Stimulating *Orobanche ramosa* Seed Germination with an *Ascophyllum nodosum* Extract

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Algit Super®, an extract of *Ascophyllum nodosum*, was found to be an effective stimulant for the germination of *Orobanche ramosa* seeds. The biological activity of Algit Super® was tested at a wide range of concentrations from 2.5 v/v to 1.2×10^{-3} v/v. The seed germination in all studied *O. ramosa* populations showed a concentration dependence, with an inhibitory effect at higher concentrations. The response of *O. ramosa* to Algit Super® resembled its response to the reference stimulant GR24. Various *O. ramosa* populations demonstrated differential response to the stimulant.
KEY WORDS: *Orobanche ramosa*; GR24; Algit Super®; suicidal germination; population variation; *Ascophyllum nodosum*.

INTRODUCTION

Orobanche ramosa (broomrape) is an achlorophyllous holoparasite that subsists on the roots of many economically important crops such as tobacco (*Nicotiana tabacum* L.) and tomato (*Lycopersicon esculentum* Mill.), especially in Mediterranean and subtropical climates. Research has been carried out on different aspects of the life cycle of *Orobanche* spp. The germination process plays an important role, since the dispersal of these plants occurs exclusively by seed. *Orobanche* seeds are usually triggered to germinate by exposure to specific chemical compounds released by the host plant roots. This chemical signal exchange between broomrape and host plants is indispensable to initiate and complete early development (12). Due to the complex relationships that exist between the parasite and its host, biological control approaches are challenging.

Taking into consideration the importance of chemical stimuli for broomrape seed germination, the simulation of 'suicidal germination' may be considered an attractive approach for keeping the *Orobanche* seed bank in the soil below a certain threshold. A considerable number of research activities have been directed towards the introduction of natural or synthetic chemical stimulants into the soil in order to induce suicidal germination in the absence of the host plants. The stimulation of *Orobanche* seed germination was extensively reported as a promising method for the control of the parasite (5,14,22). Various compounds originating from biological sources were tested as potential stimulants. Both strigolactones (22) and growth regulators (2) have been well documented to facilitate *Orobanche* germination.

Received May 14, 2006; accepted Feb. 27, 2007; http://www.phytoparasitica.org posting Aug. 5, 2007.
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The efficacy of a natural product, extracted from the alga *Ascophyllum nodosum* (Algit Super®), was evaluated for the induction of *O. ramosa* seed germination, suggesting its potential use as a germination stimulant for broomrape control. *A. nodosum* is the most widely utilized alga for the production of the commercial extracts in Europe and North America. It can generally be used as a soil conditioner, source of trace elements, and as a growth stimulant in horticulture and agriculture. Many beneficial effects have been recorded for its use (3). Application of *A. nodosum* extracts to seeds increases germination and early growth (1). Another approach was suggested by Gonsior *et al.* (10), who reported that the control of branched broomrape is possible by application of *A. nodosum* as a means to induce disease resistance to hemp and tobacco.

Much evidence has accumulated regarding the amount and type of growth-regulating substances that exist in seaweed materials, which play an important role in certain plant physiological responses and crop production (4,20). Many registered sea plant products frequently emphasize their effectiveness to plant growth in terms of hormone concentrations (15,16). Meier *et al.* (18) revealed the presence of appreciable quantities of thio-arsenosugars, a novel group of arsenosugars present in the commercial algal product Algit. According to these authors, the presence of arsenosugars in marine biota defines a new era for the environmental arsenic chemistry due to their possible involvement in the biosynthesis of arsenobetaine, the major form of arsenic in marine fauna. Francesconi (9) reported the beneficial effects to plants and animals at low but controlled arsenic exposure. In addition, Evans *et al.* (7) found that arsenic at low levels might be beneficial for corn roots grown in culture.

The present paper examines the stimulatory action of the natural product Algit Super® on the germination of *O. ramosa* seeds.

MATERIALS AND METHODS

Seed sources *Orobanche ramosa* seeds were collected in two provinces of the main tobacco zone in central Greece, from six plant populations (A, B, C, D, E and F) of different physiological maturity, parasitizing tobacco in Greece. Populations A/B, C/D, which originated from Domokos (39°07'30" N lat.), were collected during the years 2002–2003; populations E/F were collected in 2004 from Tithorea (38°36' N lat.).

Germination stimulants The seaweed extract used in the experiments was the commercial algal product Algit Super® (Bioergex), a liquid extract derived from *A. nodosum* (23). Three ppm of the artificial stimulant GR24 (an analog of strigol) was used to promote germination of conditioned *Orobanche* seeds as a reference compound.

Conditioning *Orobanche* seeds were surface-sterilized by immersion in a solution of 2-phenylphenol, Na-salt, a broad spectrum fungicide (13) obtained from Serva Ltd. (Heidelberg, Germany) (0.05% in sterile tap water) for 1 h. After sterilization, the seeds were rinsed with sterile tap water for 30 min and placed in 12-cm-diam petri dishes containing two layers of filter paper wetted with 5 ml of distilled water. All water and filter paper were sterilized by autoclaving before use. The petri dishes were sealed with Parafilm (Pechiney Plastic Packaging Inc., Saint Louis Park, MN, USA) and wrapped with aluminum foil to provide absolute darkness. They were then placed in dark, controlled growth chambers at 24°C for seed conditioning.

Germination tests After 9 days of 'conditioning', the seeds were blotted to remove excessive water. *Orobanche* seeds were then transferred to separate new 9-cm-diam petri dishes with a thin paint brush under aseptic conditions. Each dish contained two layers of filter paper moistened with different concentrations of exogenously applied stimulants. Each disc contained approximately 100 seeds. The dishes were examined and photographed under a binocular stereoscope every 4 days and germination percentage was calculated. One petri dish at a time was taken from the controlled growth chambers for counting, after which the dish was sealed again and immediately put back in its original place. Each counting took less than 5 min per dish. The final radicle length was recorded at the end of the experiment, using Image-Pro Plus version 2.0 (Media Cybernetics). Three separate replicates of each treatment were carried out. Treatments were replicated twice and lasted for up to 30 days.

Two experiments were conducted using *O. ramosa* seeds. The first experiment was designed to investigate the induction of *Orobanche* seed germination using aqueous solutions of Algit Super® at the following concentrations: 2.5 v/v, 1.25 v/v, 0.3 v/v, 0.15 v/v, 0.078 v/v, 0.038 v/v (v/v corresponds to volume of solute per volume of solvent, water in this case). The solutions were applied after the conditioning period as mentioned previously. This experiment was conducted for populations A, B, C and D. The second experiment was designed mainly to investigate to what extent *Orobanche* seeds could respond to lower concentrations of Algit Super®: 2.5 v/v, 1.25 v/v, 0.3 v/v, 0.078 v/v, 0.019 v/v, 0.0048 v/v, 0.0012 v/v. Additionally, IAA (0.01%), cytokinins (0.02%), GA (0.0025%), betaines [$C_5H_{11}NO_2.H_2O$ (0.04 %)] , and a mixture of all four growth regulators were tested at concentrations equivalent to their concentations in Algit Super®, as reported by its producer. The second experiment included also populations E and F, in addition to populations A – D.

Algit Super® cleanup The commercial product was cleaned up to investigate the possible action of the novel group of arsenosugars included in the active fractions. For isolation of the active fraction of Algit Super®, the solution passed through a C_{18} solid-phase extraction (SPE) cartridge (Bakerbond spe™ C_{18}; J.T. Baker Inc., Phillipsburg, NJ, USA). A manifold (SPE-12G glass manifold) was used carrying up to four SPE cartridges. C_{18} columns were activated with 20 ml methanol and washed with 20 ml of deionized water. 2 ml of the commercial product Algit Super® was loaded onto the C_{18} column. The cartridge was washed with 4 × 10 ml solution of 20 mM $NH_4H_2PO_4$ (18) and four fractions were collected. All fractions were then evaluated for their capacity to stimulate *Orobanche* seed germination.

Statistical analysis The percentage of final germination was calculated as the ratio between the number of seeds germinated and the total number of seeds used. The results for the first experiment are expressed as mean values of three replicates ± S.E. In the second experiment the results were analyzed using analysis of variance (ANOVA), as a factorial combination of the treatments. Furthermore, mean separations were determined by the least-squares means (LSM; Tukey). In order to allow comparison between seed populations that differ in viability, the germination data should be compared to germination response to GR24 rather than to the total number of seeds.

Algit Super® concentrations were Log-transformed for the dose response analysis. Visual inspection of the dose-response scattergrams showed clearly that our concentration–

germination curves were bell-shaped, exhibiting a dip after reaching a maximum germination level. Therefore, the following Gaussian curve equation was used to fit the data *via* nonlinear regression (19):

$$G = Basal + Range + e - \frac{10^{Log[A]} - midA}{slope}$$

$$midA = LogEC_{50} + slope\sqrt{-In(0.5)}$$

where G denotes germination, Basal denotes the minimum germination and it was constrained to zero, Log[A] is the logarithm of the concentration, slope is a slope factor, $LogEC_{50}$ is the logarithm of the midpoint location parameter and Range represents the maximum germination range over the Basal value. Data are shown as means ± S.E. $LogEC_{50}$ values, maximum germinations and slopes between the different curves were compared using an extra-sum-of-squares (F-test) and values of $P<0.05$ were considered significant. Statistical analysis was performed using the statistical package GraphPad Prism version 4 (19).

RESULTS AND DISCUSSION

Seed germination *Orobanche ramosa* seeds obtained from six populations were assayed for their ability to germinate after Algit Super® treatment. The first experiment indicated that populations (A, B, C, D) responded to Algit Super® concentration of at least 1.25 v/v, with maximum germination values at a concentration of 0.038 v/v (Table 1). The maximum germination percentage was population-dependent. In particular, populations A and C demonstrated higher germinability at 0.038 v/v, whereas populations B and D showed a moderate response at the same rate. In all cases the germination induced by the aqueous control solution was practically zero. It is notable that Algit Super® usually did not stimulate *O. ramosa* germination to the extent reached after GR24 stimulation (Table 1).

In the second experiment, the biological activity of Algit Super® was tested at a wider range of concentrations, beyond the active concentration of 0.038 v/v. In particular, populations A, C and E exhibited a high germination rate at 0.019 v/v, with 78.5%, 65.8% and 60.8% germination, respectively. These populations responded to GR24 treatment with germination rates of 100%, 71.5% and 74.3%, respectively. On the other hand, populations B, D and F had a lower germination rate. Interestingly, all populations assayed showed similar germination response, not statistically differentiated, compared with that induced by GR24 (Table 2). Maximum germination was obtained at 0.019 v/v Algit Super.

Response of *O. ramosa* populations to Algit Super® The differential response of *O. ramosa* seeds to the various Algit Super® concentrations shows a typical Gaussian curve (Fig. 1). The data indicated a significant influence of Algit Super® on populations A, C and E, whereas populations B, D and F responded in a lower rate (Fig. 1). These data are in accordance with the dose response curves of other germination stimulants, like GR24 and Nijmegen-1 (22). The estimated regression parameters ($LogEC_{50}$, Potential Maximum Germination and Slope) are shown in Table 3. $LogEC_{50}$ and slope values between populations A, B, D and F did not differ significantly, but were significantly higher than the corresponding $LogEC_{50}$ values of populations C and E. Moreover, populations C and

E demonstrated significantly steeper slopes, with maximum germination at approximately 65% and 95%, respectively.

TABLE 1. Seed germination (mean values±S.E) of *Orobanche ramosa* populations as influenced by Algit Super at different concentrations; 2.5 v/v concentration led to zero germination (GR24 and water were used as controls)

Population	Concentration, v/v					H_2O	GR24
	1.25	0.3	0.15	0.075	0.038		
A_{2002}	2.8±3.5	9.2±2.6	26.0±5.4	36.5±6.8	47.7±8.9	7.3±5.2	84.3±4.7
B_{2002}	3.4±2.8	10.5±4.6	15.9±5.3	23.9±9.3	29.8±2.8	6.2±4.2	25.0±5.2
C_{2003}	9.7±6.2	26.7±6.5	29.7±6.4	22.2±10.2	59.7±10.4	0	75.8±10.2
D_{2003}	6.8±2.3	23.1±2.8	18.5±4.8	19.9±3.7	19.2±5.4	2.5±2.8	28.0±1.6

TABLE 2. Seed germination of *Orobanche ramosa* populations as influenced by Algit Super at different concentrations; 2.5 v/v concentration led to zero germination (GR24 and water were used as controls)

Population	Concentration, v/v						H_2O	GR24
	1.25	0.313	0.078	0.019	0.0048	0.0012		
A_{2002}	24.3cdef[z]	38.9bcdef	39.8bcdef	78.5ab	32.7bcdef	31.7bcdef	15.0def	100.0a
B_{2002}	17.4def	15.5def	13.5def	20.4def	14.9def	14.8ef	16.4def	31.5bcdef
C_{2003}	3.6ef	10.9def	3.3ef	65.9abc	36.5bcdef	13.5def	4.9ef	71.5abc
D_{2003}	17.1def	19.9def	13.9def	15.1def	11.8def	9.1def	1.4ef	18.5def
E_{2004}	2.8ef	5.6ef	5.8ef	60.8abc	8.5ef	2.8ef	0.9f	74.3ab
F_{2004}	18.1def	23.7cdef	28.1bcdef	20.6def	11.2def	16.9def	18.7def	33.7bcdef

[z]Within rows, values followed by a common letter do not differ significantly at P=0.05 (LSM, Tukey).

TABLE 3. Best fit values (Log EC_{50}, Maximum Germination and Slope) (±S.E.) from the concentration – germination curves, according to non-linear regression, of *Orobanche ramosa* populations after Algit Super treatment

	Population					
	A_{2002}	B_{2002}	C_{2003}	D_{2003}	E_{2004}	F_{2004}
Log EC_{50}	-0.5±0.3a[z]	-0.1±0.3a	-1.5±0.1b	-0.2±0.2a	-1.4±0.1b	0.0±0.3a
Max Germ.	61.6±10.5b	21.5±3.0a	64.3±5.6b	18.3±2.4a	95.6±21.3b	27.8±3.8a
Slope	-1.5±0.3a	-1.8±0.4a	-0.6±0.1b	-1.5±0.2a	-0.4±0.0b	-1.6±0.3a

[z]Within rows, values followed by the same letter do not differ significantly (P<0.05).

Aitken and Senn (1) treated several species of seeds (tobacco, pea, radish, cotton, white pine and *Ligustrum*) with a range of seaweed extract concentrations from 20 v/v to 0.2 v/v and observed increased respiratory activity of the seeds in high as well as in low extract concentrations, which may correspond to our results with Algit Super®. These researchers also refer to a species dependence effect, because the optimum concentration of extract activity on seed germination varied considerably with the different species.

The data obtained from Algit Super® showed different requirements of the various *O. ramosa* populations for germination activity. Taking into consideration the different populations' origins, we may assume that differentiation in the germinability of *Orobanche*

TABLE 4. Seed germination (%) of *Orobanche ramosa* populations after treatment with growth regulators (mean values±S.E.) (mixtures of growth regulators led to zero germination)

Population	Growth regulator			
	GA	IAA	Cytokinins	Betaines
A_{2002}	29.2±4.2	33.9±5.0	51.6±11.6	12.8±2.5
B_{2002}	20.6±6.2	28.1±10.8	19.4±2.1	9.8±4.4
C_{2003}	10.5±0.7	8.4±2.4	12.3±1.8	3.3±1.5
D_{2003}	18.9±4.3	18.5±1.8	11.9±2.0	8.9±2.2
E_{2004}	7.6±2.8	11.7±4.5	22.8±3.2	2.7±0.4
F_{2004}	0.8±0.9	5.5±0.6	4.7±1.1	0.4±0.8

TABLE 5. Seed germination (%±S.E.) of *Orobanche ramosa* populations after Algit Super®cleanup using the Solid Phase Extraction method (SPE) (seeds responded only to the second fraction)

Population	2nd Fraction of Algit Super®	Algit Super®= 0.019 v/v
A_{2002}	15.1±2.2	78.5±9.4
B_{2002}	8.2±1.2	20.4±6.1
C_{2003}	19.7±3.1	65.9±4.1
D_{2003}	5.9±1.9	15.1±1.2
E_{2004}	22.4±4.6	60.8±6.8
F_{2004}	5.9±1.9	20.6±3.1

seeds may be attributed to the local environmental factors prevailing in these regions. Local conditions may increase differentiation in seed maturity during development, suggesting that these populations have obtained different environmental signals for secondary dormancy or 'wet dormancy' (11,21). The differences in the expression of 'wet dormancy' may also be due to seed age. Matthews *et al.* (17) have also reported differentiation in the germination response of broomrape seeds at the interspecific level.

Algit Super® components Our interest focused on the activity of growth regulators incorporated in Algit Super® such as IAA, GA, cytokinins, betaines and their mixture, at concentrations registered by the Algit Super® producer. Populations A and B germinated at a moderate level after the phytohormone treatments ranging from 20% to 50%, whereas the other populations demonstrated lower seed germinability. Betaines also proved only slightly effective, while the mixture of all four growth regulators was insufficient to trigger seed germination (Table 4). Egley (6) reported the effect of exogenous stimulants, GA_3 or ethylene, that apparently increased seed germination of *Striga lutea*, while the application of a growth regulator mixture on *Orobanche* seeds was also evaluated with contradictory results (2,24). Foy *et al.* (8) observed a moderate response of *Orobanche* spp. to gibberellic acid and an even lower response to cytokinins and auxins.

TABLE 6. Radicle length of *Orobanche ramosa* populations as influenced by Algit Super at different concentrations (2.5 v/v concentration led to zero germination), at the end of the measurements (GR24 and water were used as controls)

Population	Concentration, v/v						H_2O	GR24
	1.25	0.313	0.078	0.019	0.0048	0.0012		
A_{2002}	2.1abc[z]	2.8abc	1.9abc	2.3abc	2.7abc	3.7abc	3.2abc	2.6abc
B_{2002}	3.4abc	3.8abc	4.7a	2.8abc	3.0abc	3.6abc	4.3ab	4.1ab
C_{2003}	2.5abc	3.5abc	3.4abc	2.1abc	3.0abc	2.9abc	4.5ab	3.0abc
D_{2003}	2.3abc	3.1abc	3.4abc	2.8abc	3.2abc	3.1abc	2.1abc	2.8abc

[z]Within rows, values followed by a common letter do not differ significantly at P=0.05 (LSM, Tukey).

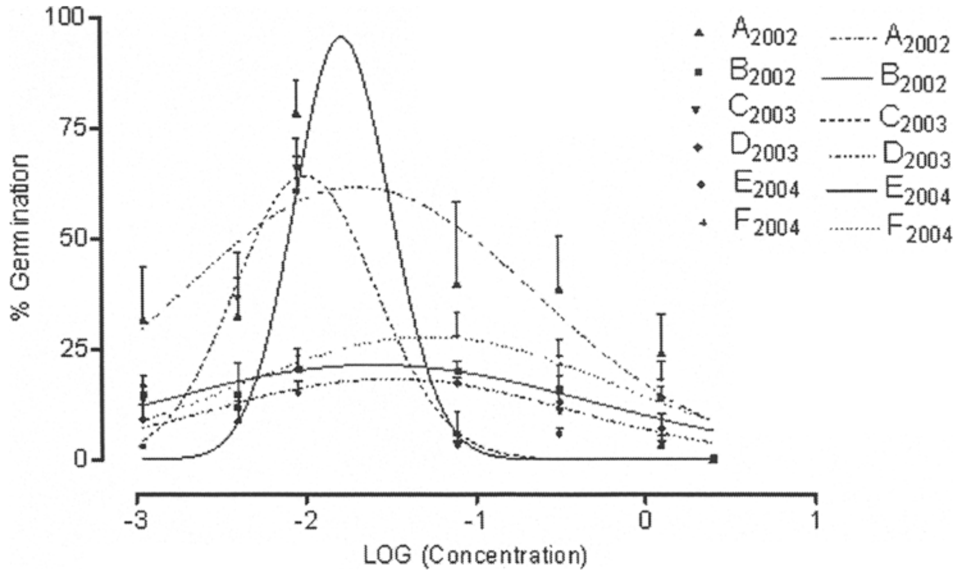

Fig. 1. Germination curves for six populations (A, B, C, D, E, F) of *Orobanche ramosa* seeds after treatment with Algit Super at concentrations of 2.5, 1.25, 0.313, 0.078, 0.019, 0.0048 and 0.0012 v/v (log-transformed). The lines were fitted according to non-linear regression analysis and the data are presented as means ± S.E.

After the investigation of the stimulatory activity of four Algit Super® fractions, we found that seeds of *Orobanche* populations responded to the second fraction (Table 5), with lower germination values compared with the active dose of Algit Super® (0.019 v/v). Taking into consideration previous studies (7,9,18), this fraction may be enriched with arsenosugars, so it is interesting to speculate on their possible stimulatory role on *O. ramosa* seeds. However, pure arsenosugars have so far not been tested for an effect on *Orobanche* seed germination.

***Orobanche ramosa* radicle elongation** Algit Super® promotes the radicle's elongation of all *O. ramosa* populations tested. In particular, the radicle length observed in the populations ranged from 1.374 mm [Algit Super®=0.0012 v/v] to 4.67 mm [Algit Super®=0.078 v/v]. Similar rates were recorded in all populations studied, following GR24 treatment (Table 6). However, it should be noted that Algit Super® activity was slightly higher when compared with that of GR24. It was previously reported that seaweed materials, when applied to ornamentals, contribute significantly to a denser and elongated root system (1). The combination of growth regulators (auxins, gibberellins, cytokinins, betaines) and probably the higher concentrations of nutrients and minerals that exist in *A. nodosum*, may stimulate a faster radicle growth (7).

It can be derived from the data obtained that the stimulatory activity of Algit Super® is based entirely on the interacting components in their original relative concentration. This assumption is reinforced by the lower stimulatory effect of its second active fraction in comparison with the data from the commercial product. Future work should investigate the biochemical involvement of arsenosugars in the behavior of Algit Super®. However, the results obtained in the present research should be of great value in establishing the groundwork for explaining the specific mechanism of stimulation by *A. nodosum.* Additional research is needed in order to study the efficacy of Algit Super® on other *Orobanche* species (work in progress). The encouraging outcome of this study is that Algit Super® has seed stimulant activity comparable to the synthetic strigol analog GR24. The differentiation which is observed among *Orobanche* populations when specific doses are applied, also broadens our spectrum of study. Algit Super® can be applied in the field in order to induce or inhibit the germination of *Orobanche* seeds resulting in depleting the soil of broomrape seeds. Another long-term prospective is to examine its effectiveness when used in a parallel way to trap or catch crops, taking into consideration the longer radicle obtained when seeds are stimulated by Algit Super®. In conclusion, it is apparent that our plans for future research are multi-faceted.

ACKNOWLEDGMENTS

We thank Professor Klaus Wegmann who kindly provided the stimulant GR24 and our colleagues from COST Action 849, Professor J. Streibig, Dr. D.M. Joel and Dr. A. Murdoch, for their useful advice and guidance.

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