Dose−Response of Seeds of the Parasitic Weeds *Striga* and *Orobanche* toward the Synthetic Germination Stimulants GR 24 and Nijmegen 1

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Striga and *Orobanche* seeds germinate in response to a host-derived germination stimulant. Dose−response curves of the synthetic strigolactone analogues GR 24 and Nijmegen 1 were determined, and their activities were compared to that of the naturally occurring stimulant sorgolactone. Typical sigmoidal curves were obtained. ED_{50} values for GR 24 were in the order of 10^{-9}−10^{-8} mol/L; for Nijmegen 1 these values were 3 orders of magnitude higher. Both synthetic stimulants are appreciably active at low concentrations and merit investigation as agents for the suicidal germination approach (i.e., treatment of the soil with stimulant in the absence of a host).

Keywords: *Germination; GR 24; Orobanche; parasitic weeds; Striga*

INTRODUCTION

The root parasitic weeds *Striga* (witchweed) and *Orobanche* (broomrape) are serious pests in agriculture (Musselman, 1987; Parker and Riches, 1993). *Orobanche* parasitizes dicotyledonous crops, such as legumes, tomato, and sunflower, and predominantly occurs in the Mediterranean region. *Striga* mainly infects cereals such as sorghum, maize and millet in tropical and subtropical areas. The lives of millions of people in Africa, India, and the Middle East are directly affected by the severe harvest reductions due to heavy infestations of susceptible crops with these parasites (Sauerborn, 1987). *S. hermonthica* and *S. asiatica* are the species that cause the most economically significant damage to cereals (Butler, 1995).

The life cycle of the parasitic weeds is closely adapted to that of their host plants. The seeds of the parasites germinate only if they are exposed to stimulant molecules, which are present in the root exudate of a suitable host plant (Musselman, 1987; Parker and Riches, 1993). Thus far, three naturally occurring germination stimulants, belonging to the class of the strigolactones (Butler, 1995), viz., strigol (**1**) (Cook et al., 1966), sorgolactone (**2**) (Hauck et al., 1992), and alectrol (**3**) (Müller et al., 1992) have been isolated (Figure 1). [Recently, a compound with the proposed structure for alectrol was synthesized by Mori et al. (1998). They concluded that the structure proposed by Müller et al., **3**, is incorrect. An alternative structure (**3b**) was synthesized, but its spectral data were also not in accordance with the ^{1}H NMR spectrum of the natural compound. On the basis of these results and the original spectral data for alectrol, reported by Müller et al., we propose that **3c** is the structure of the naturally occurring stimulant from cowpea (Figure 2). In this compound, the tertiary alcohol in the structure originally proposed for alectrol (**3**) is now part of the C-ring lactone.]

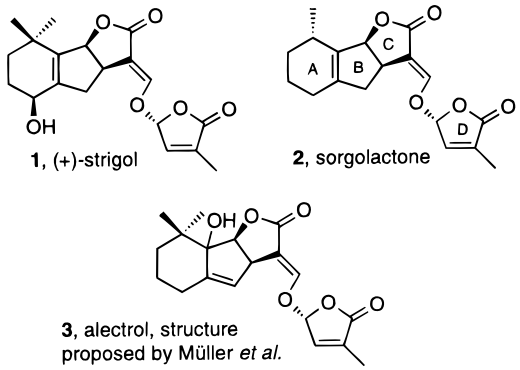

Figure 1. Structures of the naturally occurring germination stimulants strigol (**1**), sorgolactone (**2**), and alectrol (**3**).

To control parasitic weeds, various methods have been developed over the years, among others, hand pulling, fallow, crop rotation, resistant crops, and the use of herbicides. Hand weeding is not very practical, as when the parasite plant emerges from the soil, most of the damage to the host crop has already been done. Crop rotation with nonhost crops that do stimulate *Striga* seed germination, but which are not parasitized themselves (e.g., cotton), in addition to periods of fallow have been used to keep *Striga* infestations at tolerable levels (Lagoke et al., 1991). However, the growth of the human population and the increasing demand for production of food lead to intensified use of land, with more monocropping and little or no fallow, thus enlarging the numbers of parasite seeds. Although the development of resistant crops is quite difficult, the approach is promising and recently some success has been achieved (Mann, 1997). The use of herbicides is, in general, not

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10.1021/jf981006z CCC: $18.00
Published on Web 03/25/1999

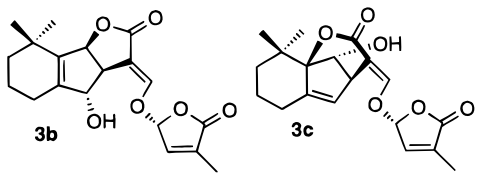


Figure 2. Alternative structures for the naturally occurring germination stimulant isolated from cowpea exudate.

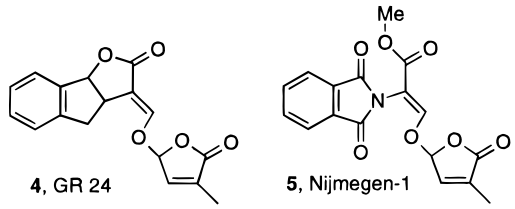


Figure 3. Structural formulas of the synthetic germination stimulants GR 24 and Nijmegen 1.

attractive from an environmental point of view, and the cost of sophisticated chemicals limits their use in the developing countries. So far, none of the above-mentioned methods is very effective in eradicating *Striga* and *Orobanche* (Parker and Riches, 1993; Berner et al., 1995, 1996).

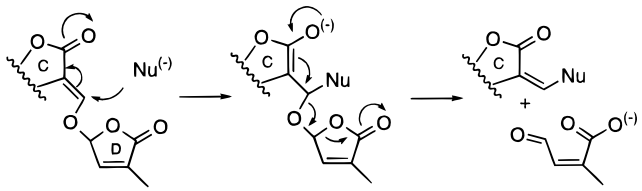
In principle, an alternative approach to control *Striga* infestations is the reduction of the amount of parasite seeds in the soil by suicidal germination, that is, the application of a synthetic germination stimulant to the soil, before the desired crop is planted (Johnson et al., 1976; Eplee and Norris, 1987). The *Striga* seeds will germinate, but, in the absence of a suitable host plant, the seedlings will die. This method should generally be applicable for all *Striga* and *Orobanche* infestations, provided a suitable germinating agent is available. A germination stimulant with a simplified strigolactone structure, which can readily be prepared in large quantities, would be an attractive candidate in this suicidal germination approach.

In this context, the synthesis of strigol (Heather et al., 1976), sorgolactone (Sugimoto et al., 1998), and their analogues, for example, GR 24 (**4**) (Figure 3) has received much attention [among others: Johnson et al. (1981), Mangnus et al. (1992a), Kranz et al. (1996), and Thuring et al. (1997a)]. A standardized bioassay to determine the germination-inducing activity of a compound was developed and structure−activity studies were conducted (Mangnus et al., 1992b). These studies revealed that the bioactiphore resides in the CD part of the strigolactone molecule, and a tentative molecular mechanism for the induction of germination was proposed (Mangnus et al., 1992c).

This mechanism involves addition of a nucleophilic species, present at the receptor site, in a Michael fashion, followed by elimination of the D ring. The ultimate result is that the ABC part of the stimulant is covalently bound to the receptor, a chemical change that may be responsible for triggering germination (Scheme 1).

GR 24 (**4**) (Johnson et al., 1981; Mangnus et al., 1992d) is a very potent synthetic germination stimulant, which is used worldwide in parasitic weed research to stimulate germination and as a standard for comparison of new germinating agents. Despite its frequent use, dose−response curves of GR 24 have never been reported, except in one paper on *O. crenata* seeds (Bergmann et al., 1993). Multigram scale production of GR

Scheme 1. Tentative Molecular Mechanism for the Induction of *Striga* Germination



24 is not (yet) economically feasible, which prevents its use as a suicidal germination agent. Nijmegen 1 (**5**) (Nefkens et al., 1997), which has a much more simplified structure, was designed to contain the essential structural features required for bioactivity, according to the mechanism depicted in Scheme 1. Nijmegen 1 can easily be prepared in multigram quantities and is an attractive candidate for *Striga* control by the suicidal germination approach.

The aim of the research presented in this paper was to determine the dose−response curves of the synthetic germination stimulants GR 24 (**4**) and Nijmegen 1 (**5**) for several *Striga* species and *Orobanche crenata*. This information is highly relevant to the possible use of these stimulants in the suicidal germination approach in the field. The activities of **4** and **5** were compared with that of the natural stimulant sorgolactone (**3**). In addition, a modified procedure for the preparation of GR 24 is presented.

MATERIALS AND METHODS

Synthesis. General methods and instrumentation are the same as described by Nefkens et al. (1997).

Nomenclature. Systematic names were generated using the ACD/Name program, provided by Advanced Chemistry Development Inc. (Toronto, Canada).

*8(S)-Methyl-3-[4′-methyl-5′-oxo-2′,5′-dihydrofuran-2′(R)-yloxymethylene]-3,3a(R),4,5,6,7,8,8b(S)-octahydroindeno[1,2-b]-furan-2-one (**2**).* The naturally occurring diastereomer of sorgolactone was synthesized as reported by Sugimoto et al. (1998).

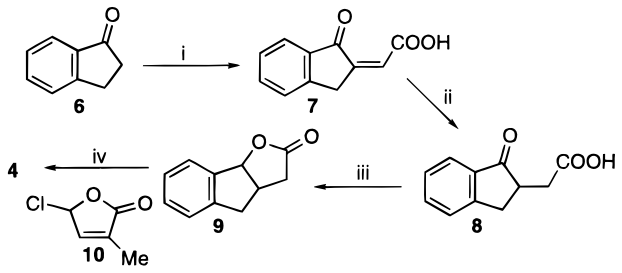
*3-[(2,5-Dihydro-3-methyl-2-oxo-5-furanyl)oxymethylene]-3,3a,4,8b-tetrahydroindeno[1,2-b]furan-2-one (**4**).* Racemic GR 24 (an equimolar mixture of four diastereomers) was essentially prepared as described by Mangnus et al. (1992d) with modifications in the synthesis of the ABC part (vide infra).

*Methyl 2-(1,3-Dioxo-1,3-dihydroisoindol-2-yl)-3-[4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy]acrylate (**5**).* Racemic Nijmegen 1 (an equimolar mixture of two enantiomers) was prepared as described previously (Nefkens et al., 1997).

*2-(1-Oxo-2,3-dihydro-(1H)-2-indanyliden)acetic Acid (**7**).* Indanone (**6**; 65.1 g, 0.5 mol) was dissolved in 150 mL of anisole. To this solution was added 50 g (0.54 mol) of glyoxylic acid monohydrate in 100 mL of benzene. The mixture was heated under reflux overnight using Dean−Stark conditions. When no more starting material was present, the reaction mixture was cooled to 0 °C, whereupon the product precipitated. The white precipitate was filtered off, washed with plenty of diisopropyl ether, and air-dried. Yield = 79%. Analytical data were in complete accordance with those reported in the literature (Fontenla et al., 1994).

*2-(1-Oxo-2,3-dihydro-(1H)-2-indenyl)acetic acid (**8**).* Acid **7** (18.8 g, 0.1 mol) was dissolved in a mixture of methanol and water (70 mL) in which 6 g (0.1 mol) of potassium hydroxide was dissolved, to enhance solubilization of the starting material. A catalytic amount of palladium on activated charcoal was added, and the mixture was hydrogenated employing a Parr apparatus until hydrogen uptake ceased. The solution was then filtered over Hyflo, and methanol was evaporated under reduced pressure; 150 mL of water was added, and the product was precipitated by slow addition of 6 N HCl. The resulting

Scheme 2. Modified Synthesis of GR 24



i) glyoxylic acid, benzene/ anisole, reflux ii) H_2, Pd/C, iii) 1) $NaBH_4$ 2)pTsOH, benzene, reflux iv)1) Na or KOtBu, ethylformate,2) chlorobutenolide **10**, THF.

white solid was washed with water (once) and diisopropyl ether (once) and dried in vacuo. Yield = 14.6 g (77%). Analytical data were in complete accordance with those reported previously (Groves and Swan, 1951; Mangnus et al., 1992d).

The steps in the reaction sequence depicted in Scheme 2 were performed as described by Mangnus et al. (1992d).

Biological Activity. *Plant Material.* Five populations of *S. hermonthica* (Del.) Benth. seeds were used. They were collected from the following host plants: pearl millet *(Pennisetum americanum* (L.) K. Schum.) in Galadima, Nigeria, in 1988; sorghum [*Sorghum bicolor* (L.) Moench] on Mbita-point Road near Homa Bay, Kenya, in 1993; sorghum [*S. bicolor* (L.) Moench] in Shire, Ethiopia, in 1996; wild sorghum [*Sorghum arundinaceum* (Desv.) Stapf.] in Kibos, Kenya, in 1993; maize (*Zea mays* L.) in Kana, Benin, in 1993. *S. asiatica* (L.) Kuntze seeds were harvested in Alupe, Kenya, from finger millet [*Eleusine coracana* (L.) Gaertn] and maize (*Z. mays* L.) in 1993 and 1995, respectively. *S. aspera* (Willd.) Benth. seeds from maize *(Z. mays* L.) were obtained in Garoua, Nigeria, in 1989. *O. crenata* Forsk. seeds were harvested from faba bean (*Vicia faba* L.) in Beheira, Egypt, in 1993. All seeds were stored in glass vials in the dark at room temperature until use in germination tests.

Preparation of Test Solutions. For the bioassay performed in 1994, exactly 5 mg of GR 24 was dissolved in 1 mL of acetone p.a. and diluted with demineralized water to 500 mL. Thus, a GR 24 solution of 10 mg/L (3.35×10^{-5} mol/L) was obtained. A stock solution of 1 mg of GR 24/L (3.35×10^{-6} mol/L) was prepared as follows: 5 mg of GR 24 was dissolved in 10 mL of acetone p.a., and 1 mL of this solution was diluted with demineralized water to 500 mL. From this stock, GR 24 test solutions of 3.35×10^{-7} to 3.35×10^{-12} mol/L were prepared. For the bioassay performed in 1998, a compound to be tested was weighed out very accurately to the amount of 1.5 mg, dissolved in 1 mL of acetone p.a., and diluted with demineralized water to 5 mL. These stock solutions of $\sim 10^{-3}$ mol/L (the exact concentration depending on the molecular mass of the compound used) were further diluted with demineralized water to obtain test solutions with concentrations ranging from 10^{-4} to 10^{-15} mol/L. All solutions were prepared 1 day prior to use.

Bioassays. All bioassays were performed at the Department of Ecology and Ecotoxicology of Plants of the Vrije Universiteit in Amsterdam, The Netherlands, in 1994 and 1998. For surface sterilization all seeds were exposed to 70% (v/v) aqueous ethanol for 5 min and then for 2 min to a 50% (v/v) aqueous solution of commercial bleach (2% hypochlorite). Subsequently, the seeds were thoroughly rinsed with demineralized water and air-dried. For conditioning, the seeds were spread on glass fiber filter paper disks (8 mm diameter, ~60−100 seeds per disk) in Petri dishes, wetted with demineralized water, and incubated in the dark at 20 °C for *Orobanche* and at 30 °C for *Striga*. Thereafter, the conditioning water was removed and the seeds were placed in new Petri dishes and exposed to the test solutions (100 μL per disk). After incubation for 24 h (*Striga*) and 5 days (*Orobanche*) in the dark at the indicated temperatures, the percentage of germinated seeds was determined under a microscope. Seeds were considered germinated when the radicle protruded through the seed coat. In each test series an aqueous solution of 0.1% (v/v) acetone was included as a negative control. For full details of the bioassay see Mangnus et al. (1992b) and Kuiper (1997). All tests were performed twice with three replicates per test solution. Each replicate contained two disks with parasite seeds. Data handling was performed using Microsoft Excel. For each replicate the mean percentage of germinated seeds was calculated. Consequently, the mean percentage of germinated seeds of all three replicates (for each test solution) was determined as well as the standard error (SE) value of the mean.

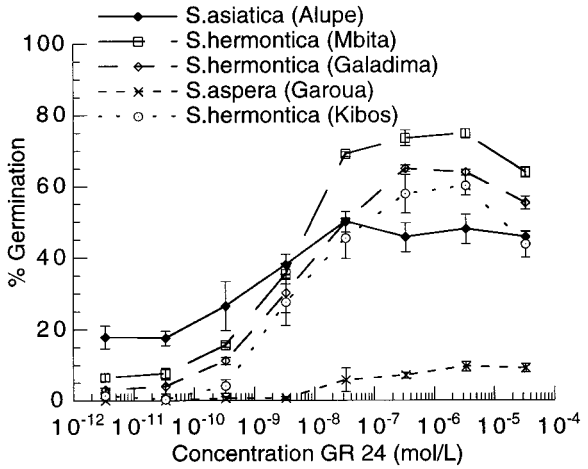


Figure 4. Percentage germination of several populations of *Striga* seeds stimulated by aqueous solutions of the synthetic germination stimulant GR 24. Data presented are the mean ± SE of one representative experiment.

RESULTS AND DISCUSSION

The strigolactone analogue GR 24 is used worldwide in parasitic weed research, often as a reference compound in bioassays in which the germination-inducing activity of a certain compound is investigated. In these assays, usually only two or three concentrations of the compounds are tested, frequently 1, 0.1, and/or 0.01 mg/L. It is important to note here that the unit moles per liter is a better way to express concentrations, because the molecular masses of the compounds tested are usually not the same. We have determined the percentage germination of five different *Striga* populations, induced by a racemic mixture of GR 24 (the form in which it is usually applied) at eight concentrations, ranging from 10^{-12} to 10^{-5} mol/L. Details of the populations used are given under Materials and Methods, and the resulting dose−response curves are depicted in Figure 4.

Figure 4 reveals that not all *Striga* populations responded equally well to stimulation by GR 24. *S. aspera* was hardly sensitive to GR 24, although it did give much higher percentages of germinated seeds (up to 40%) when stimulated with maize root exudate (Kuiper, 1997). This is an example of a seed population that is obviously very sensitive to the structure of its original stimulant, present in maize root exudate. This phenomenon was observed for a variety of *S. aspera* populations (Kuiper, 1997). The majority of the populations investigated here, however, responded well to GR 24. Germination generally began at a GR 24 concentration of 10^{-10} mol/L and reached a maximum value when [GR 24] = 10^{-7} mol/L. When the stimulant concentration became $>10^{-5}$ mol/L, a decrease in the germinating activity was observed. The concentration that induces

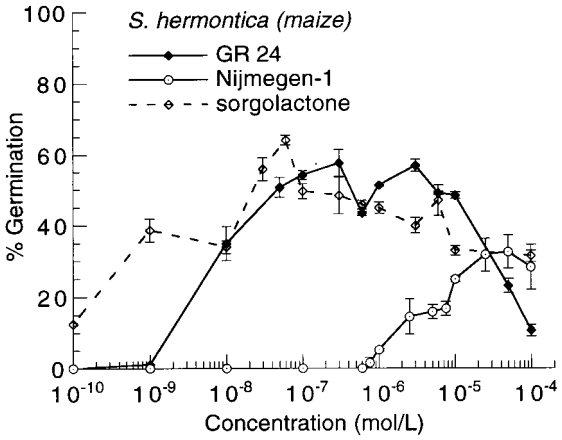


Figure 5. Percentage germination of *S. hermonthica* seeds (collected from maize) stimulated by aqueous solutions of GR 24, Nijmegen 1, and sorgolactone. Data presented are the mean ± SE of one representative experiment.

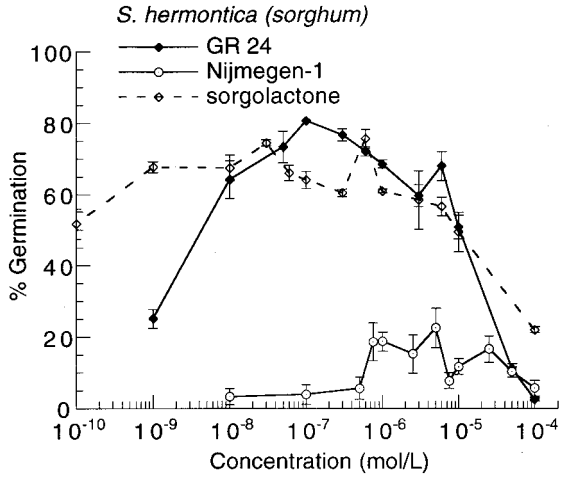


Figure 6. Percentage germination of *S. hermonthica* seeds (collected from sorghum) stimulated by aqueous solutions of GR 24, Nijmegen 1, and sorgolactone. Data presented are the mean ± SE of one representative experiment.

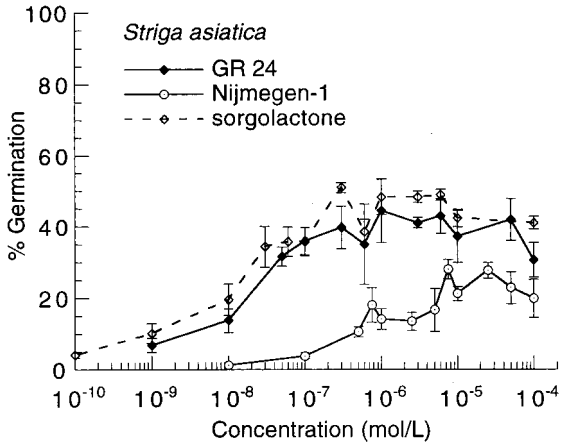


Figure 7. Percentage germination of *S. asiatica* seeds (collected from maize) stimulated by aqueous solutions of GR 24, Nijmegen 1, and sorgolactone. Data presented are the mean ± SE of one representative experiment.

half-maximal response, the ED_{50} value, is for GR 24 in all cases $\sim 5 \times 10^{-9}$ mol/L. The maximum percentages of germinated seeds are quite different and are apparently species dependent. In addition, there is also a certain host specificity, which might explain a different response of various *Striga* populations, belonging to the same species, to the same synthetic stimulant.

If GR 24 is considered as a reference compound in experimental setups, a concentration should be used in the range where maximum germination is induced (1 mg/L corresponds to 3.35×10^{-6} mol/L and is a good choice), as well as a concentration around the ED_{50} value, that is, 5×10^{-9} mol/L.

The dose−effect curves depicted in Figure 4 were determined in 1994. Recently the synthesis of all four diastereomers of GR 24 was described (Thuring et al., 1997b). Biological testing revealed that only the GR 24 diastereomer possessing the same stereochemical configuration (at all of its stereocenters) as the naturally occurring strigolactones expressed significant biological activity at the sensitive concentration of 0.001 mg/L ($=3.35 \times 10^{-9}$ mol/L). The same concentration of racemic GR 24 (which contains only 25% of the "natural configuration" isomer) induced equally high numbers of seeds to germinate. Because the racemic form is always used in parasitic weed research and its activity is comparable to that of the "natural" GR 24 diastereomer, we included only racemic GR 24 in the experiments discussed below.

In 1997, the synthesis of Nijmegen 1 (**5**) was reported by Nefkens et al. This strigolactone analogue contains only one stereogenic center, and therefore its synthesis is not complicated by the formation of diastereomers, which would require chromatographic separation. Nijmegen 1 can be synthesized in only two steps. Because of its biological activity in germinating parasitic weed seeds, and its easy preparation, Nijmegen 1 may be a potential candidate for suicidal germination in the field.

In 1998, all diastereomers of the naturally occurring stimulant sorgolactone were prepared in our laboratory (Sugimoto et al., 1998). This enabled us to compare the activities of GR 24 (**4**) and Nijmegen 1 (**5**) with the biological activity of the naturally occurring diastereomer of sorgolactone (**2**).

To this end two *S. hermonthica* populations were selected (a population from maize in Kana, Benin, and a population from sorghum in Shire, Ethiopia), as well as a population of *S. asiatica* because these species are the most economically significant ones. A population of *O. crenata* was also included. First, concentrations of 10^{-15}, 10^{-14}, 10^{-13}, etc. up to 10^{-4} mol of compound/L were assayed (data not shown). This rough test clearly indicated that in almost every case, germination started when the stimulant concentration was at least 10^{-10} mol/L and that maximum percentages of germination were generally obtained at stimulant concentrations $\geq 10^{-6}$ mol/L. Therefore, a more refined selection of test solutions was prepared with which more data points were collected in the sensitive regions of the germination curves. The resulting curves are depicted in Figures 5, 6, 7, and 8 for *S. hermonthica* (maize), *S. hermonthica* (sorghum), *S. asiatica*, and *O. crenata*, respectively. In all cases the percentage germination induced by the aqueous control solution was practically zero.

The most striking result, which is evident from all four figures, is the extremely high activity of GR 24. For every seed population that was used in this experiment, racemic GR 24 is as active as the single isomer of sorgolactone. This is in agreement with the results of Thuring et al. (1997b). ED_{50} values and maximum

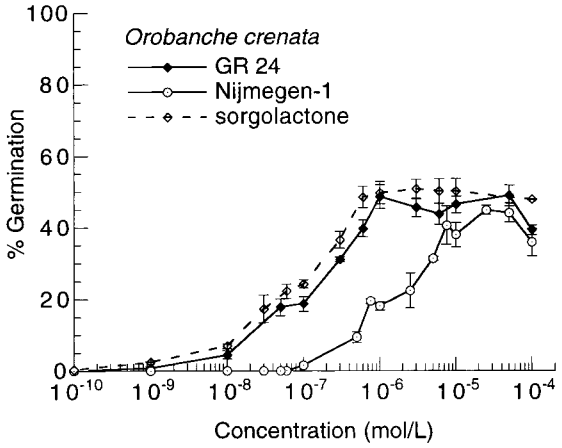


Figure 8. Percentage germination of *O. crenata* seeds (collected from faba bean) stimulated by aqueous solutions of GR 24, Nijmegen 1, and sorgolactone. Data presented are the mean ± SE of one representative experiment.

percentages of germinated seeds induced by GR 24, Nijmegen 1, and sorgolactone are summarized in Table 1.

In complete agreement with the results obtained in 1994 (Figure 3), the ED_{50} values of GR 24 are between 10^{-9} and 10^{-8} mol/L. In the case of *O. crenata* half-maximum percentages of germination are obtained at stimulant concentrations at least 2 orders of magnitude higher than in the case of *Striga*. This trend was also observed in the bioassay of all diastereomers of sorgolactone (Sugimoto et al., 1998). Sorgolactone is the major germination stimulant exuded by sorghum roots, whereas in the case of maize this is strigol (Siame et al., 1993). The lower ED_{50} value of sorgolactone in stimulating *S. hermonthica* from sorghum as compared to that for *S. hermonthica* from maize may be an example of a host specific response of parasitic weed seeds (Joel et al., 1995). From the results summarized in Table 1, it can also be concluded that *O. crenata* is less sensitive to structural modifications of the applied germination stimulant than *Striga:* in the case of Nijmegen 1, sorgolactone, and GR 24 approximately the same maximum percentages of germinated seeds were obtained, and there is also not much difference in their respective ED_{50} values. As for *S. asiatica,* its response is somewhat intermediate between those of *S. hermonthica* and *O. crenata*. The ED_{50} values of the three compounds are not as different as the maximum percentages of germination induced by each stimulant.

Nijmegen 1 is considerably less active in stimulating the germination of *Striga* seeds: its ED_{50} values are up to 3 orders of magnitude higher than those of GR 24 and sorgolactone, and also the maximum percentages of germination that were observed are significantly lower than the corresponding values determined for GR 24 and sorgolactone. This drawback could be overcome by repeated applications of the compound to soils infested with parasitic weed seeds. Its activity and the fact that Nijmegen 1 can easily be prepared in large quantities, from cheap starting materials, does merit further investigations of this compound in the field.

The results presented above clearly show that the dose−response curves of sorgolactone, Nijmegen 1, and GR 24 have a sigmoidal shape. In the case of GR 24 germination generally starts at a concentration of 10^{-9} mol/L and reaches a maximum value at concentrations of 10^{-7} mol/L. At concentrations $<10^{-10}$ mol/L no germination-inducing activity was ever observed. We therefore have doubts about the validity of the results published by Rugutt and Rugutt (1997). They reported a nonsigmoidal dose−response curve of GR 24, on *S. hermonthica* seeds, with germination values varying from 60 to 80% at concentrations between 10^{-21} and 10^{-2} mol/L. Even at the lowest concentration they observed 70% germination.

The main conclusion from the results presented above is that GR 24 is clearly the superior candidate for application as a suicidal germination agent in *Striga* control. However, the synthesis of GR 24 does not yet allow large scale production of the compound in an economically feasible manner. Recently, a few improvements were made in the synthesis of the ABC part. Following the route presented in Scheme 2, the entire ABC part can be constructed without the necessity of any distillation or chromatography procedure, which is highly desirable from an industrial point of view.

Regarding the stability and effectiveness of GR 24 and its analogue GR 7 in soil, two accounts of preliminary research have been published (Babiker et al., 1987, 1988). The conclusions were that soil moisture and pH have strong influences on stimulant activity. The compounds were stable at neutral and acidic pH, whereas in alkaline soil the activity decreased rapidly. Most likely, the instability of strigolactones under basic conditions can be explained by the reaction of hydroxide ion as the nucleophile in a Michael fashion, followed by hydrolysis of the lactone rings (see Scheme 1). These instability problems can be overcome by creating an appropriate controlled-release formulation.

Our laboratory experiments show that GR 24 and Nijmegen 1 are active germination stimulants for many species of the parasitic weeds *Striga* and *Orobanche* in the concentration range of 10^{-9}−10^{-6} mol/L. However, to evaluate the usefulness of these compounds in parasitic weed control, field studies should be undertaken to investigate the behavior of GR 24 and Nijmegen 1 in soil. Large scale preparation of Nijmegen 1 is easy, and this counterbalances its diminished activity as compared to that of GR 24. Furthermore, with the simplified synthesis of GR 24 presented above, multigram preparation of this compound is now feasible, which allows large scale field studies at relatively low costs. Both synthetic stimulants have great potential in the control of parasitic weed pests.

Table 1. ED_{50} Values and Maximum Percentages of Germinated Seeds Obtained When Four Populations of Parasitic Weed Seeds Were Stimulated with Solutions of GR 24, Nijmegen 1, and Sorgolactone

	GR 24		Nijmegen 1		sorgolactone	
population	ED_{50} (mol/L)	max % germtn	ED_{50} (mol/L)	max % germtn	ED_{50} (mol/L)	max % germtn
S. hermonthica (maize)	6×10^{-9}	57.5	5×10^{-6}	32.5	5×10^{-10}	64.0
S. hermonthica (sorghum)	2×10^{-9}	80.6	nd[a]	22.5	$<10^{-10}$	74.4
S. asiatica	2×10^{-8}	44.4	6×10^{-7}	28.0	2×10^{-8}	50.9
O. crenata	2×10^{-7}	49.0	2×10^{-6}	44.9	10^{-7}	50.8

[a] nd, not determined.

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Received for review September 8, 1998. Revised manuscript received January 14, 1999. Accepted February 9, 1999.

JF981006Z