

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

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

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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 **Keywords:** pot experiment, germination stimulants, *Striga hermonthica*, *Striga asiatica*,
46 strigolactone analogues, suicidal germination

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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
65 devastation of key agricultural produce, justifies the efforts in attempting to control them.
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

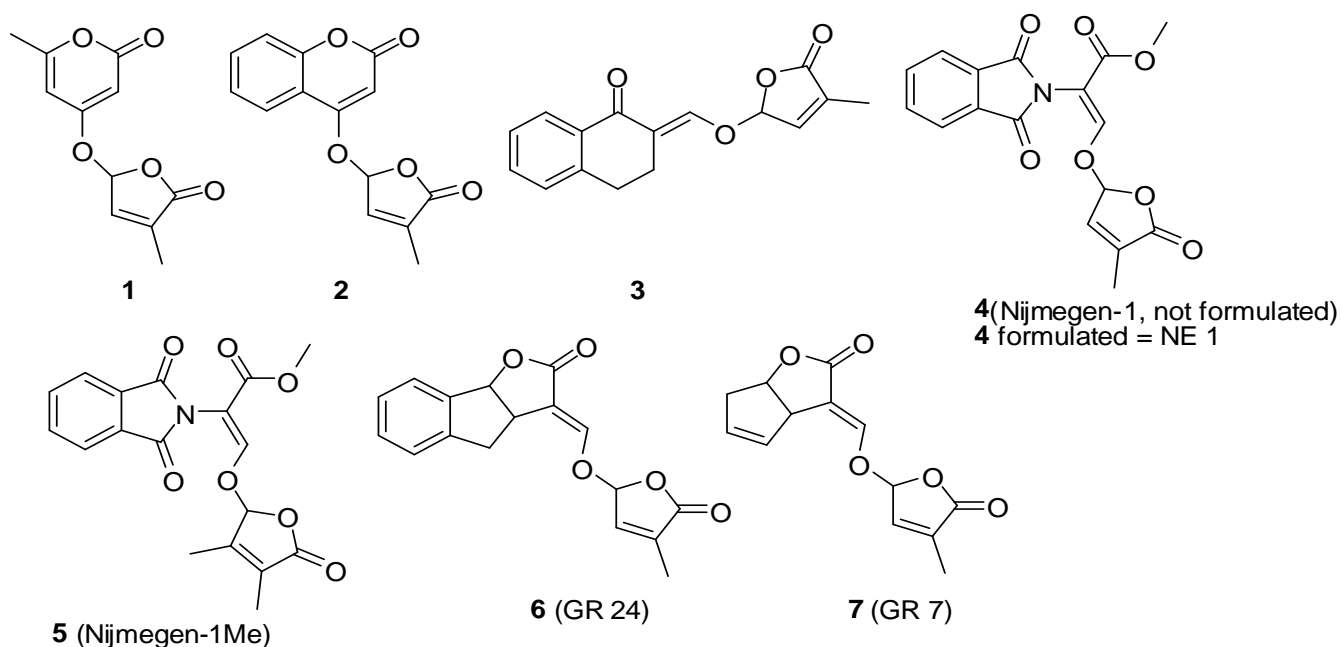
87 have been prepared (Johnson *et al.*, 1981; Hassanali, 1984; Mangnus *et al.*, 1992b; Mangnus *et al.*,
88 1992c; Nefkens *et al.*, 1997; Thuring *et al.*, 1997; Mwakaboko & Zwanenburg, 2011a;
89 Mwakaboko & Zwanenburg, 2011b; Zwanenburg *et al.*, 2009). The GR compounds constitute the
90 first series 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 ≤ 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

120 SL analogues was frequently mentioned as a potential option for parasitic weed control, it was
121 considered not realistic for many years, mainly because of the alleged instability of the stimulants.
122 Anyhow, there are no reports on attempts using SL analogues in controlling parasitic weeds since
123 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.

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142 Figure 1: Structures of SL analogues

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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
157 conditioning. Seven concentrations were used: 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} mol/L. The
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 (C₂H₄),
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

178 tests. In this formulation, NE 1 is contained in an emulsion (Zwanenburg *et al.*, 2009). Control pots
179 were similarly set up using only water (200 mL). A crop treatment was also included in the assays.
180 Sorghum (variety Segaolane) and pearl millet (variety Serere 6A) seeds, four of each were planted
181 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**.

185 The seed packets were removed from the pots for examination of germination, 7 days after
186 chemical treatment or after 17 days in the case of the crop treatment experiments. The packets
187 were rinsed from adhering soil particles and opened. Then seeds were placed carefully onto filter
188 paper and counted for germinated seeds using a binocular microscope. All experiments were
189 carried out in quadruplicate.

190 The experiments were conducted in the Nematology glasshouse of the University of
191 Reading, UK, at daily temperatures ranging between 21 and 39°C.

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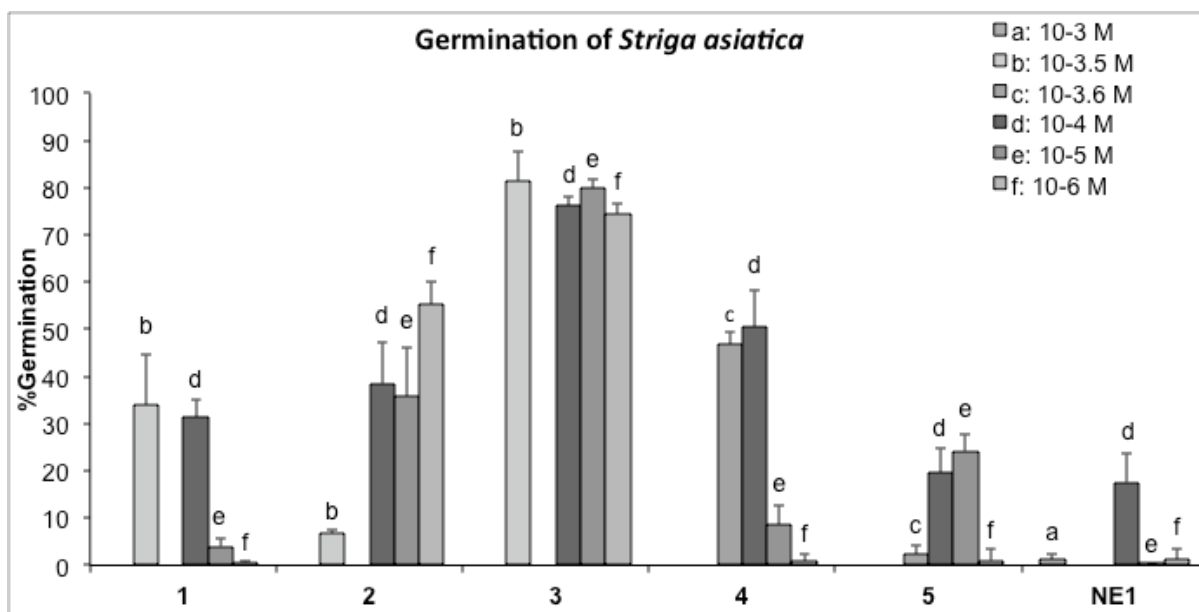
193 *Analysis*

194 Data handling and graphics were performed using Microsoft Excel and data analyses were
195 performed using the Genstat7 program. Percentage data were transformed to angles before analysis
196 (Murdoch, 1982). The results can be re-interpreted using the equation:

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

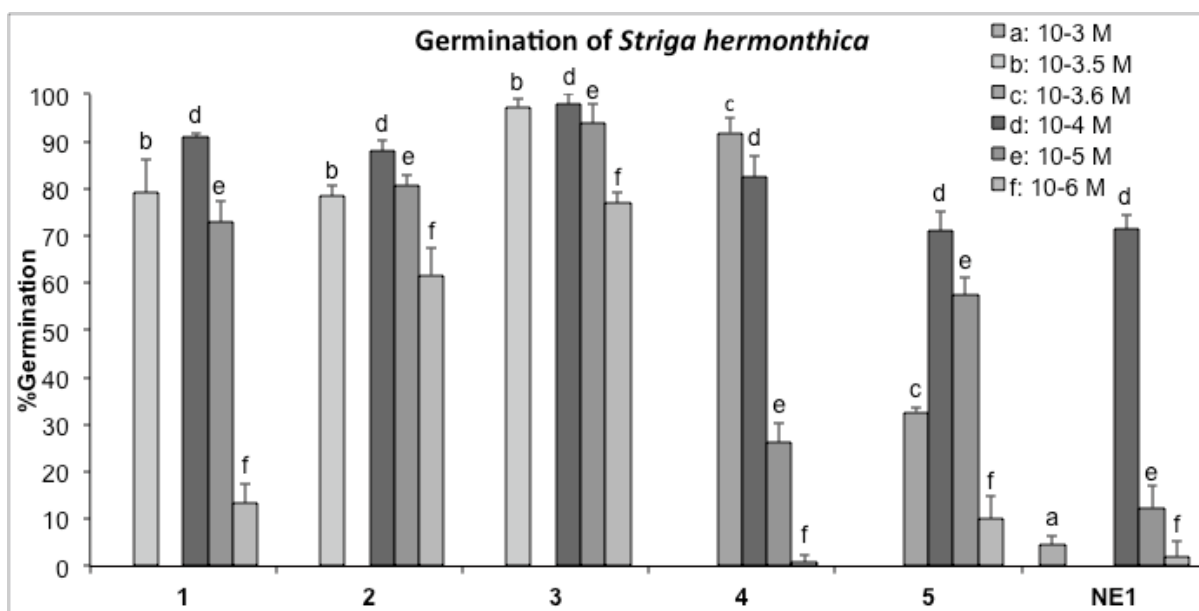
198 For the bio-assays *in vitro* the standard error of difference was 1.73 for *S. asatica* (Tanzanian
199 strain), 0.80 for *S. asiatica* (Malawi strain), and 3.02 for *S. hermonthica* (Sudan strain). The
200 germination data are not shown. For the pot experiments the s.e.d. was 2.50 for *S. hermonthica*
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.

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Figure 2: Bar graph of the percentages of germinated seeds of *S. asiatica* (Tanzania 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 ($10^{-3.5}$ M) for 1, 2, and 3, c ($10^{-3.6}$ M) for 4 and 5 and a (10^{-3} M) for NE 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.



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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 ($10^{-3.5}$ M) for 1, 2, and 3, c ($10^{-3.6}$ M) for 4 and 5 and a (10^{-3} M) for NE

221 1. Values are mean germination percentages, experiments were carried out in
222 quadruplicate. The accuracy is shown at the top of each bar. NE 1= Nijmegen 1 (4)
223 formulated as a dispersed emulsion. The water control did not induce any germination.
224
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226 **Results and Discussion**

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

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

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

247 A highly rewarding outcome of this study is that there are no noticeable signs of disturbing
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
 257 worth noting that the dose-response curve has a bell shape in both cases, whereby the maximal
 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
 264 the most active one.

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

Species	SL analogue	Maximum germination angles	Maximum germination percentage	Concentration for maximum germination
<i>S. hermonthica</i>	1	77.90	79.2 ± 7.08	10 ^{-3.5} M
	2	73.99	88.0 ± 2.28	10 ⁻⁴ M
	3	86.40	98.0 ± 2.00	10 ⁻⁴ M
	4	79.11	91.9 ± 3.00	10 ^{-3.5} M
	5	69.04	73.0 ± 4.29	10 ⁻⁴ M
	NE 1	63.93	71.5 ± 2.83	10 ⁻⁴ M
<i>S. asiatica</i>	1	43.35	33.9 ± 10.9	10 ^{-3.5} M
	2	56.20	38.5 ± 8.55	10 ^{-3.5} M
	3	79.41	81.5 ± 6.23	10 ^{-3.5} M
	4	55.49	50.5 ± 7.92	10 ⁻⁴ M
	5	40.94	24.2 ± 3.50	10 ⁻⁴ M
	NE 1	39.06	17.6 ± 6.19	10 ⁻⁴ M

267
 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.

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

297

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301

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