Consistency of inhibitory effect of metabolites produced by selected two isolates of *Streptomyces* on *in vitro* *Striga hermonthica* seed-germination

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الآية

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

وَقَلَ رَبِّ رِزْدَنِي عِلْمًا (114)

[طه 114]
DEDICATION

To my great mother who feed us the love of knowledge.

To the sole of my great father who struggled in laborious condition with no complain to grantee a better life to us.

To my sister, my friends and my relatives who give my life a wonderful meaning.

To my teachers who guided and gave us their advices as same as their knowledge, I am grateful for that and I am bragging that you were my teachers.
Acknowledgements

With immensity noun of our university and college I would like to acknowledge all the department members, whom help me to complete this project.

No words can express my great thanks to my supervisor Dr. Rashid Ibrahim M. Ahmed Mohukker for his advice, help and guidance through the period of this study.

And I also acknowledge all peoples behind the light whom they share me by their point of view.
Contents

Content

Page

آلة..........................................................i

Dedicatio ..................................................ii

Acknowledgement ............................................iii

Abstract ...................................................iv

Arabic abstract .............................................v

Chapter one

1. Introduction and literature review ..................1

1.1. Striga spp..........................................1

1.2 Streptomyces spp....................................4

1.3. Phytotoxins production by Streptomyces ...........5

1.4 Objectives of the study ..............................6

Chapter two

2. Materials and Method ................................8

2.1. Source of Striga seeds .............................8

2.2. Viability test for Striga-seeds .....................8

2.3. Conditioning of Striga-seeds .....................8

2.4. Source of Streptomyces sp..........................9

2.5. Preparation of liquid media ........................9
2.5.1. Inoculation of media ......................................................... 10

2.5.2. Filtration, centrifugation, and extraction of the inoculated media................................................................. 10

2.6. Treatment of conditioned Striga seeds with Streptomyces extracts. 11

2.7. Stimulation of Striga-seed germination with GR24 .................. 12

Chapter three

3. Results and Discussion ....................................................... 13

3.1. Viability of Striga seeds .................................................... 13

3.2. Effects of Streptomyces extracts on Striga-seed germination.................. 13

Chapter four

4. References ........................................................................... 17
# List of plates

<table>
<thead>
<tr>
<th>plate</th>
<th>topic</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plate1</td>
<td>Extraction and separation of inoculated PSB media</td>
<td>10-11</td>
</tr>
<tr>
<td>Plate2</td>
<td>Germination of control <em>Striga</em>-seeds</td>
<td>14</td>
</tr>
</tbody>
</table>

# List of Figures

<table>
<thead>
<tr>
<th>figure</th>
<th>topic</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Effect of 4 weeks <em>Streptomyces</em> extracts on <em>Striga</em>-seed germination (2011)</td>
<td>15</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Effect of <em>Streptomyces</em> extracts from different media on <em>Striga</em>-seed germination (2012)</td>
<td>16</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Effect of <em>Streptomyces</em> extracts on <em>Striga</em>-seed germination (2013)</td>
<td>16</td>
</tr>
</tbody>
</table>
Abstract

This study was initiated as part of a proposed project in the Botany department to screen potential of Pseudomonas spp. and Streptomyces spp. and their secondary metabolites to inhibit seed-germination of the parasitic weeds Orobanche spp. and Striga spp.

This study focused on the consistency of inhibitory effect of metabolite of Streptomyces on in vitro S. hermonthica seed germination which has been pointed out in similar studies two years ago (2011, 2012). For this purpose, two different isolates of Streptomyces spp. from Ashymalia and Marawi were used. The two isolates were grown in Peptone Succrose Broth (PSB) and incubated at (37°C) for four weeks. Ethyl Acetate (EtOAc) extracts of the metabolites were used in the Bioassay on Striga-seeds. In the bioassay on Striga seeds using the Streptomyces extracts, the control treatment, which included EtOAc, and EtOAc-extracts of non-inoculated PSB, showed Striga-seed germination of 50% and 31%. EtOAc extracts of Marawi and Al Shamalya Streptomyces isolates caused 1% and 0.0% Striga-seed germination. That means such inhibitory effect on Striga-seed germination is coming from metabolites produced by the bacteria in the broth media. These results are highly, and significantly consistent with the previous findings. Thus, in vitro inhibition of Striga-seed germination using metabolites of indigenous Streptomyces spp. have been consistent and successful for consecutive three years (2011-2013). This finding encourages transmission to the next phase which includes Glass-house experiments.
المستخلص

أعدت هذه الدراسة كجزء من مشروع متخرج في قسم علوم النبات لقياس القدرة الكامنة للبكتريا Streptomyces spp. و Pseudomonas spp. و Striga spp. و Orobanche spp. إنتاج بذور النباتات القاتلة للأجناس. ركزت هذه الدراسة على ثبات تأثير منتجات الأيض للبكتريا Streptomyces على نبات Streptanthina الذي تم إشتهيه في دراسات مشابهة في العامين الماضين 2012-2011. استعملت عزلتين من البكتريا Streptomyces والأخري من مروي يتم تزويج البكتريا في وسط غذائي سائل واحده فقط هو وتم تجربتها بدون تحريك عند درجة حرارة (37°C) لمدة أربعة أسابيع. استخدم Broth (PSB) درجات استباتي (EtoAc) لمنتجات الأيض للبكتريا لمعالجة بذور البوذا في تجربة معالجة بذور البوذا، المعاملات الحاكمة، و التي اشتملت على مستخلص الأليل أستباتي (EtoAc) لمنتجات الأيض للبكتريا، الترالي. مستخلصات الاستباتي أظهرت بذور البوذا بنسبة 5% و 31% على التوالي. مستخلصات الاستباتي أظهرت المنتجات الأيضية للبكتريا الشمالي لثبات بذور البوذا بنسبة 1% و 0% على التوالي. هذا يعني أن التأثير المثبط لأشكال بذور البوذا يأتي من منتجات الأيض البكتريا المفرزة في الوسط الغذائي. هذه النتائج لها درجة عالية من الثبات مقارنة بالتجارب في العامين السابقين. وهذا يوضح أن التأثير المثبط المكمل لبذور البوذا بإستخدام منتجات الأيض البكتريا كان ثابتا و ناجحا لمدة ثلاث سنوات متتالية (2013-2011) و يشجع هذا على الانتقال للمرحلة القادمة التي تنضم إلى أسباب الزيادة.
1. Introduction and Literature Review

1.1 *Striga* spp.

*Striga* spp. (Witchweed), recently placed in the Orobanchaceae (Olmstead *et al.*, 2001), are endemic obligate root parasitic weeds on the staple food of the poor in sub-Saharan Africa. Among them, three *Striga* species cause the greatest damage to important crops in Africa: *Striga asiatica* (L.) Kuntze and *Striga hermonthica* (Del.) Benth parasitize cereals such as sorghum (*Sorghum bicolor* (L.) Moench), maize (*Zea mays* L.), pearl millet (*Pennisetum glaucum* (L.) R.Br), upland rice (*Oryza sativa* L.), sugar cane (*Saccharum officinarum* L.) while *Striga gesnerioides* (Willd.) vatke parasitizes legumes such as cowpea (*Vigna unguiculata* (Walp.)), tobacco (*Nicotiana tabacum* L.) and sweet potato (*Ipomea batatas* (L.) Lam.) (Sauerborn, 1991a; Elzein and Kroschel, 2004b; Ejeta, 2007). As obligate parasite, *Striga* is dependant on its host and therefore modulates development to correspond with its host life cycle. For example, *Striga* seeds have specific dormancy and environmental conditioning requirements that must be met before they germinate. Germination of *Striga* proceeds in response to various chemicals exuded by host plants. Differentiation of radicle cells into haustorium is also cued by host rhizosphere chemistry. Both germination and haustorial initiation need to occur very near host roots for parasitic attachment. Post-attachment haustorial development allows the parasite to establish vital vascular connections as well as metabolic and
osmotic linkage with the host plant. Finally, the *Striga* matures and produces numerous seeds completing the life cycle (Rich and Ejeta, 2007).

The problem is further compounded by the replacement of the local low yielding *Striga* tolerant or resistant varietics by the high yielding *Striga* susceptible ones. In the moist savannah of West Africa *Striga* has become a major problem due to replacement of the tolerant local maize varieties and the resistant pearl millet by susceptible high yielding maize cultivars (Parker and Riches, 1993).

The occurrence of economically important *Striga* species is reported from 59 countries especially in East and West Africa as well as Asia (Sauerborn, 1991b). The Food and Agricultural Organization (FAO) of the United Nations estimates that, across the continent, *Striga* causes annual losses in excess of US $7 billion, adversely affecting over 300 million people (http://www.fao.org/wairdocs/tac/x5749e/5749-Oc.htm#1.2.%20cereals).

The grain area in Africa, actually, infested by *Striga*, is estimated to be about 21 million ha. The overall loss in grain production amounts to 4.1 million tons. Grain production in Africa might be endangered, potentially, on 44 million ha (Sauerborn, 1991a). Losses in grain yield due to *Striga* infestation vary from 5 to 75% according to the crop, the climatic condition, infestation level and the nature of soil (Salle et al., 1987). In countries such as Ethiopia and Sudan, losses of 65 – 100% are common in heavily infested fields (Ejeta et al., 1993). Severe infestation of sorghum fields leads smallholders to switch to lesser economic crops such as millet or even abandon the land when infestation is too heavy (Salle et al., 1987). The farmers may migrate from their location to other locations (Obilana and Ramaiah, 1992).
Striga hermonthica has probably been the first species to shift from native vegetations to crops, as early as the beginning of agriculture (Raynal-Roques, 1996). In Sudan, S. hermonthica occurs throughout the country and is expected anywhere millet or sorghum is grown (Musselman, 1984). Two physiological strains of S. hermonthica have been recognized in the Sudan. One occurs in the heavy clay soils and is virulent on sorghum, while the other is found mainly in the sandy soils of Kordofan in western Sudan and is virulent on millet only (Wilson-Jones, 1959; Omer and Nafie, 1984).

Limited work has been done with bacteria. Azospirillum brasilense, isolated from a sorghum field in Mali, significantly inhibited in vitro germination of S. hermonthica (Bouillant et al., 1996). One strain also exhibited a plant growth promoting effect, suggesting that under field conditions these bacteria might reduce parasite infestation and enhance crop growth. Oswald et al. (2000) found that inoculation of maize seedlings (Kenyan landrace "local white") with plant growth promoting rhizobacteria, significantly reduced Striga germination. In contrast, this effect was not observed with the commercial maize hybrid (Pioneer 3152), suggesting that the Kenyan plant growth promoting rhizobacteria were specific for the local maize variety. This makes it necessary to have indigenous biocontrol agents for Striga that suit the native crop varieties and the local environmental conditions. A laboratory test was developed to screen Pseudomonas syringae pv. glycinea, an ethylene-producing bacteria, for its ability to promote suicidal germination of Striga seeds. The bacterium was found to be highly effective in promoting seed germination in Striga spp. providing practical mean of biological control of the parasite (Berner et al., 1999). In addition to this, saprophytic soil-borne fluorescent Pseudomonas spp. significantly inhibited germination of S. hermonthica in an in vitro
investigation (Ahonsi et al., 2002). Bacillus subtilis and Pantoaea agglomerans (Beijerinck) Gavini were consistently isolated from diseased S. hermonthica plants (Abbasher, 1994). In the Sudan P. agglomerans caused 60% suppression of S. hermonthica seeds whereas Pseudomonas aeruginosa (Schrotoeter) Migula was less effective and caused 10% inhibition of germination (Idris, 1997). The phytotoxic potential of secondary metabolites produced by these bacteria is, however, not investigated to any extent.

1.2. Streptomyces spp.

Streptomyces is the largest genus of Actinobacteria. Over 500 species have been described. As with the other Actinobacteria, streptomyces are gram-positive, and have genomes with high GC- content (in microbiology, GC content is the percentage of nitrogenous bases on a DNA molecule which are either guanine or cytosine). The name Streptomyces mean (Twisted fungus). They are found predominantly in soil and decaying vegetation. Most Streptomyces produce spores, and are noted for their distinct "earthy" odor which results from production of a volatile metabolite, geosmin. Streptomyces are infrequent pathogens, though infections in human such as mycetoma can be caused by S. somaliensis and S. sudanensis, and in plants can be caused by S. caviscabies and S. scabies.

Streptomyces is the largest antibiotic-producing genus in the microbial world discovered so far; of the nine thousand antibiotics used against bacteria and fungi, 66% are produced by members of Streptomyces (Korn-Wendisch and Kutzner; 1992). Fungi produce a large number, contributing approximately 18% of the antibiotic producers and yielding about 8% of the total (Champness, 2000). Reports have shown that this group of
microorganisms will remain an important source of antibiotics (Wattve et al., 2001). Generally, new bioactive products from microbes continue to be discovered at an amazing rate: 500 per year (Dworkin et al., 2006). Among the different types of drugs prevailing in the market, antifungal antibiotics are very few but significant and have an important role in the control of mycotic plant and animal diseases (Dhanasekaran et al., 2008). The search for new, safer, broad-spectrum antifungal agents with greater potency has been progressing. The reason for this is that when compared to antibacterial, fungi, like plant cells, are eukaryotes and therefore agents that inhibit protein, RNA or DNA biosynthesis in fungi have greater potential for toxicity on plant as well (Georgopapadakou and Tkacz, 1994). Continuous screening of Streptomyces for secondary metabolites production can possibly reveal novel compounds that can be used as antifungals or herbicides.

1.3 Phytotoxins Production by Streptomyces spp.

Herbicidal activities of the compounds secreted by bacteria come into the limelight with the discovery of bialaphos (glufosinate) from Streptomyces hygroscopicus and S. viridochromogenes in 1973 (Ogawa et al., 1973). It is the first fermentation product to achieve the status of a commercial herbicide. Glufosinate is the only commercial herbicide that targets glutamine synthetase (GS). It is also a selective fungicide for Botrytis cinerea and Rhizoctonia solani. Streptomyces also produces phenazine type antibiotic active agent against certain fungal plant pathogens and also inhibit seed-germination of some crops at certain doses (Slininger et al., 1996; Khan et al., 2007).
There is currently some question whether phytotoxins produced in culture and applied alone as a bioherbicide are as effective in controlling weeds compared to application of the intact organism. (Durbin, 1983) pointed out that some bacterial pathogens are unable to produce phytotoxins in culture but only produce them in planta. Therefore, a complete understanding of the conditions required for optimum and effective phytotoxin production is necessary. Work with rhizobacteria on leafy spurge illustrates that some strains are more effective in causing plant injury when intact, whole cells are used as inoculum compared to cell free culture filtrate containing phytotoxic metabolites (Souissi, 1994; Souissi and Kremer, 1994). Investigations are needed to develop methods to maximize phytotoxin production both in vitro and in planta. Determination of phytotoxin structure would indicate the type of precursor compounds useful for enhancing phytotoxin production in culture media and/or inoculum carriers added to soil.

**Objective of the study**

**Background**

In a previous dissertation (2011), metabolites of a *Streptomyces* sp. isolated from Al Shamalya State was found to have the highest inhibitory effect on *in vitro* *Striga*-seed germination (100%) among four other *Streptomyces* spp. isolated from different areas in Sudan (Fig. 1). In the next year (2012) media enhance production of metabolites produced by Al Shamalya *Streptomyces* isolate with the highest inhibitory effect on *in vitro* *Striga*-seed germination were selected. These media included Starch Casein Broth (CKB), Peptone Succrose Broth (PSB), and King's B Broth (KMB) (Fig. 2)
Objective of the current study (2013)

The objective of this study was to investigate consistency of inhibitory effect of metabolites produced by selected two isolates of *Streptomyces* on *Striga hermonthica* seed-germination compared with the previous findings of 2011, 2012.
2. Materials and Method

2.1. Source of Striga seeds

Striga seeds were collected from, Weed Section of the Gezira Research Station, Central Sudan (Wad Madani), season (2004/2005).

2.2. Viability test for Striga-seeds

The tetrazolium red test is used to detect the presence of a hydrogenase enzyme, which indicates the Striga seed is alive (Eplee and Norris, 1987). Tetrazolium chloride solution was prepared. About (200-250) Striga seeds were placed in sterile Petri dish covered with tetrazolium solution and incubated in the dark at 37°C for 48 hrs. After the end of incubation period, tetrazolium solution was poured through a funnel lined with a 9 cm filter paper. The seeds were then examined under the light microscope and viability percentage was recorded.

2.3. Conditioning of Striga-seeds

In this experiment a modification of the method described by (parker et al. 1977) was used. Striga seeds were surface sterilized using (1%) Clorox, thoroughly washed three times with sterile distilled water before drying on a sterile filter paper. For preconditioning, Striga seeds were sprinkled on moist glass fiber filter paper disc (9 mm diam.) located in a lid of a 9 cm sterile Petri-dishes lined with two layers of moist filter papers. The entire petri-dish
was then wetted with more sterile distilled water to sufficiently moisten the *Striga* seeds. The base of each petri-dish was used to cover the lid containing the discs and then the petri-dish was covered with aluminium foil and incubated horizontally in the dark at 30°C for 10 days.

2.4. Source of *Streptomyces* sp.

In a previous study five different isolates of *Streptomyces* sp. (from Al Shamalya state, Marawi dam district, Red Sea area, Al Gadari, and Al Damazin) were used in a similar study. Ethyl Acetate extracts of the isolate from Al Shamalya, and of the isolate from Marawi dam district proved to be highly inhibitory to *in vitro Striga*-seed germination (100% inhibition) (Fig. 2). Therefore this two isolate were selected to be used in the current study. Al Shamalya *Streptomyces* isolate was kindly provided by Dr. Nazik Hamad, whereas Marawi *Streptomyces* isolate was kindly provided by Dr. Marmar Abdel Rahman in 2011, both from Botany Department, University of Khartoum. The two *Streptomyces* isolates were sub-cultured on Peptone Sucrose Agar.

2.5. Preparation of liquid media

Peptone Sucrose Broth (PSB) (Peptone 20 g, Sucrose 30 g, and Distilled water 1 L) was used in this since it proved in a previous study to be highly stimulatory to production of metabolites inhibitory to *In Vitro Striga*-seed germination and also easy to prepare. The ingredients of the media were incorporated together in 1 L distilled water, boiled in water bath until completely dissolved and autoclaved at (151 bs) pressure (121°C) for (15
minute). After being cooled the sterile broth media were aseptically poured into 250 ml flasks (2 flasks / isolate).

2.5.1. Inoculation of media

Five 250 ml flasks of the same media were inoculated under sterilized conditions with the two isolates growing on Peptone Sucrose Agar medium. The inoculated flasks were then incubated under stationary conditions at (37°C) for four weeks.

2.5.2. Filtration, centrifugation, and extraction of the inoculated media

At the end of incubation period, the broth cultures were centrifuged and then filtered using Wattman filter paper no 1. to exclude the bacterial cells. The filtrates were then extracted for two hour at room temperature using 50 ml ethyl acetate (EtOAc) by mean of a magnetic stirrer. After extraction, the upper layer of the EtOAc containing the metabolites was separated from the liquid broth using a separating funnel. The organic extracts were then concentrated into 1ml after let it about two days at room temperature to evaporate the used EtOAc.

![Magnetic stirrer](image)

**a: Magnetic stirrer**
2.6. Treatment of conditioned *Striga* seeds with *Streptomyces* extracts

Forty μl of each of *Streptomyces* extracts were applied onto 4 discs each 9mm diameter and were left to dry for 1 h aseptically at room temperature. Discs treated with sterile distilled water and EtOAc were considered as controls. A disc containing preconditioned *Striga* seeds was placed on top of each treated disc and moistened with 10 μl of Sterile distilled water and incubated at 30°C for 48 h to allow diffusion of the extracts into the discs containing the conditioned *Striga* seeds.
2.7. Stimulation of *Striga*-seed germination with GR24

GR24, which is a synthetic growth regulator, was used instead of sorghum root exudates. GR24 was kindly provided by Dr. Rasha Ali, Weed Section, Gezira Research Station, Wad Madani (Central Sudan).

The discs containing the wetted *Striga* seeds were placed in a sterilized Petri dish lined with double layer filter papers (9 cm diameter) which was wetted with sterile distilled water. After that GR24 was added to the discs and incubated at room temperature for 48 h and examined for germination under the Microscope (10x).
3. Results and Discussion

3.1. Viability of *Striga* seeds

More than 60% of the tested *Striga* seeds showed strong red pigmentation under 10x of the compound microscope and thus considered viable. The relatively low percentage of *Striga*-seed viability might be attributed to the long period since the seed were collection (2004/2005). During this period the *Striga*-seeds experienced different storage conditions especially temperature and moisture which might have decreased its germination percentage.

3.2. Effects of *Streptomyces* extracts on *Striga*-seed germination

In the bioassay on *Striga* seeds using the *Streptomyces* extracts, the control treatment, which included EtOAc, and EtOAc extracts on non-inoculted PSB, showed *Striga*-seed germination of 50% and 31% respectively (table 1; figure 3). EtOAc extracts of Marawi and Al Shamalya *Streptomyces* isolates caused 1% and 0.0% *Striga*-seed germination. Thus, the two isolates showed almost complete inhibition of *Striga*-seed germination compared with the results obtained with the controls. That means such inhibitory effect on *Striga*-seed germination is coming from metabolites produced by the bacteria in the broth media. These results are highly, and significantly consistent with the previous findings of the dissertations of 2011 and 2012 (Fig. 1 and Fig. 2). Thus, *in vitro* inhibition of *Striga*-seed germination using metabolites of indigenous *Streptomyces* spp. have been consistent and
successful for consecutive three years. This finding encourages transmission to the next phase which includes Glass-house experiments.

Besides being able to produce various types of antibacterial and antifungal metabolites, *Streptomyces* spp. are also capable of producing compounds having phytotoxic properties, the best example is the commercially used herbicide bialaphos (glufosinate) from *Streptomyces hygroscopicus* and *S. viridochromogenes* (Ogawa *et al.*, 1973). *Streptomyces* also produces phenazine type antibiotic active agent against certain fungal plant pathogens and also inhibit seed-germination of some crops at certain doses (Slininger *et al.*, 1996; Khan *et al.*, 2007). The above reports support the findings of this study of *Streptomyces* extracts being able to inhibit *in vitro* *Striga*-seed germination. However, future detailed investigations are needed to confirm these finding.

**Plate 2: Germination of control Striga-seeds**
Table 1: Effect of *Streptomyces* extracts on *Striga* seed germination

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Germination %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont. (1)</td>
<td>50 %</td>
</tr>
<tr>
<td>Cont. (2)</td>
<td>31 %</td>
</tr>
<tr>
<td>Extr. A</td>
<td>1 %</td>
</tr>
<tr>
<td>Extr. B</td>
<td>0.0 %</td>
</tr>
</tbody>
</table>

Extr. A = from Marawi *Streptomyces* isolate., Extr. B = from Al Shamalya *Streptomyces* isolate, Cont. (1) = EtOAc, Cont. (2) = EtOAc extract of non-inoculated PSB

![Graph showing germination percentages for different treatments.]

Figure 1: Effect of 4 weeks *Streptomyces* extracts on *Striga*-seed germination (2011)
Figure 2: Effect of *Streptomyces* extracts from different media on *Striga*-seed germination (2012)

1 = cont.1 (EtOAc); 2 = cont.2 (non-inoc. PSB); 3 = extr. of Marawi isolate; 4 = extr. of Al Shymalia isolate

Figure 3: Effect of *Streptomyces* extracts on *Striga*-seed germination (2013)
4. References


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Fao, http://www.fao.org/wairdocs/fac/x5749e/5749-Oc.htm#1.2.%20cereals


