Effect of crude Streptomyces metabolites from different media on *in vitro* *Striga*-seeds germination

A dissertation Submitted in Partial Fulfillments of the Requirements for BSc- (Honours) in Botany

By

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DEDICATION...

TO MY WONDERFUL GREAT MOTHER.
TO THE SOLE OF MY GREAT FATHER...
TO ALL MY FRIENDS..
TO EVERY ONE WHO HELP ME TO FINISH
THIS WORK...
Acknowledgements

First my great thanks to my god. I would like to express my sincere thanks to my research supervisor Dr. R.I. Mohukker for his over guidance, assistance and supervision throughout period of this study. I would feel this acknowledgement incomplete without thanking my family for their constant encouragement and support over. Thanks are due to my friends and any persons helped me to accomplish this work.

Thank you all!
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 with 25% dilute to inhibit seed-germination of the parasitic weeds *Orobanche* spp. and *Striga* spp. This study focused on the Effect of *Streptomyces* metabolites on *in vitro* germination of *S. hermonthica* seeds.

One *Streptomyces* isolates were used in this study. The isolates were grown in four types of broth media: Starch NB –CKB –KMB –and PSB and incubated under stationary conditions at (29°C) for four weeks and we use typically four broth media was used as control after that we diluted all the eight. In the bioassay of *Striga* seeds, almost all filters can not cause big inhibition of *Striga*-seed germination and the best media caused inhibition was CKB and the best media incubated with streptomycyes was NB . Treatment with the controls *striga* seed germination for 2day and 4 days resulted in 55% and 93% of incubate *Striga*-seed with sorghum exudates , respectively. *Streptomyces* spp. are reported to produce compounds having phytotoxic properties, the best example is the commercially used herbicide bialaphos (glufosinate) from *Streptomyces hygroscopicus* and *S. viridochromogenese*. 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 findings.
مستخلص الأطروحة

أنتجت هذه الدراسة كجزء من مشروع مُقترح في قسم النبات لتقصي القدرة الكامنة للبكتيريا من منتجات أيضاً ثانوية بتركيز مخفض إلى 25% Streptomyces spp. على تثبيط إناث بذور النباتات المطلقة التابعة للأجناس Striga spp. ركزت هذه الدراسة على تأثير منتجات الأيض البكتيريا S. hermonthica على الإناث المحمل لبذور حشيشة البودا S. hermonthica المنطلقة.

استعملت عزلة واحدة من البكتيريا في هذه الدراسة. تم تزريع Streptomyces Streptomyces في أربعة أوساط غذائية كما استخدمت نفس الأوساط الغذائية الاربعية دون تزريع البكتيريا واستخدمت كنواها وتم تحضين النباتات فينوع بدون تحريك عند درجة حرارة (29°C) لمدة زمنية أربعة أسابيع. بعدها تم تخفيض تركيز كل الأوساط الغذائية الثمانية. كل التراكيز لم تسبب تثبيط كبير لإناث البودا وإن أفضل وسط غذائي يحتوي على B. subtilis A. مثبط بتور البودا مع جزيز الذرة CKB، كما ان أفضل وسط غذائي اجعد تثبيط NB النامية لفتره 40 يوم وللذكرين البودا وذئب النتائج كنوبها وكانت علي التوالي 55% و93% أوضح الدراسة إن منقحة البكتيريا تثبيط البكتيريا ثمانية لذات البودا. ان تركيز البكتيريا ليس لها تأثير واضح ألعاب مثل ذلك نتائج المستخدم TJ و S. hygroscopicus من الأنواع bialaphos (glufosinate) viridochromogenense. التقرير أعلاه يدعم نتائج هذه الدراسة حول عدم وجود تأثير كبير للبكتيريا على تثبيط إناث بذور البودا معملاً.
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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 on 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, 1991; 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).
Witch-weed (*Striga* spp.) is among the most serious causes of sorghum crop loss to small farmers in the rain-fed agriculture of the semi-arid tropics (Doggett, 1988). *Striga* may have already become the greatest biological constrain on food production in Africa, and now it becomes a serious problem more than insects, birds, or plant diseases (Ejeta *et al.*, 1993). *Striga* has increased in its importance in the recent years due to increased population pressure and the subsequent intensive cereal monocropping. The problem is further compounded by the replacement of the local low yielding *Striga* tolerant or resistant varieties 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 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, 1991 a). 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 (Sallec *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 (Sallec *et al.*, 1987). The farmers may migrate from their location to other locations (Obilana and Ramaiah, 1992).
Several control methods have been tried and adapted for the control of *Striga* spp. in Africa. However, it is obvious that there is no single method that can effectively be used for *Striga* control. The approach should basically be an integrated one aimed at reducing or depleting the seeds reserve of *Striga* and producing a healthy crop (Singh *et al.*, 1991).

Recent observations of sporadic natural *Striga* death promoted further investigations into isolation and identification of the pathogens involved (Abbasher, 1994). Intensive surveys on the occurrence of micro-organisms pathogenic to *Striga* spp. were conducted in different parts of the world. Results of the surveys revealed the presence of several fungal pathogens associated with the naturally infected *Striga* spp (Nag Raj 1966; Meister and Eplee 1971; Zomme 1977; Abbasher *et al.*, 1995; 1998; Idris, 1997; Ahmed *et al.*, 2001; Mohamed, 2002; Mohukker, 2009).

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 *Pantoea 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, streptomycetes 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 Streptomycetes 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 (Wathe *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, sager, 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 (Slahinger 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.

1.4. Objectives of the study

The objectives of this study were to:

1. Investigate the effect of crude Streptomyces metabolites from different growth media on in vitro Striga hermonthica seed-germination.

2. Select the potent medium (media) for future similar investigations.
9cm 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 25°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 Gadarif, and Al Damazin) were used in a similar study. Ethyl Acetate extracts of the isolate from Al Shamalya, kindly provided by Dr. Nazik Hamad from Botany Department, U of K (Plate 1), proved to be highly inhibitory to in vitro Striga-seed germination (100% inhibition). Therefore this isolate was solely used in the current study.

2.5. Preparation of liquid media

Four different liquid media were selected to be used in this study. These media are of common use for growth and secondary metabolites production of different bacterial genera including Streptomyces. These media included:

1- Starch Casein Broth (CKB)

- Starch 10 g, KNO₃ 2 g, K₂HPO₄ 2 g, NaCl 2 g, Casein 0.3 g, MgSO₄.7H₂O 0.05 g, CaCO₃ 0.02 g, FeSO₄.7 H₂O 0.01g, 1 L sterile distilled water.

2- Peptone Sucrose Broth (PSB)

- Peptone 20 g, Sucrose 30 g, and Distilled water 1 L.
3- King's B Broth (KMB)
   > Peptone 20 g, K$_2$HPO$_4$ 1.5 g, MgCl$_2$ 1.5 g, Glycerol (10 g) and Distilled water 1 L (7.2 ± 0.2) PH.

4- Nutrient Broth (NB)
   > Nutrient Broth 13 g, Distilled water 1 L.

The ingredients of each medium 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 (3 flasks/isolate).

2.5.1. Inoculation of media

Three 250 ml flasks of each broth medium were inoculated under sterilized conditions with Al Shamalya Streptomyces isolate growing on Starch Casein agar medium. The inoculated flasks were then incubated under stationary conditions at (29°C) for four weeks (Plate 2).

Plate 1: Al Shamalya Streptomyces isolate
Plate 2: Starch Casein, Peptone Sucrose, King’s B, Nutrient broth media inoculated with *Streptomyces* isolate

2.5.2. Filtration, centrifugation, and dilution of *Streptomyces* metabolites

At the end of incubation periods, the broth cultures were centrifuged and then filtered using Wattman filter paper no. 1 to exclude the bacterial cells. The crude filtrates were then diluted with sterilized distilled water (1:3 v/v, H₂O: Crude v/v).
2.6. Treatment of *Striga* seeds with *Streptomyces* crude metabolites

Sixty μl of each of *Streptomyces* diluted crude metabolite were applied onto 3 discs each 9mm diameter and were left to dry for 24 h aseptically at room temperature. Discs treated with sterilized distilled water were considered as controls. A disc containing preconditioned *Striga* seeds (10 to 30 per disc) 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 sorghum root exudates

Sorghum seeds were surface sterilized using (2%) Clorox, thoroughly washed with sterilized distilled water. These seeds were put in a sterilized Petri dish containing lined with double layer filter papers (9 cm diameter), wetted with sterile distilled water and incubated at room temperature for (4-6) days to allow germination. After that the discs containing the wetted *Striga* seeds were placed near the germinating sorghum roots in the Petri dishes (Plates 4, 5), incubated at (30°C) and examined for germination under the Microscope (10x) 24 h later.
Plate II: Production of sorghum root exudates

Plate III: Conditioning of *Striga*-seeds with sorghum root exudates
3. Results and Discussion

3.1. Viability of *Striga* seeds

About 40% of the tested *Striga* seeds showed strong red pigmentation under 10x of the compound microscope and thus considered viable (Plate 6). The 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.

3.2. Effects of *Streptomyces* crude metabolites on *Striga*-seed germination

In the bioassay on *Striga* seeds using the *Streptomyces* crude filtrates from the four different media, the control treatment, which included sterilized distilled water, non-inoculated NB, non-inoc. KMB, non-inoculated CKB, and non-inoc. PSB showed 93, 40, 33, 34, and 66% *Striga*-seed germination (table 1; figure 1). The percentage of germination of water control is significantly high when compared with the non-inoculated broth media controls. That means there is some ingredients in the media itself that lower *Striga*-seed germination. Most of the diluted inoculated media gave even better *Striga*-seed germination compared to their controls (e.g. inocu-CKB, inocu.-KMB, and inocu.-PSB) (table 1; figure 1). It is likely that *Streptomyces* sp. Produce two different types of metabolites in the growth media. Some of which are inhibitory to *Striga*-seed germination at certain concentrations as revealed from previous and current similar studies (Pils see dissertation of my colleague Tasneem); and some of which are stimulatory to *Striga*-seed germination as revealed in this study after the metabolites were diluted. Thus, the dilution process was the key factor in reversing the effect of
Streptomyces metabolites in the different used broth media from inhibitory to stimulatory. 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 later report supports the findings of this study, that the effect of *Streptomyces* metabolites on *in vitro Striga*-seed germination is highly dose dependant. However, future detailed investigations are needed to confirm these finding.

Plate 5 Red-pigmented *Striga*-seed treated with tetrazolium solution
Plate 7: Germination of control *Striga*-seeds

Table 1: Effect of *Streptomyces* diluted filtrates on *Striga*-seed germination

<table>
<thead>
<tr>
<th>Treatment</th>
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<tr>
<td>Cont.</td>
<td>93</td>
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<tr>
<td>Cont. 1</td>
<td>40</td>
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<tr>
<td>Cont. 2</td>
<td>33</td>
</tr>
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<td>Cont. 3</td>
<td>34</td>
</tr>
<tr>
<td>Cont. 4</td>
<td>66</td>
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<tr>
<td>Dilute 1</td>
<td>33</td>
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<td>Dilute 2</td>
<td>59</td>
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<td>Dilute 3</td>
<td>59</td>
</tr>
<tr>
<td>Dilute 4</td>
<td>68</td>
</tr>
</tbody>
</table>

Cont. = Ster. Dist. H₂O; Cont.1 = non-inoculated NB; Cont.2 = non-inoculated KMB; Cont.3 = non-inoculated CKB; Cont.4 = non-inoculated PSB
Dilute.1 from NB, Dilute.2 from KMB, Dilute.3 from CKB, Dilute.4 from PSB
Cont.1 = non-inoculated CKB; Contr.2 = non-inoculated Kbb; Cont 3 = non-inoculated PSB;
Cont 4 = non-inoculated NB
Dilute.1 from CKB, Dilute.2 from Kbb, Dilute.3 from PSB, Dilute.4 from NB

Figure 1: Effect of *Streptomyces* filtrates from different media on *Striga*-seed germination
4. References


Ejeta, G., Butler, L. and Babiker, A. G. T. 1993. New Approaches to the control of *Striga*. *Striga* Research at Purdue University, Agriculture Experiment Station, Purdue University, USA. Bulletin no. 10, 27 pp.


