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

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Summary

Striga hermonthica and *S. asiatica* are obligate root parasites that cause serious problems in the production of staple cereal crops in Africa. Because of the high levels of infestation, there is an urgent need to control these weeds. An interesting option is depletion of the soil seed bank by suicidal germination which involves germination of the seeds in the absence of host plants. Suicidal germination is often mentioned as an interesting option, but not considered as realistic due to the alleged untimely decomposition of the stimulants in the soil, despite the fact that some encouraging results were reported around 1980. The alleged instability has prevented active research in this direction for the past 20-25 years. Five newly designed synthetic germination stimulants are investigated as candidates for suicidal germination. An important issue is the persistence of these stimulants in soil. Packets with *Striga* seeds were put in pots with soil and then treated with aqueous solutions of the stimulants. All five compounds induced germination under these soil conditions. There were no noticeable signs of disturbing decomposition of the stimulants. The best performing stimulant is derived from 1-tetralone. The conclusion is that synthetic SL analogues have excellent prospects for use in the field in combating parasitic weeds.

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

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Introduction

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

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. Interestingly, the biology of these three genera of parasitic weeds is closely related which suggests the use of similar control strategies. It has been proposed that the important means of controlling root holo- and hemi-parasitic weeds should focus on the seed cycle: reducing soil seed-bank, preventing seed set and inhibiting seed movement from infested to non-infested areas (Rubiales *et al*., 2009). To date, there is no single effective method of controlling these weeds partly due to their complex life cycle, their vascular connection to the host, the production of many tiny, long-living seeds (Joel *et al*., 1995; Rubiales *et al*., 2009a) and because, unlike most weeds, they damage the host inconspicuously, while still underground/subterranean.

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

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

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

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

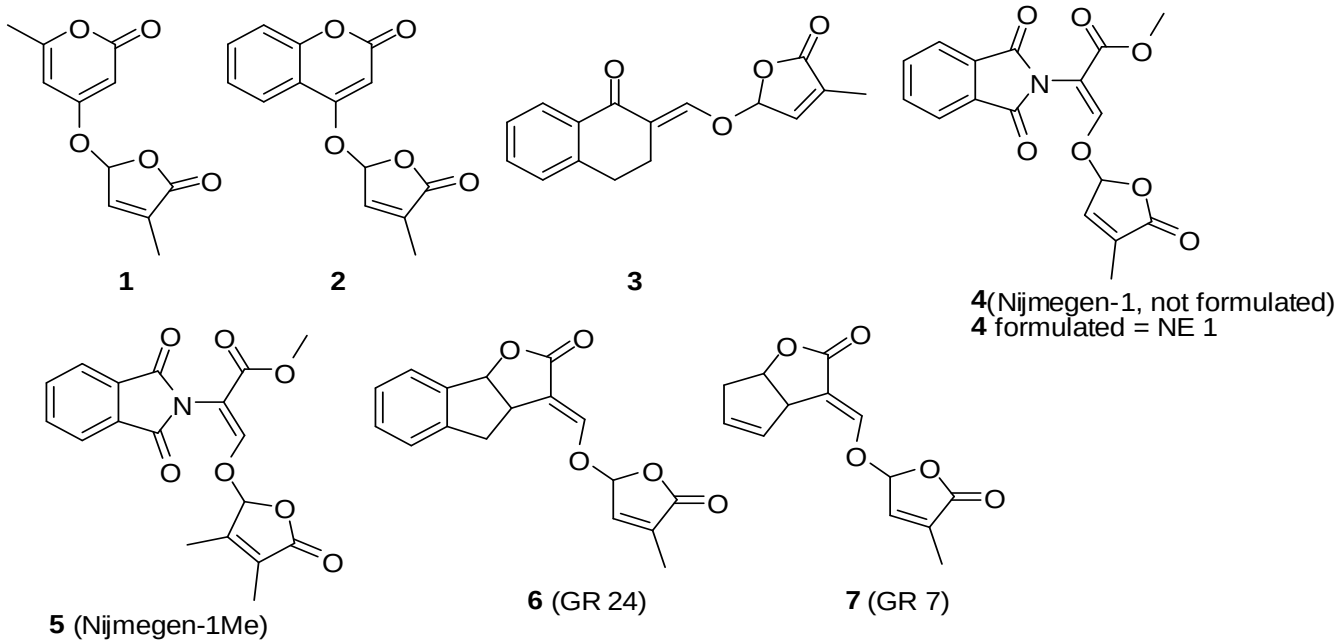

Figure 1: Structures of SL analogues

Materials and Methods

SL analogues

Analogues **1** and **2** were prepared as described by Mwakaboko & Zwanenburg (2011b) , analogue **3** as described by Mwakaboko & Zwanenburg (2011a), compound **4** (Nijmegen-1) as described by Nefkens *et al*. (1997) and Nijmegen-1Me (**5**) as described by Thuring *et al*. (1997).

Seeds

Seeds of three *Striga* species were used in the study: *S. hermonthica* (Sudan 1992, *ex* sorghum), *S. asiatica* (Tanzania 1997, *ex* sorghum) and S. *asiatica* (Malawi 1993, *ex* sorghum); all seeds had been stored at room temperature.

In vitro germination assays

All three seed species were subjected to an *in vitro* bio-assay protocol similar to that used 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 assays were carried out in duplicate. In some cases there was also germination observed during the water controls in these *in vitro* assays. This was ascribed to the enclosed environment of the tests and to the fact that the Petri dishes were stacked which could have led to cross contamination between treatments. Sometimes bio-assays are disturbed by a low concentration of ethene (C_2H_4), which also acts as a germination stimulant, in the air, thus leading to erroneous results. It is also possible that the pre-treatment of the seeds was inappropriate; traces of sterilizing agents may be disturbing the assays. Normally, no germination occurred in the water controls, thus the observed positive blanks must be artefacts and accordingly these assays were disregarded.

Details of the pot experiments

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

The germination experiments were conducted using four concentrations of compounds **1-5**. The first three were 10^{-6}, 10^{-5}, and 10^{-4} M while the fourth corresponded to the maximum solubility 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.

Analysis

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:

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

For the bio-assays *in vitro* the standard error of difference was 1.73 for *S. asatica* (Tanzanian strain), 0.80 for *S. asiatica* (Malawi strain), and 3.02 for *S. hermonthica* (Sudan strain). The germination data are not shown. For the pot experiments the s.e.d. was 2.50 for *S. hermonthica* (Sudan strain) and 3.32 for *S. asiatica* (Tanzanian strain). The germination percentages were recalculated from the germination angle analysis and are shown in as bar diagrams in Figures 2 and 3.

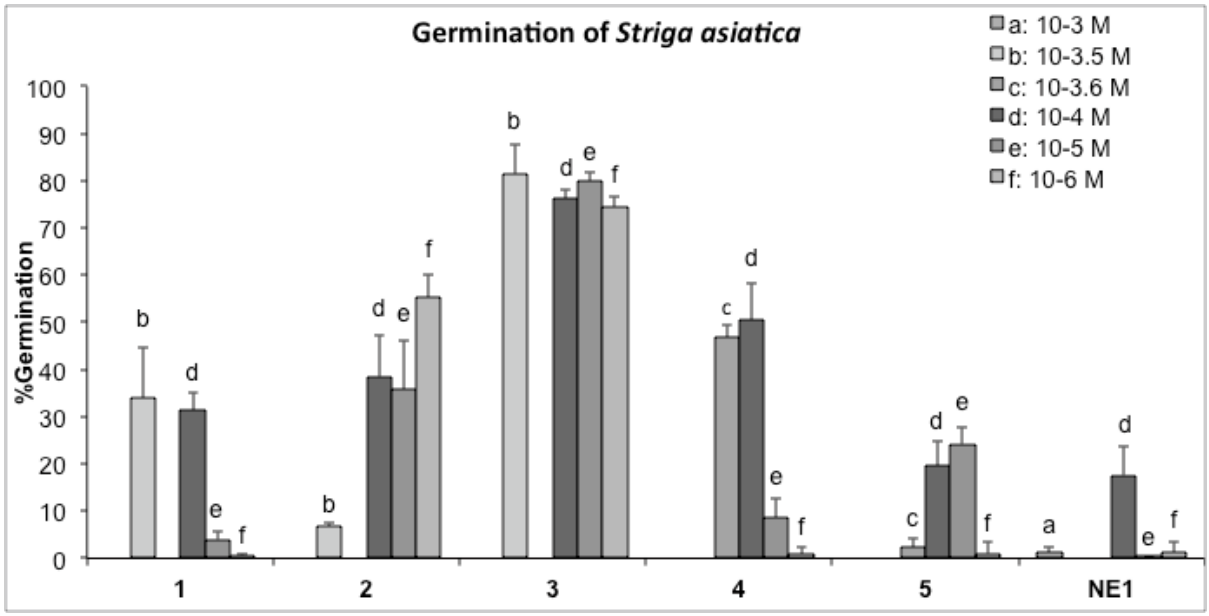

Figure 2: Bar graph of the percentages of germinated seeds of *S. asiatica* (Tanzania strain) after exposure to various concentrations of **1**-**5** in the pot experiments. All stimulants **1**-**5** and **NE1** were assayed at the concentrations **d**, **e** and **f** + the concentration of maximum 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.

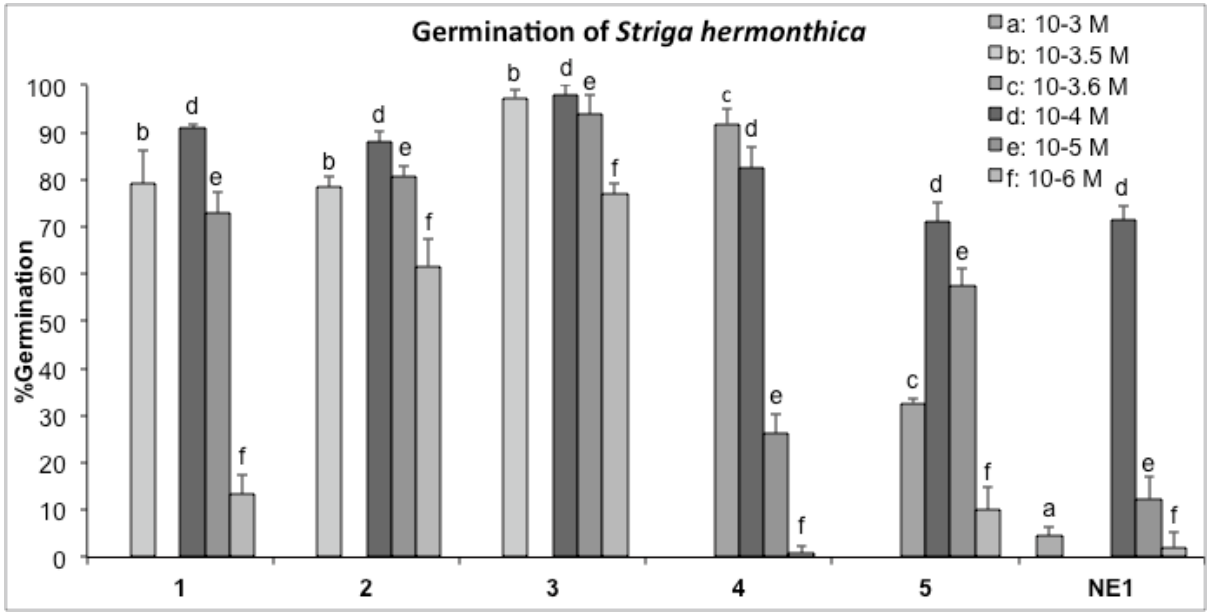

Figure 3: Bar graph of the percentages of germinated seeds of *S. hermonthica* (Sudan strain) after exposure to various concentrations of **1**-**5** in the pot experiments. All stimulants **1**-**5** and **NE1** were assayed at the concentrations **d**, **e** and **f** + the concentration of maximum 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**

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.

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.

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

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 activity is seed species and stimulant dependent. This is in line with earlier observations in *in vitro* studies, that a dose-response curve has a maximum (Wigchert *et al*., 1999). The optimum concentration for certain seed species and a stimulant can also readily be deduced from Table1 wherein the maximum germination angles/percentages and the maximum germination concentration are indicated. For the most responsive seed species *S. hermonthica* SL analogues **3** is clearly performing as the best, closely followed by analogue 2, while for *S. asiatica* analogue **3** is the most active one.

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

Species	SL analogue	Maximum germination angles	percentage	Concentration for maximum germination
S. hermonthica	**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
S. asiatica	**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

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

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

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

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