

# Strigolactone analogues induce suicidal seed germination of Striga spp. in soil

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Accepted Version

Kgosi, R. L., Zwanenburg, B., Mwakaboko, A. S. and Murdoch, A. (2012) Strigolactone analogues induce suicidal seed germination of Striga spp. in soil. Weed Research, 52 (3). pp. 197-203. ISSN 0043-1737 doi: https://doi.org/10.1111/j.1365-3180.2012.00912.x Available at https://centaur.reading.ac.uk/27608/

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To link to this article DOI: http://dx.doi.org/10.1111/j.1365-3180.2012.00912.x

Publisher: Wiley-Blackwell

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1 2	Strigolactone analogues induce suicidal seed germination of Striga spp. in soil				
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14					
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16					
17	Received				
18	Revised version accepted				
19					
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21	Running head: Strigolactone analogues for suicidal germination of Striga seeds				
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# 29 Summary

30	Striga hermonthica and S. asiatica are obligate root parasites that cause serious problems in
31	the production of staple cereal crops in Africa. Because of the high levels of infestation, there is an
32	urgent need to control these weeds. An interesting option is depletion of the soil seed bank by
33	suicidal germination which involves germination of the seeds in the absence of host plants.
34	Suicidal germination is often mentioned as an interesting option, but not considered as realistic due
35	to the alleged untimely decomposition of the stimulants in the soil, despite the fact that some
36	encouraging results were reported around 1980. The alleged instability has prevented active
37	research in this direction for the past 20-25 years. Five newly designed synthetic germination
38	stimulants are investigated as candidates for suicidal germination. An important issue is the
39	persistence of these stimulants in soil. Packets with Striga seeds were put in pots with soil and then
40	treated with aqueous solutions of the stimulants. All five compounds induced germination under
41	these soil conditions. There were no noticeable signs of disturbing decomposition of the stimulants.
42	The best performing stimulant is derived from 1-tetralone. The conclusion is that synthetic SL
43	analogues have excellent prospects for use in the field in combating parasitic weeds.
44 45	<b>Keywords</b> : pot experiment, germination stimulants, <i>Striga hermonthica</i> , <i>Striga asiatica</i> ,
46	strigolactone analogues, suicidal germination
47	
48	ZWANENBURG B, MWAKABOKO A S, KGOSI R L & MURDOCH A J (2012). Strigolactone analogues induce
49	suicidal seed germination of Striga spp. in soil. Weed Research
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#### 53 Introduction

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55 Striga, Orobanche and Phelipanche spp. are among the most damaging parasitic weeds in the 56 world. Striga spp. cause severe loss of important staple food crop (cereal) production, mostly in 57 Africa, Middle East and India while *Orobanche* and *Phelipanche* spp. have their greatest impact on 58 vegetables and legumes. According to a recent review (Parker, 2009) there is a growing concern on 59 the deleterious effects that these weeds have on the livelihood of people living in developing 60 countries because of the high levels of infestation. Over the years several reports (e.g. Parker 1994 61 and 2009; Rubiales *et al.*, 2003; Ejeta, 2007) indicate that these parasitic weeds pose a large-scale 62 problem that requires an urgent attention. For example, the infestations by these weeds are 63 estimated to cover about two thirds of the arable land in Africa.

64 The fact that these weeds threaten the subsistence of millions of people by causing serious devastation of key agricultural produce, justifies the efforts in attempting to control them. 65 66 Interestingly, the biology of these three genera of parasitic weeds is closely related which suggests 67 the use of similar control strategies. It has been proposed that the important means of controlling 68 root holo- and hemi-parasitic weeds should focus on the seed cycle: reducing soil seed-bank, 69 preventing seed set and inhibiting seed movement from infested to non-infested areas (Rubiales et 70 al., 2009). To date, there is no single effective method of controlling these weeds partly due to 71 their complex life cycle, their vascular connection to the host, the production of many tiny, long-72 living seeds (Joel et al., 1995; Rubiales et al., 2009a) and because, unlike most weeds, they 73 damage the host inconspicuously, while still underground/subterranean.

74 There are several non-chemical control methods for parasitic weeds, most of which only 75 achieve partial control when employed alone (Rubiales et al., 2009b). Induction of suicidal 76 germination is an attractive approach to reducing the soil seed-bank. It involves the introduction of 77 an appropriate natural or synthetic germination stimulant into the soil in the absence of a suitable 78 host, leading to both seed bank depletion and death of the weed seedlings because of their 79 complete dependence on the host for water and nutrition. Naturally occurring germination 80 stimulants are exuded by the roots of the host plants. Typical examples are strigol, orobanchol and 81 sorgolactone, but recently several more, structurally related stimulants, collectively called 82 strigolactones (SLs), have been isolated (Yoneyama et al., 2009; Yoneyama et al., 2010; Xie et al., 83 2010). The natural stimulants have a too complex structure to synthesize them on a multigram 84 scale (Sugimoto *et al.*, 1998; Reizelman *et al.*, 2000) and as a consequence they are not suitable 85 candidates for the use in the suicidal germination approach to eradicate parasitic weeds. Several SL 86 analogues with a simpler structure than the natural SLs, but with retention of germination activity

87 have been prepared (Johnson et al., 1981; Hassanali, 1984; Mangnus et al., 1992b; Mangnus et al., 88 1992c; Nefkens et al., 1997; Thuring et al., 1997; Mwakaboko & Zwanenburg, 2011a; 89 Mwakaboko & Zwanenburg, 2011b; Zwanenburg et al., 2009). The GR compounds constitute the 90 first series of such SL analogues with high germination activity (Johnson et al., 1981). The most 91 well known are GR 24 and GR 7 (structures are given in Fig. 1). The first mention of suicidal 92 germination dates from 1976 (Johnson et al., 1976). The experiments were carried out in boxes of 93 50 x 80 x 15 cm to simulate field conditions. The results were encouraging as a considerable 94 number of seeds of Striga asiatica germinated by applying GR 7 at low concentrations (10 mg/L 95 which equals to 750 g/ha and 1 mg/L corresponding with 75 g/ha). Field applications with GR 7 96 were simulated using plastic cups filled with soil. Using 330 g of GR 7 per ha gave good control of 97 Orobanche crenata in faba beans grown in acidic soils, whereas 1500 g/ha was required to control 98 O. ramosa (syn. P. ramosa) in alkaline soils (Saghir, 1986). The stability of the stimulant in soil is 99 clearly an important factor. At pH  $\leq$ 7.5 the half-life of GR 7 is ca 100 h, while at alkaline pH the 100 stability rapidly decreases (Johnson et al., 1976). It was also shown (Babiker & Hamdoun, 1982; 101 Babiker et al., 1987) that the germination of S. hermonthica (Del.) Benth. in response to GR 7 and 102 GR 24 was strongly influenced by soil moisture. Excessive soil moisture (±70 %, w/w) resulted in 103 a low response to GR 24. Transfer of the seeds to soil with a lower moisture content (47 %), 104 improved the response. GR 7 and an extract of *Euphorbia aegyptiaca* Boiss containing natural 105 germination stimulants were investigated using soils of varying types, collected from five different 106 locations in Sudan. Adequate persistence (6-8 days) of GR 24 was observed in acidic soils (pH 5.0-107 6.3), whereas it was short (1-3 days) in alkaline soils (Babiker *et al.*, 1988).

108 The experiments using GR compounds to reduce seed banks were discontinued, probably 109 because of the stability problems especially in alkaline soil which is common in broomrape 110 infested fields, but probably also their commercial availability played a role (Eplee & Norris, 1987; 111 Parker & Riches, 1993). It was generally assumed that the instability of synthetic SL analogues, 112 like GR 7 and GR 24, may be an intrinsic characteristic of these stimulants (Parker & Riches, 113 1993), but details were not given. However, it is known that SLs are inherently susceptible to 114 hydrolysis due to the presence of an enol ether unit conjugated with an ester (Mangnus & 115 Zwanenburg, 1992). The hydrolysis proceeds by an initial nucleophilic addition of water to this 116 enone moiety, followed by an elimination of the D-ring (Mangnus & Zwanenburg, 1992). The rate 117 of hydrolysis is strongly dependent of the SL structure and the experimental conditions. Under 118 neutral conditions the half-life for the hydrolysis of GR 24 was estimated to be 10 days and for 5-119 deoxy-strigol 1.5 days (Akiyama *et al.*, 2010). Although the suicidal germination approach using

SL analogues was frequently mentioned as a potential option for parasitic weed control, it was
considered not realistic for many years, mainly because of the alleged instability of the stimulants.
Anyhow, there are no reports on attempts using SL analogues in controlling parasitic weeds since
the mid 1980s.

124 In spite of the negative prospects (Eplee & Norris, 1987; Parker & Riches, 1993) of using 125 SL analogues in the field, we decided to examine the efficacy of some newly synthesized SL 126 analogues 1-5 (Figure 1) under soil conditions, and to test whether the germination activities were 127 different from those deduced from simple in vitro assays. An important issue is whether the 128 presence of soil allows an effective contact of the stimulant solution with the seeds to initiate 129 germination. In this context adsorption of stimulants to the soil is serious concern. The SL 130 analogues 1-5 were taken from a series of new highly active germination stimulants for the seeds 131 of Striga, Orobanche and Phelipanche spp. that were designed and synthesized (Nefkens et al., 132 1997; Thuring et al., 1997; Mwakaboko & Zwanenburg, 2011 a and b) employing a tentative 133 molecular mechanism for the mode of action of the stimulants (Mangnus et al., 1992). The 134 ultimate objective of the search for highly active stimulating agents is to apply them in the field in 135 order to deplete the soil seed bank of the parasitic weeds, to reduce the damage these weeds cause 136 to many important food crops and hence to improve the food supply in the countries currently 137 affected by them. In this paper, the results of some pot experiments with Striga seeds using 138 synthetic stimulants 1-5 which differ in structure from the known GR analogues are reported. 139



- 140 141
- 142 Figure 1: Structures of SL analogues
- 143

#### 144 Materials and Methods

## 145 SL analogues

146 Analogues **1** and **2** were prepared as described by Mwakaboko & Zwanenburg (2011b), analogue

147 **3** as described by Mwakaboko & Zwanenburg (2011a), compound **4** (Nijmegen-1) as described by

148 Nefkens *et al.* (1997) and Nijmegen-1Me (5) as described by Thuring *et al.* (1997).

149 Seeds

150 Seeds of three *Striga* species were used in the study: *S. hermonthica* (Sudan 1992, *ex* sorghum), *S.* 

- 151 *asiatica* (Tanzania 1997, *ex* sorghum) and S. *asiatica* (Malawi 1993, *ex* sorghum); all seeds had
- 152 been stored at room temperature.
- 153

## 154 In vitro germination assays

155 All three seed species were subjected to an *in vitro* bio-assay protocol similar to that used 156 previously (Mangnus et al., 1992). All test compounds induced germination after standard conditioning. Seven concentrations were used:  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$ ,  $10^{-8}$ ,  $10^{-9}$ ,  $10^{-10}$  mol/L. The 157 158 assays were carried out in duplicate. In some cases there was also germination observed during the 159 water controls in these in vitro assays. This was ascribed to the enclosed environment of the tests 160 and to the fact that the Petri dishes were stacked which could have led to cross contamination 161 between treatments. Sometimes bio-assays are disturbed by a low concentration of ethene  $(C_2H_4)$ , 162 which also acts as a germination stimulant, in the air, thus leading to erroneous results. It is also 163 possible that the pre-treatment of the seeds was inappropriate; traces of sterilizing agents may be 164 disturbing the assays. Normally, no germination occurred in the water controls, thus the observed 165 positive blanks must be artefacts and accordingly these assays were disregarded.

166

#### 167 Details of the pot experiments

168 Two of the three seed species were used in the pot experiments, namely those that responded best 169 in the vitro assays. Seeds of S. hermonthica (Sudan) and S. asiatica (Tanzania) with a minimum of 170 100 seeds per experiment were placed in a 3 x 5 cm packet made of 80 micron precision mesh and 171 the open end secured with a stapler. Each packet was placed in a one litre pot (10 x 10 x 10 cm) 172 half filled with a 1:1 mixture of sand and steam sterilized loam of neutral pH, then covered with 5 173 cm of the soil mix (Babiker et al., 1987), watered and kept moist during a 14 day conditioning 174 period. After this period the seed packets were removed from the conditioning pots and placed into 175 similar sized pots half-filled with the same type of soil mixture, covered with 5 cm of the mix and 176 then treated with 200 mL of solutions of varying concentrations of each of the stimulants 1-5. A 177 formulated version of Nijmegen-1 (4), which is designated as NE 1, was also included in these

tests. In this formulation, NE 1 is contained in an emulsion (Zwanenburg *et al.*, 2009). Control pots
were similarly set up using only water (200 mL). A crop treatment was also included in the assays.
Sorghum (variety Segaolane) and pearl millet (variety Serere 6A) seeds, four of each were planted
in pots and 3 days after emergence, the seedlings were thinned to one per pot.

182 The germination experiments were conducted using four concentrations of compounds **1-5**. 183 The first three were  $10^{-6}$ ,  $10^{-5}$ , and  $10^{-4}$  M while the fourth corresponded to the maximum solubility 184 namely,  $10^{-3}$  M for **NE 1**,  $10^{-3.5}$  M for compounds **1**, **2** and **3**, and  $10^{-3.6}$  for derivatives **4** and **5**.

- The seed packets were removed from the pots for examination of germination, 7 days after chemical treatment or after 17 days in the case of the crop treatment experiments. The packets were rinsed from adhering soil particles and opened. Then seeds were placed carefully onto filter paper and counted for germinated seeds using a binocular microscope. All experiments were carried out in quadruplicate.
- The experiments were conducted in the Nematology glasshouse of the University of
  Reading, UK, at daily temperatures ranging between 21 and 39°C.
- 192
- 193 Analysis

194 Data handling and graphics were performed using Microsoft Excel and data analyses were

performed using the Genstat7 program. Percentage data were transformed to angles before analysis(Murdoch, 1982). The results can be re-interpreted using the equation:

197 Angles =  $1/\text{sine }\sqrt{p}/100$ , wherein p is the percentage of germination.

198 For the bio-assays *in vitro* the standard error of difference was 1.73 for *S. asatica* (Tanzanian

199 strain), 0.80 for *S. asiatica* (Malawi strain), and 3.02 for *S. hermonthica* (Sudan strain). The

200 germination data are not shown. For the pot experiments the s.e.d. was 2.50 for *S. hermonthica* 

201 (Sudan strain) and 3.32 for S. asiatica (Tanzanian strain). The germination percentages were

202 recalculated from the germination angle analysis and are shown in as bar diagrams in Figures 2 and

203 3.



206Figure 2: Bar graph of the percentages of germinated seeds of *S. asiatica* (Tanzania strain) after207exposure to various concentrations of 1-5 in the pot experiments. All stimulants 1-5 and208NE1 were assayed at the concentrations d, e and f + the concentration of maximum209solubility b  $(10^{-3.5} M)$  for 1, 2, and 3, c  $(10^{-3.6} M)$  for 4 and 5 and a  $(10^{-3} M)$  for NE2101. Values are mean germination percentages, experiments were carried out in211quadruplicate. The accuracy is shown at the top of each bar. NE 1= Nijmegen 1 (4)212formulated as a dispersed emulsion. The water control did not induce any germination.



Figure 3: Bar graph of the percentages of germinated seeds of *S. hermonthica* (Sudan strain) after
 exposure to various concentrations of 1-5 in the pot experiments. All stimulants 1-5 and
 NE1 were assayed at the concentrations d, e and f + the concentration of maximum
 solubility b (10<sup>-3.5</sup> M) for 1, 2, and 3, c (10<sup>-3.6</sup> M) for 4 and 5 and a (10<sup>-3</sup> M) for NE

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## 226 Results and Discussion

The *in vitro* bio-assays revealed that the responses of *S. asiatica* (Tanzanian strain) and *S. hermonthica* (Sudan) were higher than for *S. asiatica* (Malawi), the highest response of about 90 % being observed for *S. hermonthica*. For *S. asiatica* (Tanzanian strain) the response is ca 70%. The low response of the Malawian strain is most probably due to low viability, although there was also a considerable amount of trash in the seed sample, which could introduce errors in determining the extent of germination. For that reason only the two best responding seed species were used in the pot experiments.

1. Values are mean germination percentages, experiments were carried out in

quadruplicate. The accuracy is shown at the top of each bar. NE 1 = Nijmegen 1 (4)

formulated as a dispersed emulsion. The water control did not induce any germination.

Fig. 2 shows that the SL analogue **3**, that is the analogue derived from 1-tetralone, is the most active one in the pot experiments with *S. asiatica*, followed by the analogue **2** derived from hydroxy-coumarin. Both analogues perform considerably better than the Nijmegen-1 analogues **4** and **5**. The data in Fig 3 for *S. hermonthica* show that SL analogue **3** is also the best performing one, with the coumarin analogue **2** as a good second. Here the Nijmegen-1 analogues **4** and **5**, and also analogue **1**, are appreciably active (see Fig. 3).

Part of the difference of both seeds species in their response to stimulant can be explained by taking into account the difference of viability (*vide supra*). By far the best response was observed for the 1-tetralone derived stimulant **3**. For both seed species, excellent germination has been achieved at all concentrations, thus making this stimulant a superb candidate for weed control in the field. The performance of **2** with regard to *S. hermonthica* is such that it would also be a candidate for use in the field. Nijmegen-1 (**4**) shows a rather high response at higher concentrations in the case of *S. hermonthica*, while *S. asiatica* germinates only moderately at these concentrations.

A highly rewarding outcome of this study is that there are no noticeable signs of disturbing 247 248 decomposition of the SL analogues under the soil conditions employed. No serious disturbing 249 adsorption of stimulant has been observed either. The response of the germination stimulants 250 resembles that of *in vitro* experiments. These results imply that the fear that the germination agents 251 would decompose too rapidly in the soil to be effective as germination stimulant appeared not to be 252 real. This also means that an essential requirement for a successful application of SL analogues as 253 germinating agents in the suicidal germination approach has been met. It is relevant to keep in 254 mind however that in alkaline soil the hydrolysis of stimulant may be a serious factor that cannot 255 be ignored (Babiker et al., 1987 and 1988).

256 Despite the fact that the response of the two seed species is considerably different, it is worth noting that the dose-response curve has a bell shape in both cases, whereby the maximal 257 258 activity is seed species and stimulant dependent. This is in line with earlier observations in *in vitro* 259 studies, that a dose-response curve has a maximum (Wigchert et al., 1999). The optimum 260 concentration for certain seed species and a stimulant can also readily be deduced from Table1 261 wherein the maximum germination angles/percentages and the maximum germination 262 concentration are indicated. For the most responsive seed species S. hermonthica SL analogues 3 is 263 clearly performing as the best, closely followed by analogue 2, while for S. asiatica analogue 3 is the most active one. 264

265 266

**Table 1** :Maximum germination angles and percentages of S. asiatica and S. hermonthicafor SL analogues 1-5 in pot experiments

Species	SL	Maximum germination		Concentration for
	analogue	angles	percentage	maximum germination
S. hermonthica	1	77.90	$79.2  \pm 7.08 $	$10^{-3.5} \mathrm{M}$
	2	73.99	$88.0\pm\ 2.28$	$10^{-4}$ M
	3	86.40	$98.0\pm\ 2.00$	$10^{-4}$ M
	4	79.11	$91.9\pm~3.00$	$10^{-3.5} \mathrm{M}$
	5	69.04	$73.0\pm\ 4.29$	$10^{-4}$ M
	<b>NE 1</b>	63.93	$71.5\pm2.83$	$10^{-4}$ M
S. asiatica	1	43.35	$33.9 \pm 10.9$	10 <sup>-3.5</sup> M
	2	56.20	$38.5~\pm~8.55$	$10^{-3.5}$ M
	3	79.41	$81.5\pm\ 6.23$	$10^{-3.5}$ M
	4	55.49	$50.5 \pm \ 7.92$	$10^{-4}$ M
	5	40.94	$24.2\pm~3.50$	$10^{-4}$ M
	<b>NE 1</b>	39.06	$17.6\pm\ 6.19$	10 <sup>-4</sup> M

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268 Formulated Nijmegen-1 (NE 1) exhibits a considerably lower activity than an aqueous 269 solution of the same stimulant 4. It is speculated that the formulated stimulant may need an 270 adjusted watering regime in order to transport the emulsion-containing stimulant to the seed 271 packets. An aqueous solution of the stimulant allows a more even distribution. For field 272 applications it is suggested, however, that formulation of the stimulants will be desirable to slow 273 down the movement of stimulant through the soil profile and so maximise the exposure of seeds to 274 the stimulant. Soil treatment with formulated stimulants, therefore deserves attention in future 275 research (Zwanenburg et al., 2009).

276 The experiments with sorghum and millet showed a rather low germination for both Striga 277 species, considerably lower (about half) than with synthetic stimulants. It should be noted that 278 sorghum roots exude sorgolactone (Hauck et al., 1992) as the stimulant, whereas millet roots 279 produce strigol (Siame et al., 1993). The difference in germination induction of natural stimulants 280 and possibly the difference in concentration of the exudates, may account in part for the different 281 germination percentages in the presence of these host plants. In addition, the root exudates may be 282 less evenly distributed on the seed packets than with the chemical treatments or even they may not 283 reach the seeds. The experiments with the synthetic stimulants show that, when the seeds are 284 having contact with the stimulants, induction of germination can occur quite extensively.

285 The overall conclusion of the experiments described in this paper, is that synthetic SL 286 analogues have excellent prospects for combating parasitic weeds in the field. The stimulant 3 287 derived from 1-tetralone is of particular interest because of its excellent performance at different 288 concentrations for both seed species. The SL analogues persist under soil conditions and there were 289 neither noticeable signs of disturbing decomposition of the stimulants nor disturbing adsorption to 290 the soil. A cautious remark is in place however. It has been shown that there is a sizeable group of 291 parasitic weed seeds that does not respond to GR24 (Fernandez-Aparicio et al., 2011). The SL 292 analogues used in this study are possibly inactive for such parasitic weeds.

In the present study there was no significant difference between the germination induced in *in vitro* conditions and *in vivo* pot experiments, which is suggesting that *in vitro* bio-assays can be used to predict the potency of stimulants for field applications. All in all, the outcome of this study opens new avenues for parasitic weed research.

297

### 298 Acknowledgements

We are grateful to the Joint Japan/World Bank Graduate Scholarship programme for providingfunding for RK and to the Government of Botswana for granting her study leave.

301

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