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A Standardized Bioassay for Evaluation of Potential Germination Stimulants for Seeds of Parasitic Weeds

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Abstract. A standard bioassay for the evaluation of the biological activity of potential germination stimulants for seeds of parasitic weeds has been designed by carefully reconsidering all steps of a literature procedure. Addition of a surfactant to the solution for sterilization of the seeds gave a considerable reduction of contamination during the conditioning and stimulation process. Furthermore, it was shown that the water/seed ratio during conditioning affects the germination results. For the seed conditioning and stimulation process, the "sandwich" technique was introduced, whereby the seeds were placed between two layers of glass fiber filter paper discs. The standardized bioassay has been used for the evaluation of the stimulatory activity of numerous (new) synthetic analogues of strigol (the natural germination stimulant). It is essential to include a reference compound—GR24, an analogue of strigol, is recommended—in every test series, because it was observed that, although a standard bioassay was used, germination percentages obtained with GR24 solutions vary from test to test. For seeds of *Striga hermonthica,* seasonal effects were found in the germination percentages obtained with GR24 as stimulant. Test results with strigol analogues having modifications in ring D reveal that structural variations in this part of the molecule have dramatic effects on the biological activity of strigol analogues.

Parasitic weeds of the genera *Striga* and *Orobanche* cause severe damage to graminaceous and leguminous crops, respectively, in tropical and semitropical areas. The lives of more than 400 million people in Africa, India, and the middle East are affected by severe reductions of food crop yields through heavy infestations of these parasitic weeds (Musselman 1987, Parker 1986, Ramaiah 1987).

Striga and *Orobanche* are well-adapted plants, whose life cycles are closely coupled to their environment and to their host. They produce tiny seeds in vast numbers, which remain viable in the soil as long as 6–20 years. The seeds need an after-ripening period, followed by a period of about 2 weeks under warm moist conditions, before a chemical stimulant can break dormancy and induce germination. This stimulant is exuded by the host root, thus ensuring that a suitable host is identified for further development of the parasite (Nour et al. 1986, Press and Graves 1989).

Induction of germination in the absence of a suitable host causes starvation of the young seedlings within a few days after germination. This process, often named suicidal germination, is an attractive method for controlling weed pests. Ethylene, which is a germination stimulant, has been very effective in reducing viable *Striga* seed populations in the soil (Eplee 1975).

At present, only two natural germination stimulants have been identified: Sorgoleone, isolated from sorghum roots (Chang et al. 1986) and Strigol, isolated from cotton root exudate (Cook et al. 1966, 1972). These compounds do not lend themselves for use as weed-control agents because their stability under soil conditions is limited and their complicated structures (Fig. 1) make their syntheses lengthy and uneconomic.

Since the discovery of strigol, considerable effort has been devoted to the synthesis and biological activity evaluation of precursors and analogues of strigol (Johnson et al. 1976, 1981, Mangnus et al. 1991, 1992a–c, Pepperman et al. 1982, Vail et al. 1990, Zwanenburg et al. 1986). The analogues GR24 and GR7 (see Fig. 1; Hassanali 1984) are in world-

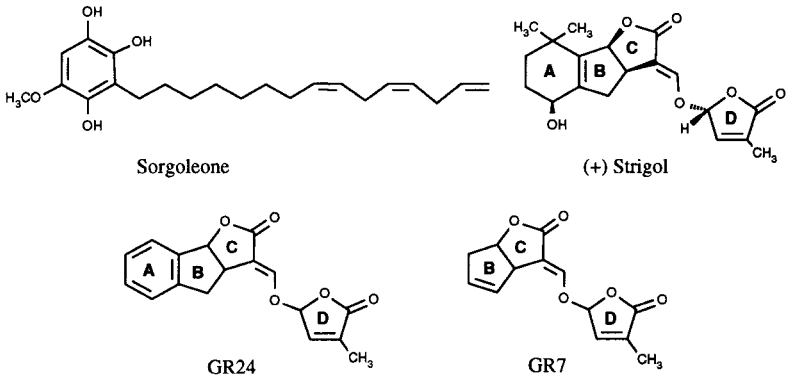


Fig. 1. Structures of some natural and synthetic germination stimulants.

wide use to induce germination of parasitic weed seeds under laboratory conditions.

Data on the biological activity of strigol analogues are not always reproducible (Pepperman et al. 1982). Furthermore, comparison between results obtained in different laboratories is often not possible through differences in bioassays.

The aim of the present study is to design a standard bioassay for the primary screening of potential germination stimulants. Also, ideas are discussed which enable comparison of results obtained in different laboratories. A series of strigol analogues has been evaluated for germination-stimulant activity using the proposed standardized bioassay.

Materials and Methods

Seeds

The seeds of *Striga asiatica* (L.) Kuntze were collected in Sudan in 1985 and those of *Striga hermonthica* (Del.) Benth. in Sudan in 1987. Both seed collections were obtained from Dr. A. G. T. Babiker (Gezira Agric. Res. Station, Wad Medani, Sudan). Seeds of *Orobanche crenata* Forsk. were collected in Egypt in 1988 and were obtained from Prof. O. A. Al-Menoufi (Alexandria University, Alexandria, Egypt). All seeds were stored in the dark at room temperature.

Sterilization of the Seeds

Striga and *Orobanche* seeds were first surface-sterilized in an aqueous solution containing sodium hypochlorite (2 g/100 ml active chlorine) and Triton X-100 (1% v/v, purchased from Aldrich). After a contact time of 5 min, with agitation, seeds were removed from the sterilization solution by filtration. The seeds were then washed thoroughly with demineralized water to remove all traces of sterilization solution and allowed to dry overnight.

Conditioning of the Seeds

For the conditioning of *S. hermonthica* seeds, two layers of filter paper (7 cm diameter) were placed in a 9 cm Petri dish and wetted with 3 ml of demineralized water. Five discs of glass fiber filter paper (1 cm diameter) were placed on the moist filter paper. Using a small paint brush, at least 25 seeds were spread on each disc. The discs with seeds were covered with a second disc of glass fiber filter paper (1 cm diameter) and an additional 3 ml of demineralized water per Petri dish was carefully added. The Petri dishes, enclosed in polyethylene bags, were incubated in the dark at 27°C for 2 weeks.

For the conditioning of *O. crenata* seeds, two filter papers (9 cm diameter) were placed in the lid of a 9 cm Petri dish and wetted with 6 ml of demineralized water. Discs of glass fiber filter paper (1 cm diameter) were placed on the moist filter paper (up to 45 discs per Petri dish). At least 25 seeds were spread on each disc. The discs with seeds were covered with a second glass fiber filter paper disc (1 cm diameter). Before putting the base of the Petri dish on top of the lid, another 4 ml of demineralized water was carefully added and dishes were incubated 'vide supra' at 23°C for 2 weeks.

Stimulant Solutions

For germination tests, freshly prepared stimulant solutions with test compounds in concentrations of 1 mg/L and 0.01 mg/L were used. The syntheses of test compounds will be described elsewhere.

A compound to be tested was weighed out very accurately to the amount of 10 mg, dissolved in 10 ml of acetone p.a., and diluted with demineralized water to 100 ml. From this stock solution, a dilution series was made using demineralized water exclusively, to give a solution with 1 mg/L of stimulant and 0.1% of acetone (v/v), and a solution with 0.01 mg/L of stimulant and 0.001% acetone (v/v). For the controls, solutions containing 0.1 and 0.001% of acetone (v/v), respectively, were prepared.

Stimulation Treatment of the Seeds

The double-discs with seeds ("sandwiches") were removed from the conditioning Petri dishes and placed on dry filter paper for 1 h to remove surplus moisture. Two rings of filter paper of 9 cm outer diameter and 7 cm inner diameter were placed inside the lid of a 9 cm Petri dish and wetted with 2 ml of demineralized water. Five "sandwiches" with seeds were placed within the moist ring of filter paper, and each disc was moistened with 100 µl of stimulant solution. For each stimulant to be tested, three replicate

Petri dishes (each containing five "sandwiches" with seeds) were used. The reference compound GR24 (chemical name: 3-[(2,5-Dihydro-3-methyl-2-oxo-5-furanyl)oxymethylene]-3,3a,4,8b-tetrahydroindeno-[1,2-b]furan-2-one; see Fig. 1 for structure) and the controls were tested in the same manner. The Petri dishes were covered, enclosed in polyethylene bags, and stored in the dark in an incubator. For *S. hermonthica*, the dishes were placed in an incubator at 27°C for 5 days, and *O. crenata* seeds were placed at 23°C for 5–7 days. After this incubation period, the percentage of germinated seed for each "sandwich" was determined using a microscope. A seed was considered to be germinated if the radicle protruded through the seed coat.

Results and Discussion

In 1977, Parker et al. described a procedure to evaluate host root exudates for the presence of germination stimulant. This procedure is used in most laboratories as the basis to evaluate solutions of stimulant for bioactivity. However, local laboratory conditions and introduction of small modifications may be responsible for different results obtained in various laboratories with the same stimulant. For example, *O. aegyptiaca* seeds collected in Israel exhibit significant spontaneous germination in laboratories in the United States and the Netherlands, whereas no spontaneous germination was observed in Israel (R. Jacobsohn, personal communication).

In order to design a standard bioassay for the primary screening of potential germination stimulants, each step of Parker's method was carefully reconsidered.

Sterilization of Seeds

Contamination during the conditioning and stimulation process will influence the germination percentages obtained at the end of the bioassay, and this makes surface sterilization of the seeds necessary. When aqueous solutions containing sodium carbonate or hydrogen peroxide were used, no sterilization was achieved. Aqueous calcium hypochlorite or sodium hypochlorite (2% active chlorine) were only effective for some seed types. No contamination was observed after surface sterilization of the seeds for 5 min with an aqueous solution of sodium hypochlorite (2 g/100 ml active chlorine) and detergent Triton X-100 (1% v/v) followed by extensive washing with water to remove all chlorine.

Detergents have been previously used in seed pretreatment, but the seeds were treated with detergent solution (Tween 20, 0.04% v/v) and hypochlorite solution in separate steps (Fate et al. 1990). According to Okonkwo (1987) detergents facilitate the submergence of tiny seeds, but in our tests *O. crenata* seeds remained floating even when Triton X-100 was present. It is more likely that detergents enhance the effect of the sterilization solution by dissolving fats at the outer layer of the seed coat. Measurement of the direct effect of seed sterilization on the final response to germination stimulants is difficult because, without sterilization, contamination interferes with germination.

Table 1. Germination percentages of parasitic weed seeds after treatment with GR24 (1 mg/L). The effect of conditioning the seeds under different seed/water ratios.

	% Germination ± SE	
	Seed/water ratio during conditioning	
	High[a]	Low[b]
S. asiatica	11 ± 5	29 ± 4
O. crenata	60 ± 6	49 ± 4

[a] Approximately 1500–2000 seeds in 10 ml water (Petri dishes were loaded with the maximum number of "seed sandwiches").
[b] Approximately 150–200 seeds in 6 ml water (Petri dishes were loaded with five "seed sandwiches" according to Parker's procedure).

Conditioning

The physiological mechanisms involved in conditioning which make the seed responsive to a germination stimulant are not at all well understood. It was observed that a low seed/water ratio during conditioning of *Striga* seeds (*S. asiatica*) resulted in higher germination percentages than a high seed/water ratio. For *Orobanche* seeds (*O. crenata*) a high seed/water ratio gave higher germination percentages (Table 1). For this reason the *Striga* seeds were conditioned with five discs (5 × 25 to 50 seeds) in 6 ml of water, whereas *Orobanche* seeds were conditioned with approximately 45 discs (45 × 25 to 50 seeds) in 10 ml of water. The effect of the seed/water ratio on germination of *S. asiatica* seeds has also been observed by Hsiao et al. (1979) and Pavlista et al. (1979a). These authors obtained more reproducible germination results with lower seed/water ratios.

Since it was our aim to design a facile, reproducible, standard bioassay, no attempt was made to improve results by refreshing the conditioning solution after one day or even every day during the conditioning period as suggested by other authors (Okonkwo 1987, Pavlista et al. 1979a).

For *S. hermonthica* it was found that the optimum temperature for conditioning was 23°C, whereas the optimum temperature for the stimulation treatment was 33°C (Reid and Parker 1979). In

Table 2. Germination percentages of parasitic weed seeds after treatment with GR24 (1 mg/L). The effect of covering the seeds with a second glass fiber filter paper disc during conditioning and stimulation.

	% Germination ± SE	
	Without cover[a]	With cover[a]
S. asiatica	18 ± 7	26 ± 4
S. hermonthica	36 ± 7	43 ± 7
O. crenata	41 ± 7	71 ± 3

[a] Cover = second glass fiber filter paper disc to cover the parasitic weed seeds which are spread on top of another glass fiber filter paper disc ("sandwich technique").

order to conduct conditioning and stimulation in the same incubator, a temperature of 27°C was chosen for both treatments of *S. hermonthica* seeds.

It was found also that germination percentages were higher when the seeds were placed between two glass fiber filter paper discs ("sandwich technique"; Table 2). In Parker's procedure (1977), seeds were spread on glass fiber filter paper discs without covering them with a second disc. Another advantage of the "sandwich technique" is that seeds are prevented from floating away from the discs during additions of water or stimulant solution.

The reason for higher germination percentages obtained with the "sandwich technique" may be attributed to an improved contact between water and seed during conditioning and between stimulant solution and seed during the stimulation process. A glass fiber filter paper layer on top of the seeds may also reduce the harmful effect of light during early stages of germination (Worsham 1987).

Stimulant Solutions

Strigol and its analogues show only limited solubility in water and, as a consequence, at least one co-solvent is needed to obtain an aqueous solution of the compounds. Good results are obtained with 0.1% and 0.001% of acetone (v/v) in aqueous solutions of stimulant with 1 and 0.01 mg/L of potential stimulant, respectively (see Figs. 2 and 3). Pepperman et al. (1982) obtained slightly better germination response with dimethylsulfoxide (DMSO) as the carrier; however, since DMSO may also affect membrane permeability, we recommend the use of acetone. Still, the insolubility of some analogues in water (at a concentration of 100 mg/L) remains a problem, even when 10% acetone (v/v) is added. For these compounds, solutions of stimulant may be prepared using pure organic solvents, and after

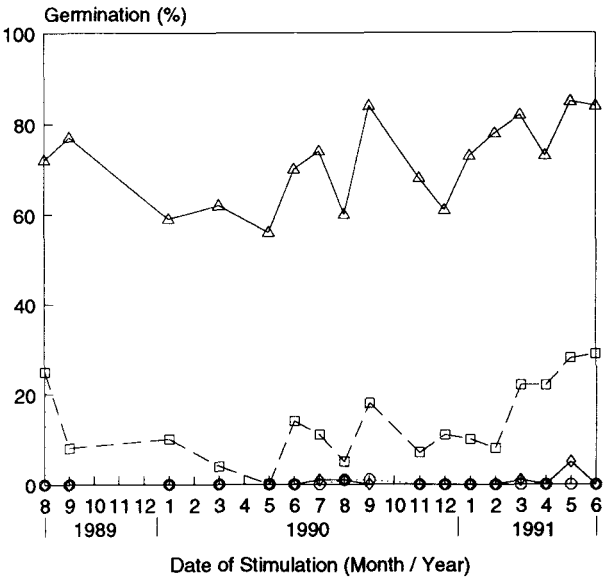


Fig. 2. Germination of *O. crenata* seeds stimulated by solutions of GR24 in the concentration of 1 mg/L (△) and 0.01 mg/L (□), and the corresponding controls with 0.1% (◇) and 0.001% of acetone (○), respectively. During some months more than one test series was run and, in these cases, the experimental points represent the average germination percentage of all tests run during that month.

addition of an aliquot of stimulant solution to a filter paper disc, the organic solvent is allowed to evaporate before pretreated seeds and water are added (Visser 1975, 1982). However, in such a bioassay, it is not clear whether germination is triggered by direct contact between seeds and stimulant crystals or by stimulant molecules which do dissolve in water. Moreover, the concentration of stimulant cannot be specified accurately in these tests.

In a comparison experiment we treated one portion of *S. hermonthica* seeds in the standard manner with a solution of 1 mg/L of GR24 (see Materials and Methods). A second portion of preconditioned seeds was treated with a solution of 1 mg/L of GR24 dissolved in pure acetone and after evaporation of acetone, water was added. For the third portion of seeds, contact between acetone and seeds was prevented by wetting a third glass fiber filter paper disc with GR24 in pure acetone (1 mg/L). After evaporation of acetone, a "sandwich" with seeds was put on top of this "GR24-coated disc" and water was added to the obtained triple layer of glass fiber filter paper discs.

Only the stimulant treatment as described in the standard bioassay gave significant germination. In the case of the second seed portion, acetone may have been toxic for the seeds; whereas for the third seed portion, the poor solubility of GR24 in water

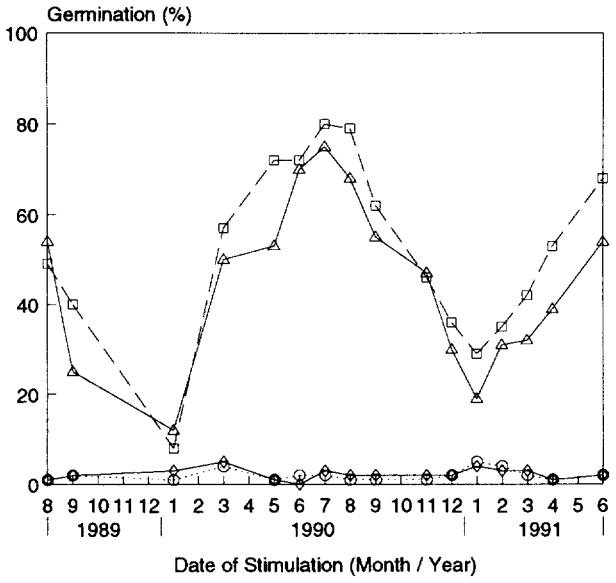


Fig. 3. Germination of *S. hermonthica* seeds stimulated by solutions of GR24 in the concentration of 1 mg/L (△) and 0.01 mg/L (□), and the corresponding controls with 0.1% (◇) and 0.001% of acetone (○), respectively. During some months more than one test series was run and in these cases the experimental points represent the average germination percentage of all tests run during that month.

probably prevented the GR24 from contacting the seeds.

Recently, saturated solutions of strigol analogues in water without any carrier were prepared and dilutions of these saturated solutions were used to stimulate germination of parasitic weed seeds (Vail et al. 1990). The disadvantage of this procedure is that the concentration of stimulant in the solutions is unknown. In Vail's bioassay the solubility of the stimulant in water may be a more important factor in the ultimate germination percentage than the actual biological activity of the compound.

During the present study it was noted that in a stock solution of GR24 (100 mg/L in water/acetone 9:1) within 4 h at room temperature, a solid material appeared at the bottom of the flask, indicating that GR24 crystallized out of the solution. Therefore, we recommend the use of freshly prepared stimulant solutions. Storage of stimulant solutions in a freezer or refrigerator, as described by other authors (Pepperman et al. 1982, Pavlista et al. 1979b), might lead to changes in stimulant concentrations due to precipitation of the compound.

Treatment with Stimulant

The procedure for the actual germination stimulation was essentially the same as described by Parker et al. (1977), except that "sandwiches" of glass fiber filter paper discs with seeds in between were used. In order to ensure good contact between seeds and stimulant solutions, 100 μl of test solution was added to every "sandwich" with seeds.

Control and Reference Compound

In every test series, a control (without stimulant) and a reference stimulant solution (GR24) were included. This is absolutely essential, because sometimes tests can give unreliable results. For *S. hermonthica* seeds, sometimes the same germination percentages were obtained with the control as with the GR24 solution or any other stimulant solution included in that particular test. It was also observed that, although a standard procedure was used, germination percentages obtained with GR24 solutions may vary from test to test (see Figs. 2 and 3).

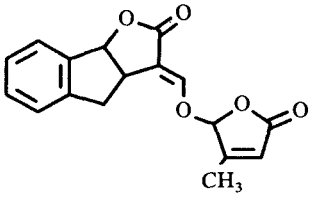
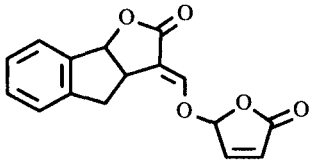
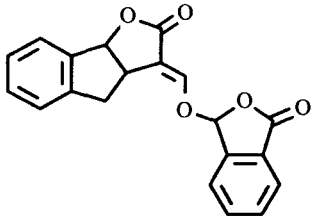
For this reason, a direct comparison between germination percentages obtained with compound X in one test series and compound Y in another test series is impossible. However, by including GR24 in every test series, activities can be expressed with respect to the activity of GR24 and this enables comparison of activities obtained in different test series. Parker et al. (1977) treated results obtained with different root exudates in the same manner. The germination percentages obtained with the root exudates were presented as percentages of the germination caused by the standard root exudate.

A large variation in germination percentages obtained with a standardized stimulating solution was also reported for *O. crenata* seeds (Edwards et al. 1976). The germination percentages varied between 35% and 65%, and for a period of 6–8 weeks in any given year the seeds even failed to germinate. The latter was not observed during the present investigations with *O. crenata* seeds (see Fig. 2). On the other hand, our results indicate that the response of *S. hermonthica* seeds to germination stimulants is season-dependent (see Fig. 3). During wintertime germination, percentages were lower, although seeds were stored under the same conditions throughout the whole year. Since our results cover only approximately 2 years, conclusions about season dependency of germination response of *S. hermonthica* seeds are still premature. Further investigations in this area are needed.

Germination Stimulants

In the standardized bioassay, several strigol ana-

Table 3. Stimulant activities of GR24 analogues with modifications in ring D. The analogues were evaluated using seeds of *S. hermonthica* and *O. crenata*.

No.	Sample/concentration (mg/L)	*S. hermonthica*		*O. crenata*	
		1	0.01	1	0.01
		Relative activity[a] (%) [Absolute activity[b] (% ± $t_{0.05} \cdot (s/\sqrt{n})$)]			
1		106 [40.7 ± 5.7]	—[c] [2.4 ± 1.2]	3 [2.1 ± 1.0]	—[c] [0.5 ± 0.5]
2		53 [20.5 ± 6.4]	—[c] [1.9 ± 1.1]	—[c] [0.3 ± 0.4]	—[c] [0.5 ± 0.5]
3		13 [5.0 ± 1.7]	—[c] [2.3 ± 1.2]	—[c] [0.3 ± 0.4]	—[c] [0.5 ± 0.5]

[a] For the calculation of relative activities, the germination percentages obtained with GR24 (see Fig. 1 for structure) at the same concentration and under the same conditions was fixed at 100%.
[b] The absolute germination percentages are the mean of three replicate tests. In each test the percentage was determined at least 10 times by counting the number of germinated striga seeds in a sample of 25 seeds.
[c] The germination percentages obtained were not significant compared to results obtained in control experiments (without stimulant).

logues have been evaluated for germination-stimulant activity using seeds of *S. hermonthica* and *O. crenata*. Germination percentages have been presented in separate reports for pure diastereomers of GR24 (Mangnus et al. 1992a), for all four optical pure enantiomers of GR7 (Mangnus and Zwanenburg 1992b), and for several AD-, ABD-, and ACD-ring analogues of strigol (Mangnus et al. 1992c) have been presented in separate reports. In Table 3, germination percentages of some analogues of GR24 with modifications in ring D are given. These analogues have previously been described by Johnson et al. (1981) and Hassanali (1984), but exact data on their biological activity were never reported.

In Table 3, the activities have been expressed as percentage of the germination caused by a GR24 solution of the same concentration. In addition, the absolute germination percentages obtained in our standard bioassay are given between brackets. These absolute germination percentages may be of more practical use. However, it should always be realized that these absolute values depend on the bioassay used and on the local conditions as has been illustrated above.

It is obvious from the results presented in Table 3, that modifications in ring D of strigol and analogues have a dramatic effect on the biological activity. *O. crenata* seeds are extremely sensitive to variations in the D-ring, and none of the analogues evaluated gave reasonable germination percentages. For *S. hermonthica* seeds, the 4-methyl analogue (1) and the analogue without methyl group (2) are still active at a concentration of 1 mg/L, but here also the effect of the modifications is significant. From a synthetic point of view, these results are very disappointing, because the analogues presented in Table 3 are much easier to prepare than GR24 itself.

Conclusion

It has been demonstrated that each modification in the procedure for the evaluation of bioactivities of potential germination stimulants for seeds parasitic

weeds may influence the final germination percentages. Even local laboratory conditions and seasonal effects may interfere with the germination response of parasite seeds (as shown in Fig. 3). For this reason, it is *essential* to include a reference compound in every test series. For this purpose, strigol analogue GR24 is recommended; because its synthesis is less complicated than that of strigol, it has better stability properties than the natural germination stimulants, and it is generally accepted as the most active, synthetic germination stimulant. The use of a reference compound in every test series makes it possible to present bioactivities as relative germination percentages with respect to the reference compound. This method of bioactivity presentation enables the comparison of test results obtained in different bioassay runs. However, absolute germination percentages should be reported in addition to relative values in order to show the effective germination of a given stimulant. This can be illustrated by the results obtained with the most active enantiomer of GR7 (Mangnus and Zwanenburg 1992b). For *O. crenata* seeds, the relative germination percentages (with respect to GR24) obtained with this compound at concentrations of 1 and 0.01 mg/L were 98% and 215%, respectively. These values correspond, however, with absolute germination percentages of 77% and 23%, respectively. It is obvious that in such a case neither relative activity nor absolute activity alone effectively presents the results.

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