfer 100uL of this solution into 60mls of agar and pour into 3 9 petri dish plates to produce 3 plates per strain for in-vitro testing. When the agar has solidified place 10 annual ryegrass seeds on each plate. Results of seed germination and annual ryegrass leaf length is observed and recorded after 5 days.

The positive control are the three concentrations of the S.M. and the negative control were M4 blank, ISP2 blank and agar with 100uL 70% ethanol.

2.4. Greenhouse assay for active extracts of first round actinobacteria strains

Annual ryegrass grown for 2-3 weeks provided at University of Adelaide. Active extracts of actinobacteria from marine sponge were used for testing in the greenhouse against annual ryegrass. 0.019 g of the extract dissolved in 500uL of 10% methanol. Completely dissolve actinobacteria extract then add 50uL of surfactant (Agral). This solution was sprayed to pots with grown annual ryegrass with three replications. Observe changes on the annual ryegrass after 7-10 days. After final observations the plants were used for dry weight analysis.

2.4.1. Dry weight of annual ryegrass

Leaves from the annual ryegrass sprayed with actinobacteria extract were cut leaving the roots. These leaves are placed inside the oven to dry for 72 hours. After leaves were oven dried take the weight of each sample and record.

2.4.2. Second round of seed germination of annual ryegrass with actinobacteria from marine sponge

15 strains for the second round of seed germination testing will be utilized. From each strain, weigh 10mg of extract and transfer to Eppendorf tube for reference. The remaining extracts are dissolved in 600uL (m4) and 300uL (ISP2) 100% methanol. Centrifuge the solution and transfer liquid from the solution in clean Eppendorf tube. Take 100uL from the liquid solution and add to 60mls of agar. Pour 20mls of agar solution in 3 9 petri dish plates.

2.5. Thin layer chromatography (TLC) for second round of actinobacteria strains

The 10mg extract for the second round of testing is diluted with 100ul 95% methanol. Prepare 2 eluting solvents ethyl acetate and butanol:acetic acid:water (4:1:1). Transfer 20uL of diluted extract and blot on 10x20 silicone paper. Do this for both M4 and ISP2 actinobacteria strains. Place silicone paper on tanks with eluting solvents and run test.

2.5.1. Seeding of active strains from the second round actinobacteria strains in Thin layer chromatography

5 strains of actinobacteria showing activity in thin layer chromatography is used for seeding in IM-22 medium and ISP2 medium. The five strains are YA-1, YA-20, YA-32, YA-12b-152 and RB-128. Inoculate active strains from ISP2 agar plates grown with strains of actinobacteria into IM-22 and 20 plates of ISP2 media.

2.5.2. Fermentation of strains from IM-22 seeding medium into different liquid media

The active actinobacteria strains in IM-22 seeding media is cultivated for 3 days. On the 3rd day 2mls of the inoculum culture from IM-22 is transferred to 50mls of ISP2 broth, F26 and F28 fermentation media in 250ml baffled flasks.

2.4.2. Thin layer chromatography for actinobacteria using liquid medium

The thin layer chromatography for the active strains of actinobacteria based on the TLC results obtained from the powdered extracts from the ISP2 solid medium is done by using samples incubated for 14 days in the shaker at 27ºC with 150rpm. From the incubated cultures 2mls of sample were taken from F26, F28 and ISP2 broth on Day 5 to Day 14 and were transferred to 2mls Eppendorf tubes. these samples are centrifuged at 2500rpm for 5mins and supernatant is transferred in 5mls tube and stored for 24hours in -80ºC freezer. The samples were freeze dried to remove excess water for 5-6 hours. The powdered extract after freeze drying was used for further TLC assay.

2.6. High performance liquid chromatography

The high performance liquid chromatography was done for the detection of compounds present in the actinobacteria strains as based on the results from the TLC. The system for the HPLC (Shimadzu LC-10AS) used the eluting solvent acetonitrile:1% a onium acetate (2:8) and acetonitrile:water with 0.1% Tetrafluoroacetic acid (2:8) run for 35 minutes with a flow rate of 0.7ml/min. 25ul sample from the actinobacteria extracts were injected into the Kinetex C18 6u column (length 50x2.10 , ID 2.1 , SPD-20A, UV-VIS detector).

CHAPTER 3: RESULTS

3.1 Seed germination assay for first round of actinobacteria from marine sponge

The test is to determine the herbicide activity of the actinobacteria extract in comparison to the positive control sulfometuron methyl. The test for the seed germination is performed in vitro by observation of the leaf length and germination of the annual ryegrass seeds. The actinobacteria strains used for the first round are based on the previous study on the herbicide activity of actinobacteria on marine sponge actinobacteria.

In using the medium ISP2 for the seed germination assay, one actinobacteria extract presented herbicide activity on annual ryegrass. This is based on the comparison of the tests for the germination and leaf length of the annual ryegrass after 5 days of incubation. SP-H-12 was selected to be used for further testing for greenhouse assay.

In using the medium ISP2 for the seed germination assay, one actinobacteria extract presented herbicide activity on annual ryegrass. This is based on the comparison of the tests for the germination and leaf length of the annual ryegrass after 5 days of incubation. SP-H-12 was selected to be used for further testing for greenhouse assay.

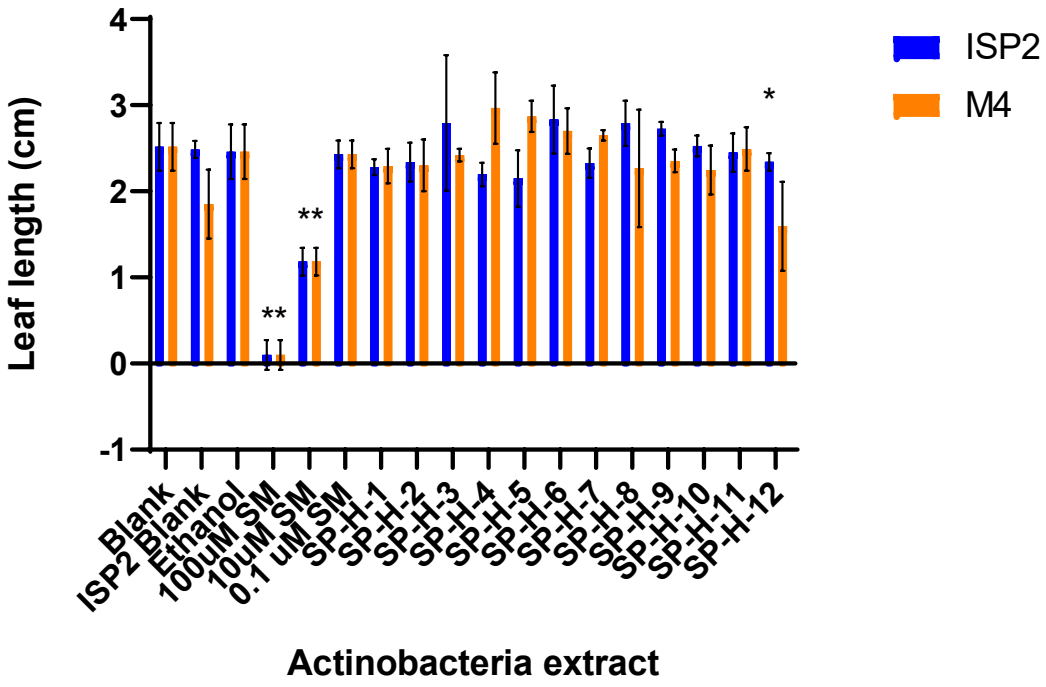

Figure 3.1.1. Average leaf length of annual ryegrass with the positive control S.M in ISP2 and M4 solid media
*Significant at P=<0.05

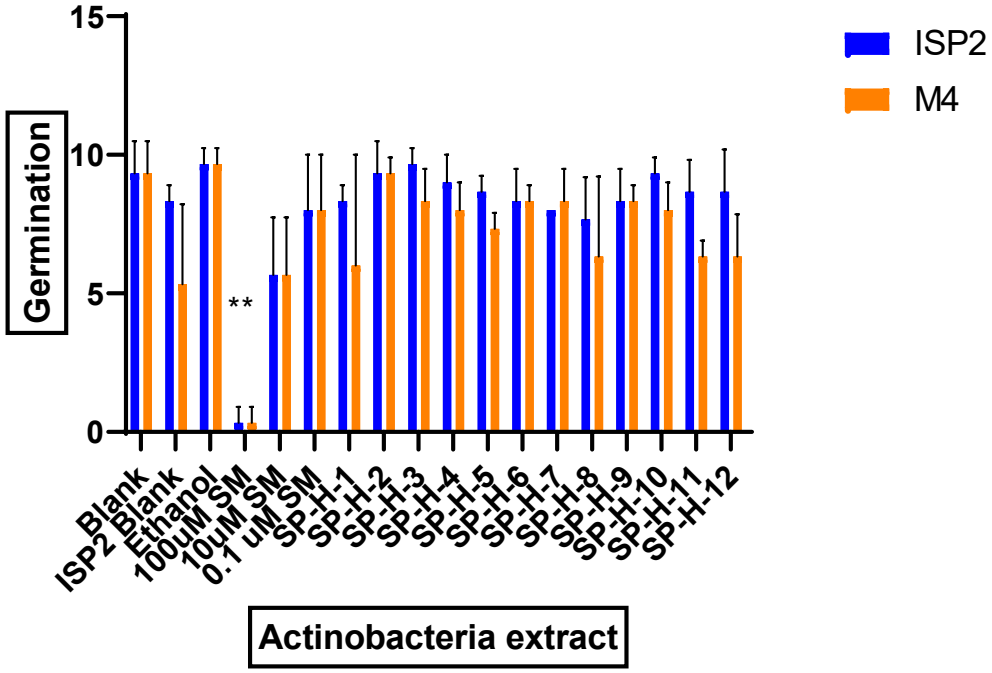

Figure 3.1.2. Average germination of annual ryegrass with the positive control S.M in ISP2 and M4 solid media
*Significant at P=<0.05

3.2. Seed germination assay for second round of actinobacteria from marine sponge

The seed germination for the second round will test 15 new strains of marine actinobacteria using leaf length determination and germination.

The seed germination test is performed using a modified assay from the first round of testing. There is very low herbicide activity from the candidate strains from this round of testing no further testing of greenhouse assay has been employed.

The seed germination testing for the annual ryegrass with actinobacterial extracts on M4 media showed very low herbicide activity therefore no further testing on greenhouse assay is performed

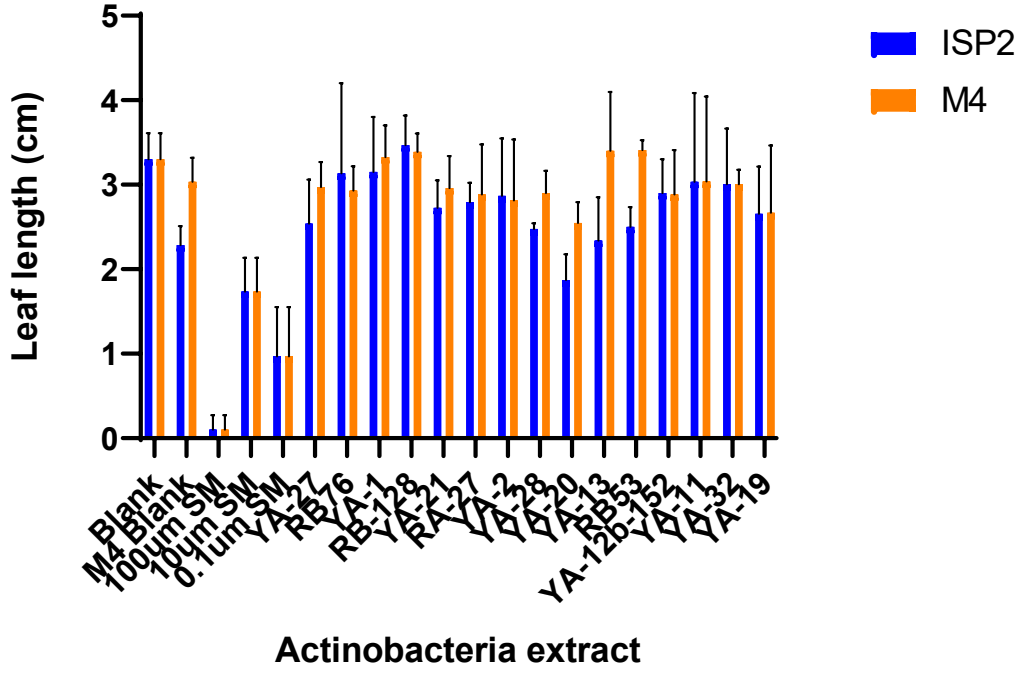

Figure 3.2.1. Average leaf length of annual ryegrass with the S.M control in ISP2 and M4 solid media
*Not Significant at P=<0.05

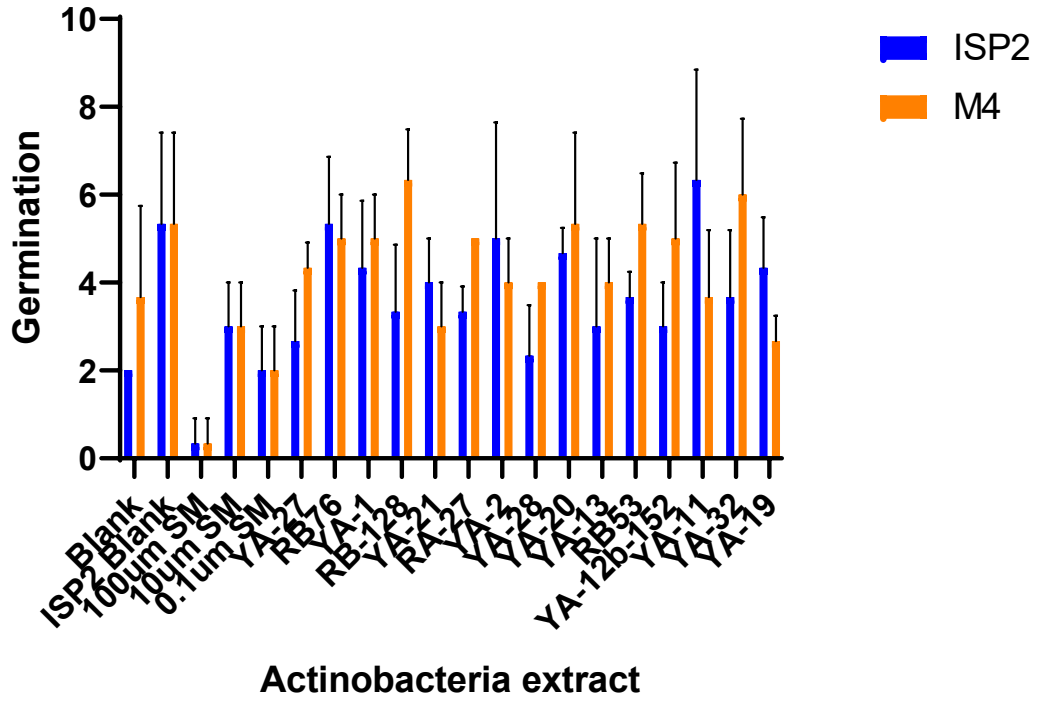

Figure 3.2.2. Average germination of annual ryegrass with the S.M control in ISP2 and M4 solid media
*Not Significant at P=<0.05

3.3. Greenhouse assay for first round of actinobacteria from marine sponge with herbicide activity with *in vitro* testing. Testing for the herbicide activity of the actinobacteria extract against annual ryegrass showed that there is low activity to inhibit the growth and necrosis of the weed by the actinobacteria extracts.

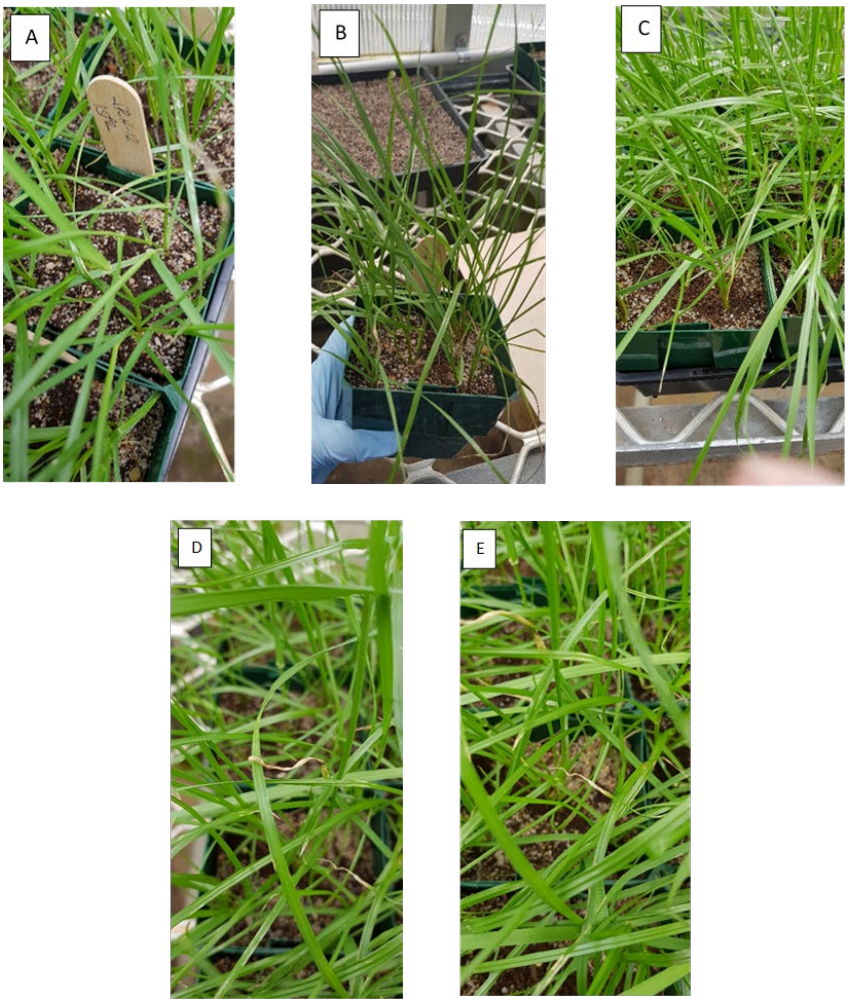

Figure 3.3.1. Greenhouse assay using 1-spray method of actinobacteria extracts against annual ryegrass

Figure 3.3.2. Greenhouse assay using 2-spray method of actinobacteria extracts against annual ryegrass

3.3.1. Dry weight determination of annual ryegrass with herbicide activity

The dry weight determination is performed to ensure that herbicide activity from the actinobacteria extract can be based on the biomass of the annual ryegrass leaves compared to the control. The annual ryegrass leaves exposed to the actinobacteria extract spray and the methanol showed that both 1-spray and 2-spray method has higher biomass than that of the control.

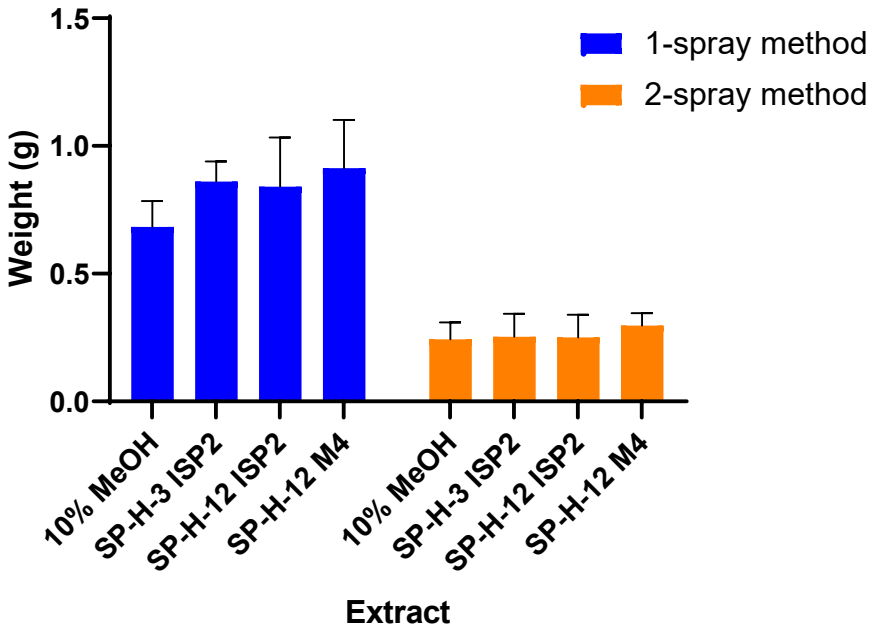

Figure 3.3.1.1. Average dry weight of annual ryegrass using actinobacteria extracts
*Not Significant at P=<0.05

3.4. Thin layer chromatography (TLC) of Second round of actinobacteria Extract

3.4.1 Thin layer chromatography for actinobacteria extracts

The actinobacteria extracts from the second round of testing is assayed for thin layer chromatography using ethyl acetate and butanol:acetic acid:water (4:1:1) as the eluding solution. The TLC results of the actinobacteria extracts showed extracts inoculated in the ISP2 solid medium have higher active compounds.

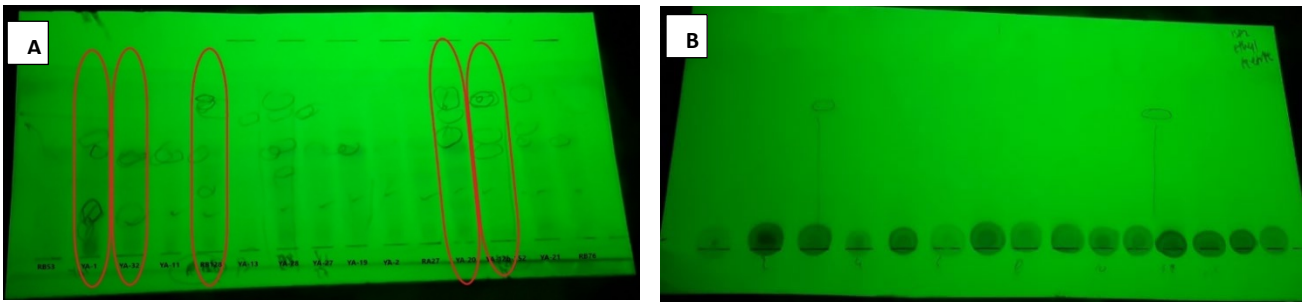

Figure 3.4.1.1. TLC of actinobacteria extracts in ISP2 solid media using A) BAW (4:1:1) and B) ethyl acetate

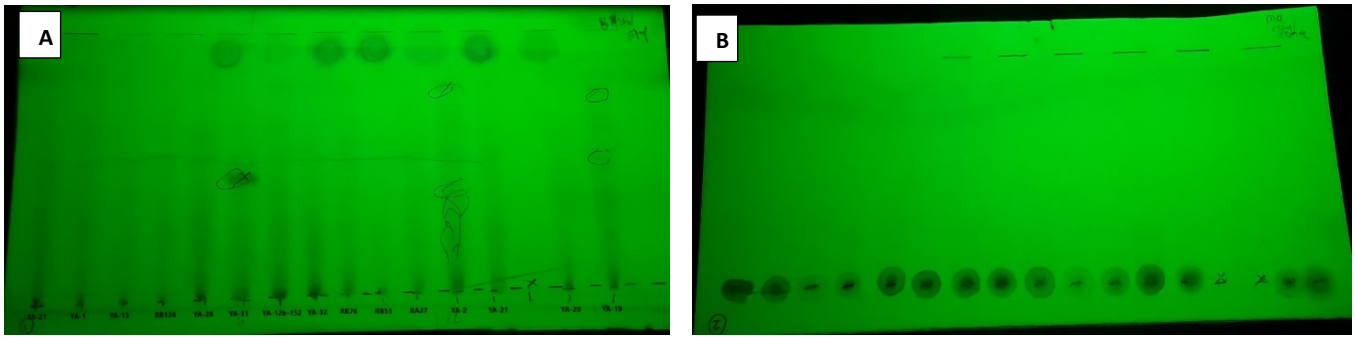

Figure 3.4.1.2. TLC of actinobacteria extracts in M4 solid media using A. BAW (4:1:1); B. ethyl acetate

3.4.2. Thin Layer chromatography of active extracts of actinobacteria from different liquid medium

Five active strains of actinobacteria in ISP2 solid medium are used for the assay using three liquid medium ISP2 liquid medium, F26 and F26 medium using extracts from day 5 to day 14 of incubation.

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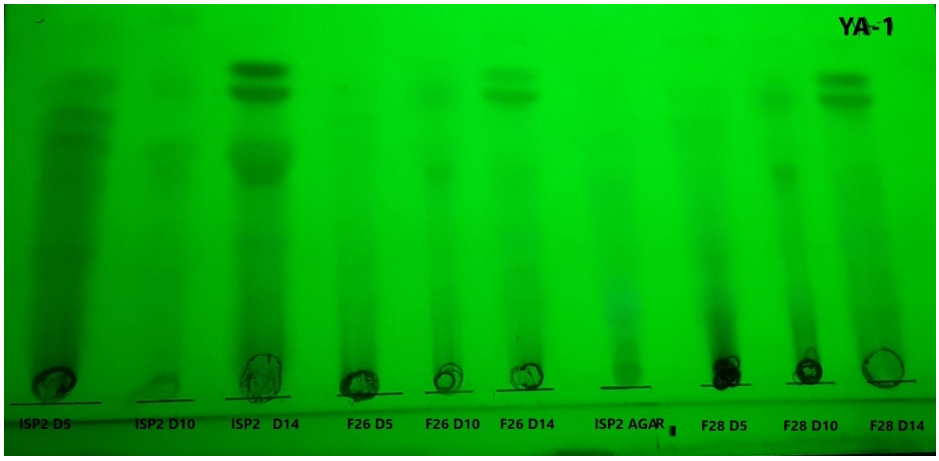

Figure 3.4.2.1. YA-1 strain of actinobacteria tested for TLC assay for Day 5, Day 10 and day 14 for 3 liquid media

3.4.3. TLC of actinobacteria extracts in ISP2 liquid medium from Day 5 to Day 9 of incubation

Based on the previous results of the TLC it is found that actinobacteria in ISP2 liquid medium produced more compounds as compared to the other media used. Therefore, additional chromatography test for Day 5- Day 9 of incubation is performed. The incubation time showing the highest compound production are used for the HPLC analysis.

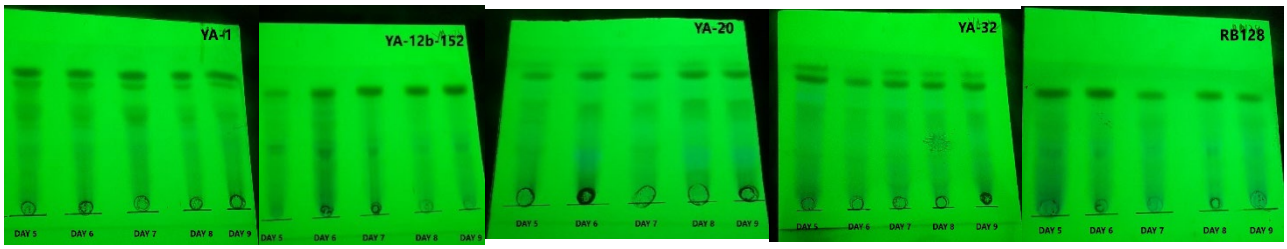

Figure 3.4.3.1. Result of the TLC of the active strains of actinobacteria from ISP2 liquid medium.

3.5. High performance liquid chromatography (HPLC)

High performance liquid chromatography was used to detect and confirm the presence of compounds based on the TLC results. The HPLC is done on active actinobacteria from samples that showed highest intensity of bands from the TLC results for Day 5-Day 9 incubation. The HPLC results showed that some strains of actinobacteria from different incubation periods can produce different number of compounds.

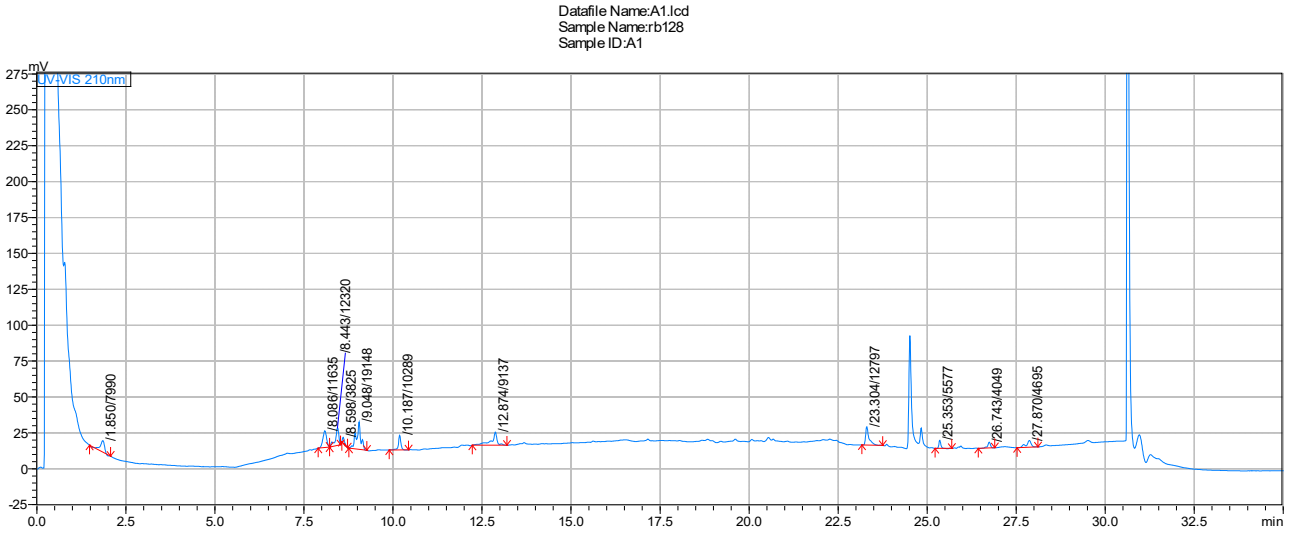

Figure 3.5.1. Chromatogram result for actinobacteria strain RB128 in ISP2 liquid medium.

YA-1			
Peak#	Ret. Time	Area Day 5	Area Day 7
1	8.811	207240	189507
2	10.334	89091	88169
3	14.735	79330	63603
4	15.999	232303	158756
5	21.921	2066169	2609250
6	24.384	324421	363508
Total		2998553	3472792

Table 3.5.1. HPLC peak area for actinobacteria strain YA-1 in ISP2 liquid medium collected on Day 5 and day 7.

The results of the HPLC from the actinobacterial strain YA-1 showed that at 21.921 minutes there is the highest peak of and from Day 5 there is an increase of production from the sample on Day 7.

Table 3.5.2. HPLC peak area for actinobacteria strain YA-12b-152 in ISP2 liquid medium collected on Day 5 and day 6.

YA-12b-152			
Peak#	Ret. Time	Area D5	Area d6
1	5.098	4326106	7455188
2	8.854	186214	208337
3	10.374	97717	116569
4	14.758		85878
5	16.03	172786	195916
6	24.411	441429	351727
Total		5224253	8425040

The results on the HPLC on the actinobacterial strain YA-12b-152 showed that on 5.098 minutes the compound has the highest intensity. On day 6 it has increased production on sample obtained on Day 5 of inoculation.

Table 3.5.3. HPLC peak area for actinobacteria strain YA-20 in ISP2 liquid medium collected on Day 6 and day 9.

YA-20			
Peak#	Ret. Time	Area Day 6	Area D 9
1	8.227	223186	288655
2	8.856	434873	516780
3	10.375	116392	96438
4	12.453	287757	286031
5	12.857	134288	137641
6	13.942	92230	92802
7	14.519	135173	20719
8	16.024	184097	170217
9	24.418	335010	319827
Total		1943005	1929110

The results on the HPLC on the actinobacterial strain YA-20 showed that on 8.856 minutes the compound has the highest intensity. On day 6 it has increased production on sample obtained on Day 9 of inoculation.

Table 3.5.4. HPLC peak area for actinobacteria strain YA-32 in ISP2 liquid medium collected on Day 5 and day 9.

YA-32			
Peak#	Ret. Time	Area D5	Area D9
1	8.892	161727	200057
2	12.447	945121	893034
3	12.861	350998	802986
4	14.543	190042	268125
5	15.48	250672	107936
6	16.048	200031	212181
7	24.437	278286	316373
Total		2376877	2800743

The results on the HPLC on the actinobacterial strain YA-32 showed that on 12.447 minutes the compound has the highest intensity. The sample obtained from day 9 of inoculation is lower compared to the sample obtained in Day 5. It was also observed that there is a high difference on the compound peak area on 12.861 minutes.

Table 3.5.5. HPLC peak area for actinobacteria strain RB128 in ISP2 liquid medium collected on Day 5 and day 6.

RB128			
Peak#	Ret. Time	Area D5	Area D6
1	3.776	88082	
2	5.464	3807295	2525181
3	8.923	179277	178396
4	10.428	89201	73322
5	14.802	79408	
6	16.064	171105	146981
7	24.454	337213	343784
Total		4751581	3267665

The results on the HPLC on the actinobacterial strain RB128 showed that on 5.464 minutes the compound has the highest peak area. The sample from day 5 of inoculation has higher peak are than the sample obtained from day 6 of inoculation.

CHAPTER 4: DISCUSSION

This study aims to test the herbicide activity of different strains of actinobacteria grown on different culture media against annual ryegrass in greenhouse trial and in-vitro testing.

The study is in part a continuation of a previous study in the development of herbicide products from marine sponge specific on Actinobacteria against annual ryegrass. On in vitro testing actinobacteria strains are found to feature inhibitory properties against annual ryegrass thus further greenhouse trial was done in order to find evidence of commercial viability of these microorganisms' herbicide activity.

4.1 Growth of actinobacteria in different culture media

The growth medium used for actinobacteria is a determining factor in the optimum production of different compounds (Davis et al., 2005). The use of the medium ISP2 and M4 in this study is based on the previous project on the herbicide activity of actinobacteria from marine sponge against annual ryegrass. Using both medium, the study aims to determine the reproducibility of the previous test method employed. In this study there has been a modification on the M4 medium using RO water with NaCl. The modification produced more growth on the M4 medium than in the ISP2 medium as previously reported. This may be due to the presence of salt in the medium that attribute to more products.

This study also employed using both solid and liquid medium for growth of actinobacteria. The importance to produce actinobacteria in both the solid and liquid media is to determine the best medium to produce compounds in the most optimum state to be used in other bioactive test determination (Mbiyu et al., 2012).

4.2 Development of germination assay

In this study, two rounds of testing have been employed using two batches of actinobacteria strains. The strains from the first round were a follow up from the previous study performed on herbicidal activity of marine sponge actinobacteria against annual ryegrass. It has been found from this study that the same strain from the previous study also showed herbicidal activity against annual ryegrass. The strains for the first round of testing showed very low herbicidal activity against annual ryegrass, therefore the need to test new marine sponge actinobacteria is employed for the second round of testing.

The first round of actinobacteria 2.16 mg was dissolved in 70% ethanol and mixed with 60mls of agar. The positive control for this study sulfometuron methyl was also dissolved in 70% ethanol and further diluted to produce different concentrations to be employed in the germination assay.

The strains to be tested for the second round were chosen based on the results of antibacterial and antifungal testing. Strains with bacterial and antifungal activity and strains that do not show any activity were used in order to compare the results of potential herbicide activity. The second round of actinobacteria produced is done by taking 10mg from each strain used as reference and all powder extracts is dissolved with 100% methanol.

From this dilution diluted extracts are used for in-vitro testing of actinobacteria against annual ryegrass using the same positive control. Based on the results of the seed germination assay in-vitro first round strains with 70% ethanol has shown more inhibitory herbicide activity as compared to using 100% methanol even at the highest concentration. The positive control employed on both first and second round of testing produced significant result and may be due to the presence of salt in the medium. The second round of actinobacteria strains showed lower inhibiting herbicide activity on annual ryegrass. Therefore it was decided to

obtain larger amounts of the compounds and then test these larger amounts so that there s a better possibility of a positive result.

4.3 Detection of compounds from marine actinobacteria

Thin layer chromatography for the second round of actinobacteria strains was employed in this study to determine the most optimum media and time that compounds from the strains which have activity on the in-vitro seed germination test. Both solid and liquid media were used for the Thin layer chromatography. Results of the TLC showed that ISP2 broth was the most optimum medium for the actinobacteria between Day5-Day9 of incubation at 27^0C. from the TLC results HPLC was done to check the variation of the compounds produced from the different date of sample collection. Two compounds, RB128 and YA-12b-152 produced compounds not present on both collection days. We can identify from the HPLC results that incubation times for actinobacteria can produce different compounds. From the results of the 24minute peak area of the HPLC, we compare the results with the chromatogram of the TLC of the darkest band for each of the actinobacterial strain.

4.4 Greenhouse assay

The greenhouse assay for the study was employed to be able to produce results on the basis for the effects in terms of commercial viability. The trial was performed under natural conditions to produce results that can be comparable to the growth of the annual ryegrass in its natural habitat.

We employed the spray method of treating the annual ryegrass with the actinobacterial extracts. The annual ryegrass for the trial was checked for possible symptoms of necrosis and leaf drying after 7-10 days after spraying. To further evaluate the effectivity of the herbicide potential of the actinobacteria extract the leaves are subjected to biomass investigation.

The results for the greenhouse trial have showed low inhibition of growth and no significant leaf necrosis and drying of the leaves of the annual ryegrass. The biomass results showed that the actinobacteria extracts have no significant herbicide effect.

4.5 Future directions

This study showed in-vitro seed germination assay that there is potential for actinobacteria from marine sponge to produce herbicide activity. The greenhouse assay showed little herbicide activity using the spray method. To establish the potential of active strains tested on this study further optimization of the concentration of the extract should be used. It has also been identified that different media produce different concentrations of compounds on different incubations periods. The strains from the first round of actinobacteria should be assayed for fungal and bacteria assay to be able to provide a relationship for the microbial activity for the active strain.

CHAPTER 5: CONCLUSIONS

This study investigated the herbicidal activity of 27 marine actinobacteria strains against annual ryegrass and detected the presence of compounds with herbicidal activity by applying different types of inoculation media and collection time. TLC patterns and HPLC chromatograms showed that actinobacteria strains produced different metabolites when growing on different media under different conditions. Extracts produced from marine sponge-associated actinobacteria SP-H-12 showed low herbicidal activity against annual ryegrass both *in vitro* and greenhouse assays. However, investigation on dry weight of annual ryegrass leaves showed the tested extract on the leaves have no herbicidal activity. However, this study is limited to very low powdered extract production from the actinobacteria from solid growth medium preparation. The production in liquid medium was successful, however, there was insufficient time to scale up the production and obtain more of each compound for testing.

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Appendix

1. Culture media

IM-22

Composition per 1L RO water:

Glucose	15.0g
Soyatone	15.0g
Pharmamedia	5.0g
Calcium carbonate	5.0g
Sodium chloride	5.0g

F26

Composition per 1L RO water:

Glucose	10g
Soybean flour	10g
$CaCO_3$	4g
$CoCl_2.6H_2O$	1mg
pH	7.0-7.4

F28

Composition per 1L RO water:

Glucose	10g
Soluble starch	10g
Malt extract	7.5g
Peptone	7.5g
$MgSO_4.7H_2O$	1g
NaCl	3g
Trace elements	
$CuSO_4 .5H_2O$	7mg
$FeSO_4.7H_2O$	1mg
$MnCl_2.4H_2O$	8mg
$ZnSO_4.7H_2O$	2mg
pH	7.0-7.4

2. Tables for germination and dry weight determination

Data for the results of Table 3.1.1.

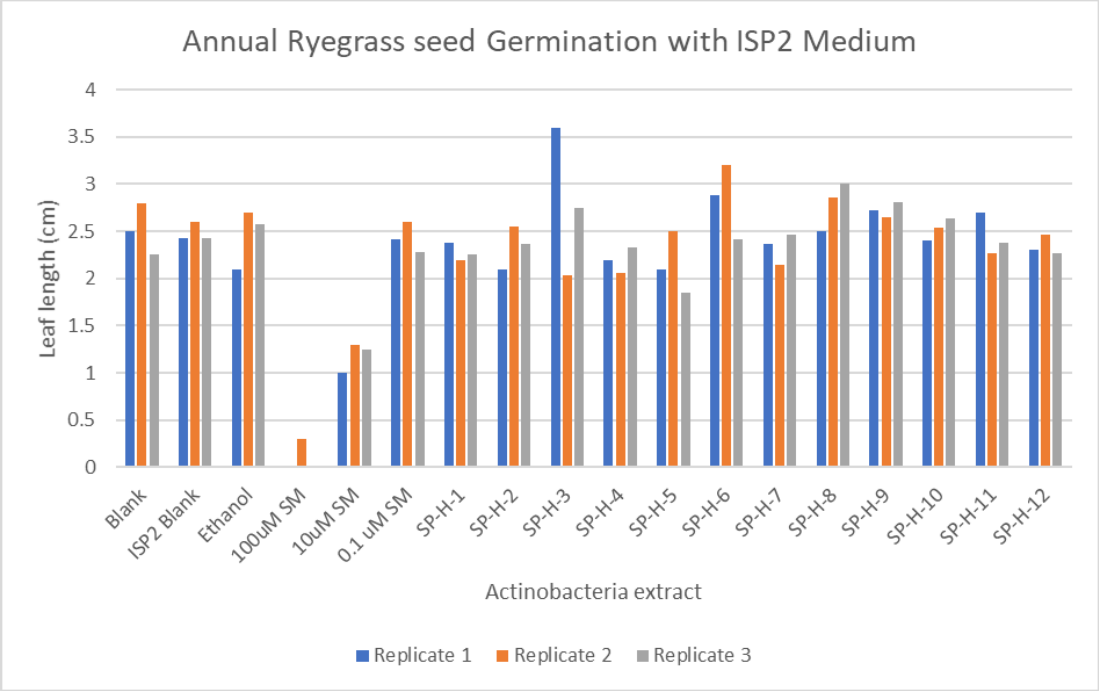

Figure A. Leaf length of Annual ryegrass on Actinobacteria extracts grown on ISP2 medium for the first round of testing

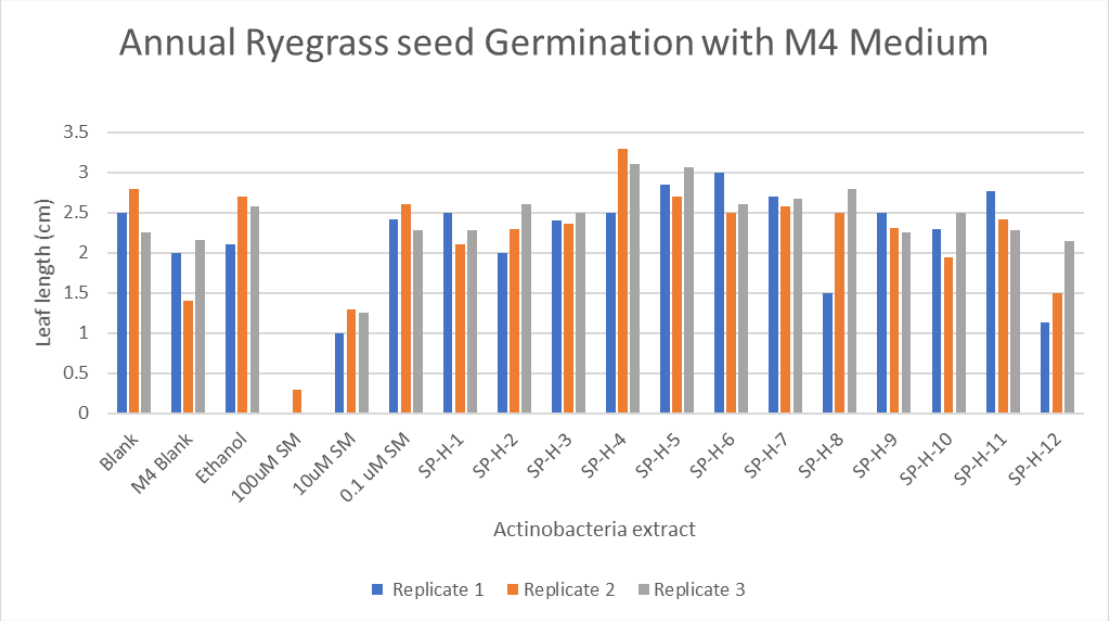

Figure B. Leaf length of Annual ryegrass on Actinobacteria extracts grown on M4 medium for the first round of testing

Data for the results of Table 3.1.2.

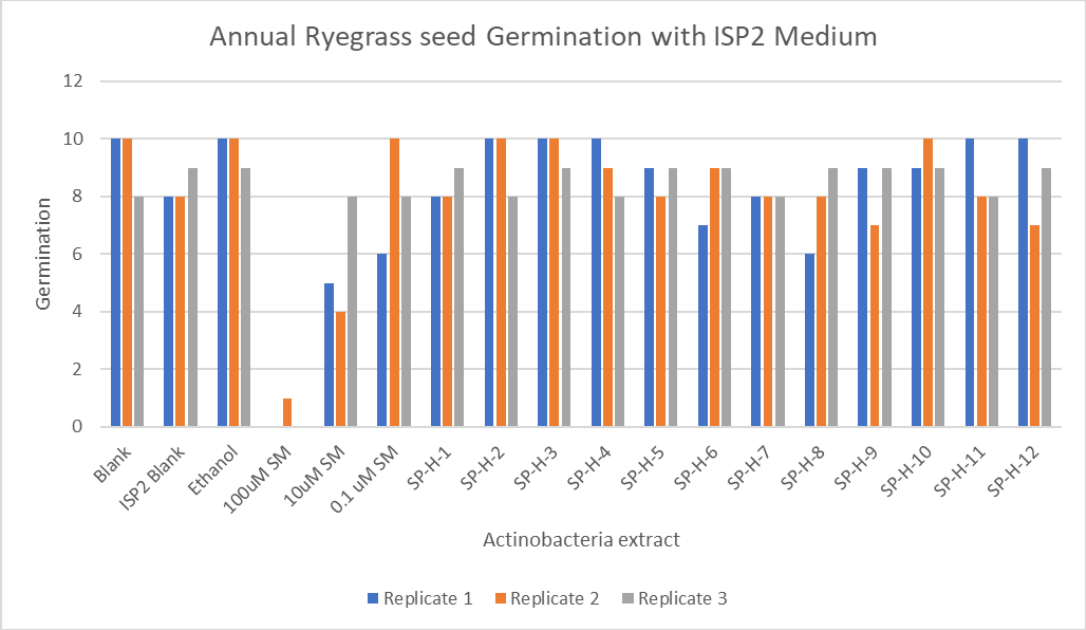

Figure C. Seed germination of Annual ryegrass on Actinobacteria extracts grown on ISP2 medium for the first round of testing

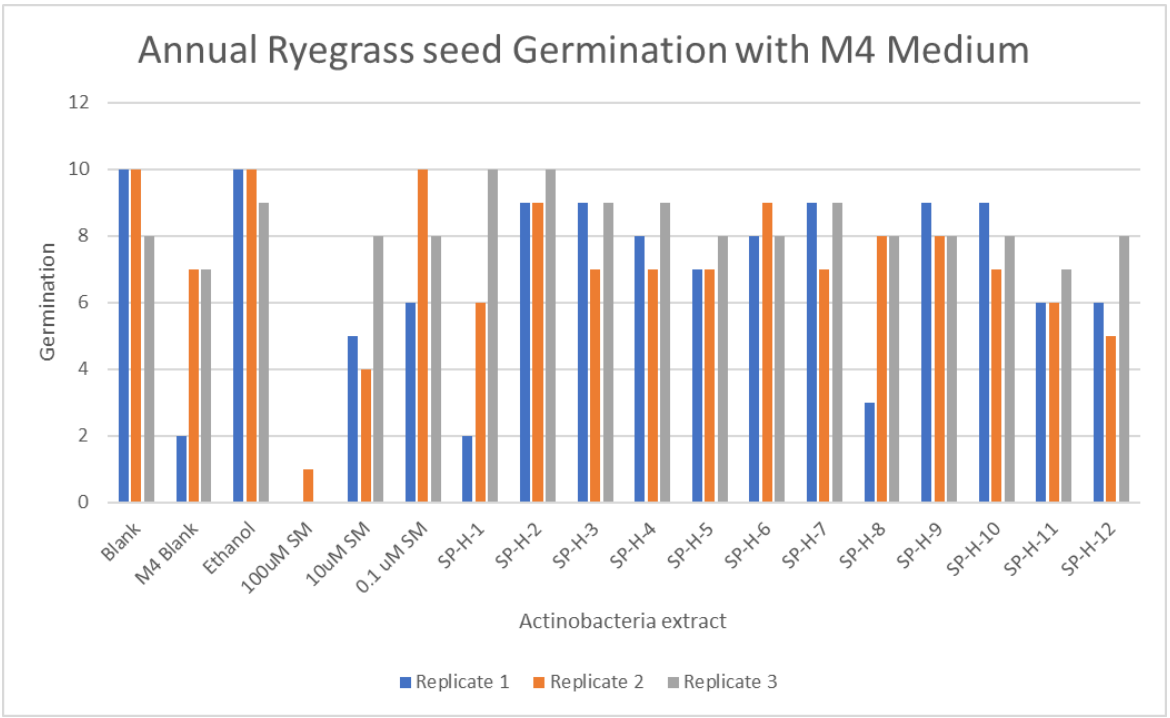

Figure D. Seed germination of Annual ryegrass on Actinobacteria extracts grown on M4 medium for the first round of testing

Data results for Table 3.2.1

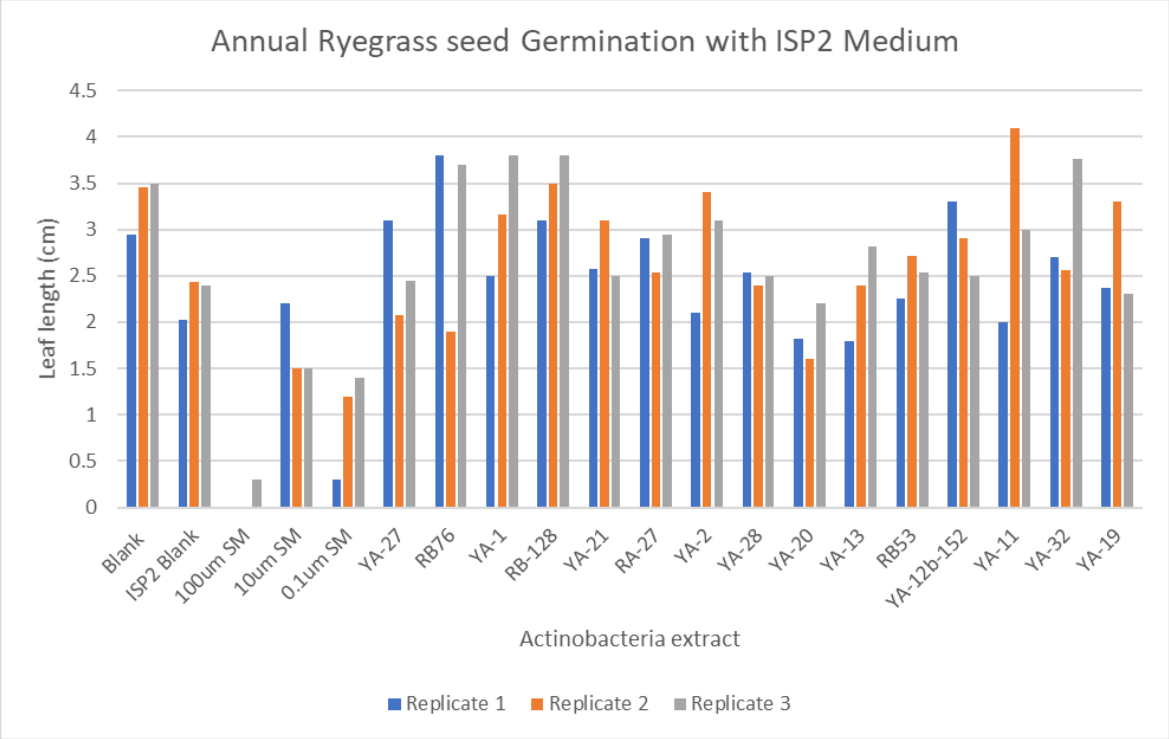

Figure E. Leaf length of Annual ryegrass on Actinobacteria extracts grown on ISP2 medium for the second round of testing

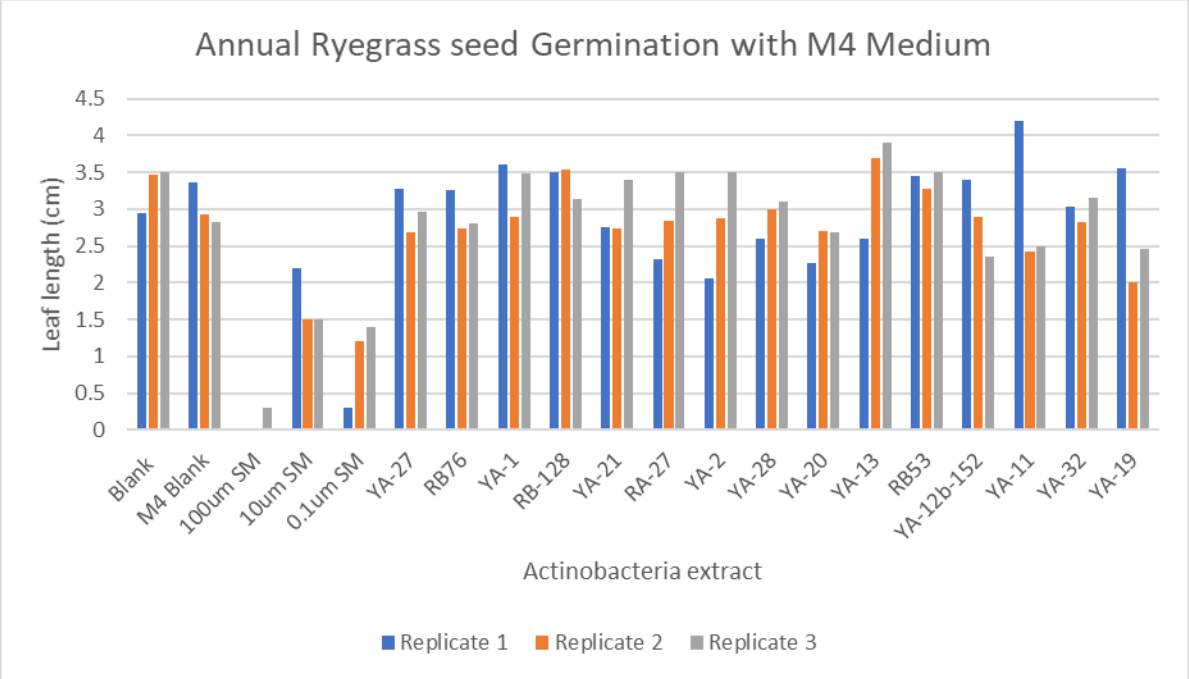

Figure F. Leaf length of Annual ryegrass on Actinobacteria extracts grown on M4 medium for the second round of testing

Data results for table 3.2.2.

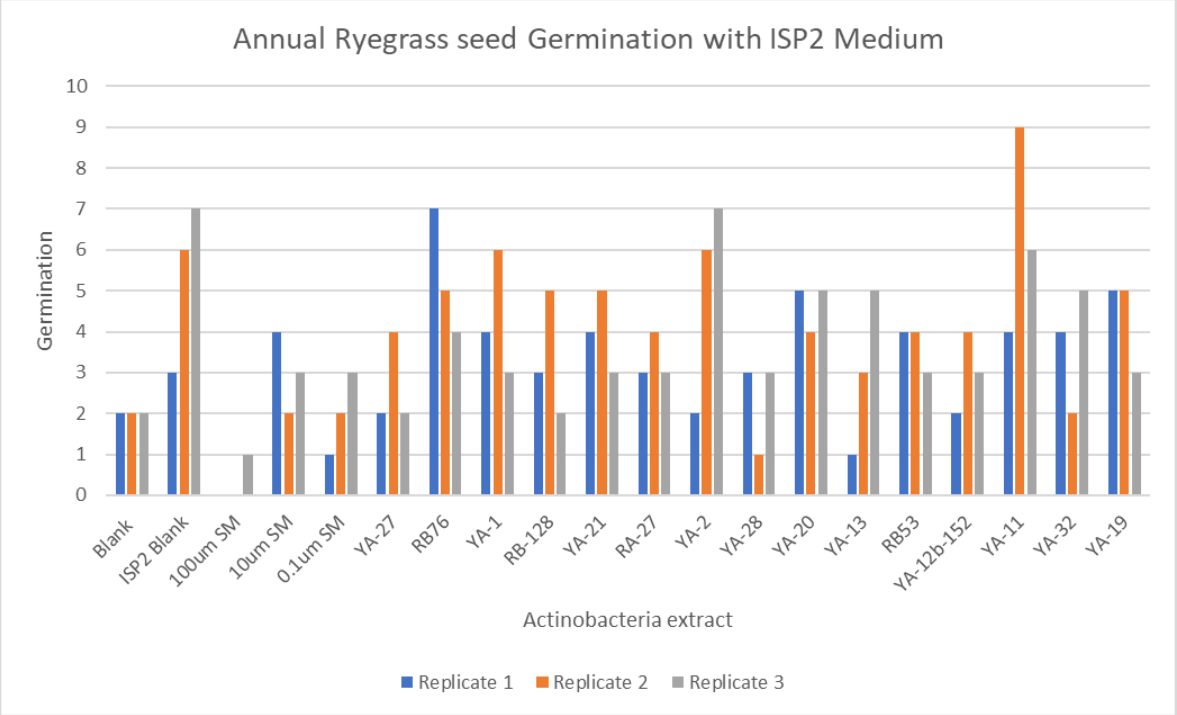

Figure G. Seed germination of Annual ryegrass on Actinobacteria extracts grown on ISP2 medium for the second round of testing

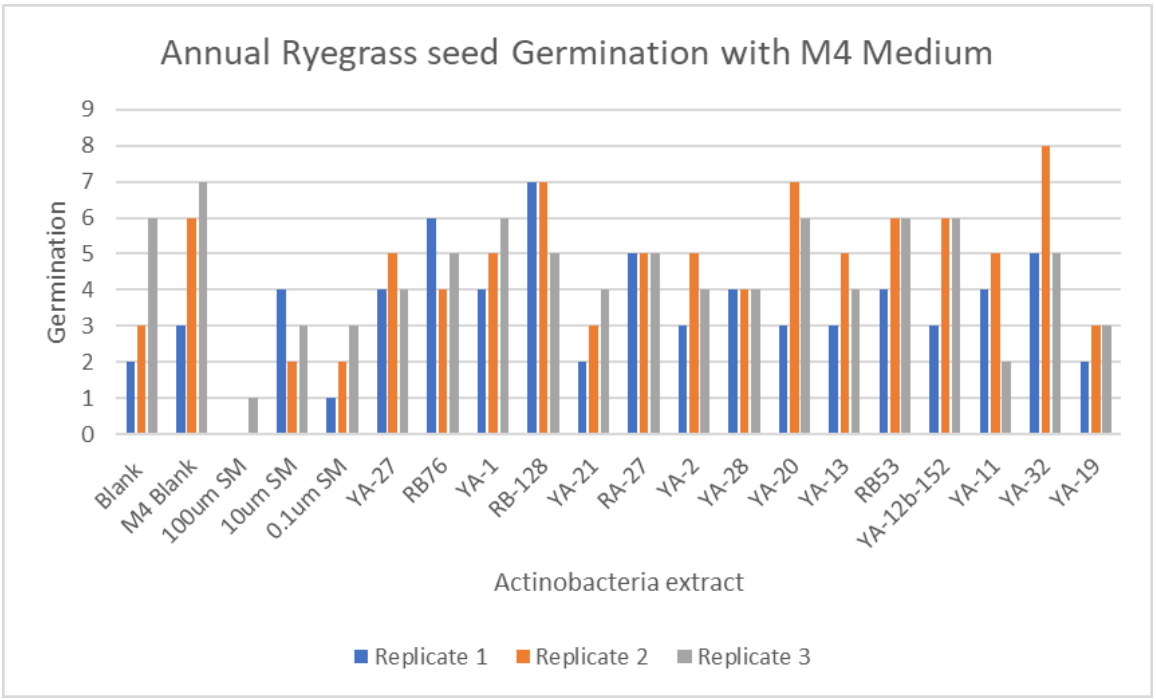

Figure H. Seed germination of Annual ryegrass on Actinobacteria extracts grown on M4 medium for the second round of testing

Data results for Table 3.3.1.1.

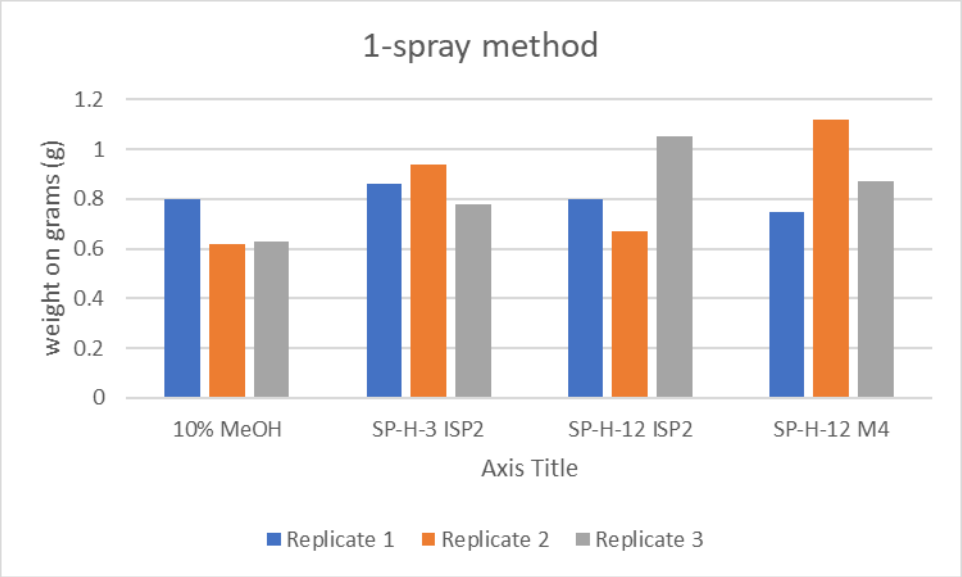

Figure I. Biomass of annual ryegrass using 1 spray method of actinobacteria extract from marine sponge

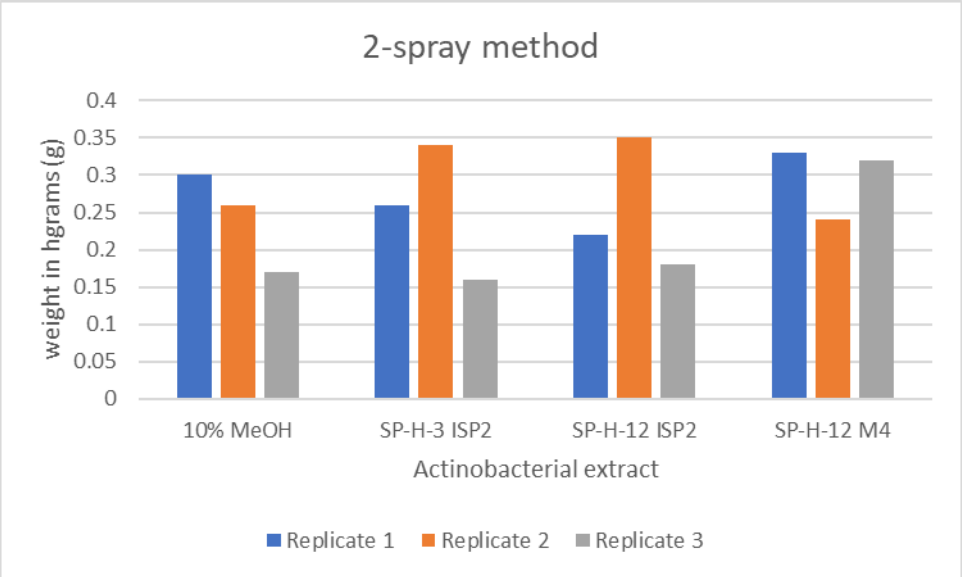

Figure J. Biomass of annual ryegrass using 2 spray method of actinobacteria extract from marine sponge

3. Statistical analysis

Repeated measures ANOVA summary					
Assume sphericity?	No				
F	15.86				
P value	0.0217				
P value summary	*				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.096				
R square	0.888				
Was the matching effective?					
F	0.3723				
P value	0.6919				
P value summary	ns				
Is there significant matching (P < 0.05)?	No				
R square	0.002447				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	21.78	17	1.281	F (1.632, 3.264) = 15.86	P=0.0217
Individual (between rows)	0.06016	2	0.03008	F (2, 34) = 0.3723	P=0.6919
Residual (random)	2.747	34	0.08079		
Total	24.59	53			
Data summary					
Number of treatments (columns)	18				
Number of subjects (rows)	3				
Number of missing values	0				

Table A. ANOVA single factor for ISP2 leaf length measurement for 1st round testing