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Differential induction of *Orobanche* seed germination by *Arabidopsis thaliana*

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Abstract

Parasitic plants, including the root holoparasites *Orobanche* spp., cause devastating damage to crops worldwide. *Arabidopsis thaliana* (L.) is widely used an amenable model for the study of plant biology, including plant–pathogen interactions. Bringing the two plants together in a controlled system will enable the study of the molecular and genetic basis involved in host-parasitic plant interactions and should provide tools for the detection of genes responsible for incompatibility and resistance responses. The objective of this study was to screen *Arabidopsis* lines for reduced germination of *Orobanche* seeds. A 96-cell well bioassay was developed to test the potential of lines, ecotypes and mutants of *Arabidopsis* to induce germination of *Orobanche*. Screening of 50 *A. thaliana* ecotypes did not reveal non-inducing ecotypes. Screening of 13 000 *A. thaliana* fast neutron mutated M2 plants detected 94 non-inducing mutant plants of which 34 were rescued, self pollinated, and M3 seeds collected. M3 seedlings from five lines were reduced in their ability to induce germination. In a separate assay, we determined that the reduced germination rates corresponded with reduced distance from the roots at which germination occurred. While further studies are necessary to determine the segregation of low germination phenotypes, these lines might prove useful for studying the genetic basis of variation in germination stimulant production in *A. thaliana*.

Keywords: *Arabidopsis thaliana*; *Orobanche ramosa*; *Orobanche aegyptiaca*; Fast-neutron mutagenesis; Parasitic plants; Seed germination induction

1. Introduction

Parasitic plants account for approximately 1% of extant angiosperm species and are represented in 22 plant families [1]. Several of the parasitic species are important agricultural weeds, particularly parasitic weeds in the closely related families Scrophulariaceae and Orobanchaceae. While genetic host resistance is generally considered a critical component of integrative pest management, there is currently little known about genetic resistance against parasitic weeds. The parasitism of plants by other plants is a multi-step process requiring signal exchanges between both players in the association [2]. The elimination or modification of host factors required by the parasite may prove a useful strategy for engineering resistance against these agricultural pests. This study was directed toward identifying host genes in *Arabidopsis* that contribute towards its susceptibility towards *Orobanche*. The family Orobanchaceae includes 14 genera of chlorophyll-lacking root holoparasites of which the most important genus is *Orobanche*, known in its common name as broomrape. Among the different *Orobanche* species, *O. ramosa* and the very similar *O. aegyptiaca* have the widest host range and inflict heavy damages to numerous important agricultural crops such as tomato, potato, tobacco, eggplant, faba bean, vetch, lentil, peanut, carrot, celery, parsley, sunflower and *Brassica* spp. [3,4].

The *Orobanche* lifecycle is highly specialized for parasitism [5,6]. Seeds germinate in the soil only

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after a preconditioning period of moist and suitable temperatures, and only in response to a specific chemical germination stimulant exuded by host plant roots, ensuring that only seeds within the host root rhizosphere will germinate. The parasite seedling radicle grows only a few mm and must contact a host root to ensure its existence. Upon contact with the host root, the radicle develops a specialized parasitic-plant organ, the haustorium, which adheres to the root, penetrates the epidermis and cortex tissues of the root, and ultimately establishes connections to the host vascular system [7–9]. Through these connections the parasite draws water and its nutritional needs from the host. This connection leads to the swelling of a parasite tubercle followed by a floral shoot that emerges above the soil surface. The plants rapidly develop flowers that produce capsules containing hundreds of thousands of tiny seeds per plant [10,11].

One of the first stages in plant parasitism that can be targeted for genetic control is seed germination, a highly complex procedure in *Orobanche* and other obligate root parasites. Because the seeds are so small, once they have germinated they must rapidly attach to a host plant root before the seed resources are exhausted, typically within a few days. One of the parasites strategies for ensuring the presence of a potential host root is to require chemical signals released by the host roots, called xenognosins, for germination [12]. This allows the seeds to germinate within a few millimeters of a suitable host root [3]. Specific xenognosins identified for *Striga* germination include three tetracyclic sesquiterpenes: strigol isolated from cotton, a non-host of *Orobanche* and *Striga* [13], sorgolactone, released by *Sorghum bicolor* [14] and alectrol, identified in *Vigna unguiculata* roots [15]. An additional *Striga*-germinating xenognosin, *the* hydroquinone sorgoleone, was also detected in roots of *S. bicolor* [16]. Strigol and its synthetic analogue GR24 are widely used to induce germination of *Striga* and *Orobanche* seeds in laboratory studies. Recently an *Orobanche minor* xenognosin named orobanchol has been identified in the roots of *Trifolium pratense* [17]. However despite recent progress, the number, identities and mode of action of most xenognosins remain unknown [18].

In at least one case, resistance to the parasitic weed *Striga* results from the low germination stimulant production by host roots. The sorghum cultivar 1S-7777 is more resistant to *Striga* apparently because of the presence of a single recessive mutation in germination stimulant production [3,19]. There is little information on mechanisms of tolerance or resistance to *Orobanche* due to low germination stimulant production by host roots [20]. Racovitza [21] found a wide range of *O. ramosa* susceptibility and germination stimulant production in tobacco cultivars in Romania. The most resistant tobacco cultivar 'Joiner' showed relatively low exudation of germination stimulant. Alders and Pieters [22] report reduced production of germination stimulants as one of the possible mechanisms for the low number of *O. crenata* spikes on resistant faba bean cultivars. Hershenhorn et al. [23] report that tolerant pepper varieties stimulate low germination of *O. aegyptiaca*.

Arabidopsis thaliana (L.) Heyhn is a small annual plant belonging to the mustard family (Cruciferea or Brassicaceae) found throughout temperate regions of the world in Europe, Asia and North America [24]. *Arabidopsis* is an attractive model organism for plant genetic studies because of its small size, the lack of repetitive DNA in its genome, and because of the number of genetic variants assembled by workers around the world. The eminent release of the entire *Arabidopsis* genomic sequence further advances its potential as a model organism (TAIR (2000) TAIR: The *Arabidopsis* Information Resource. NCGRI and Carnegie Institute of Washington. http://www.arabidopsis.org/index.html). Bringing *A. thaliana* and *Orobanche* plants together in a controlled system enables the study of physiological, molecular and genetic basis involved in host-parasitic plant interactions, thus providing practical tools for the detection of genes responsible for incompatibility and resistance responses.

In earlier studies we have demonstrated the susceptibility of *A. thaliana* 'Columbia' to *O. aegyptiaca*, *O. ramosa and O. minor* [25]. While there are multiple developmental stages in the parasitism of *Arabidopsis* by *Orobanche* that could be targeted for mutagenesis, in this manuscript we present results screening *Arabidopsis* for defective *Orobanche* germination. Our hypothesis was that inactivation of host genes responsible for synthesis, modification, or release of germination factors by *Arabidopsis* by mutagenesis will result in loss of parasite seed germination potential. The objectives

of the present study were to screen *Arabidopsis* ecotypes and mutants for differential germination induction of *O. ramosa and O. aegyptiaca* seeds.

2. Materials and methods

2.1. Plant material

2.1.1. Arabidopsis

Seeds of wild type and Fast Neutron M2 populations of *A. thaliana* Columbia with the *gl*1 marker were purchased from Lehle Seeds (Round Rock, Texas) and stored at room temperature. The *Arabidopsis* ecotypes seeds were obtained from the *Arabidopsis* Biological Resource Center at The Ohio State University (ABRC (1999) Arabidopsis Biological Resource Center. http://aims.cps.msu.edu/aims/).

2.1.2. Orobanche

O.ramosa L. seeds were collected in 1998 from flowering plants parasitizing a tomato field near Courtland California. *O. aegyptiaca* Pers. seeds were obtained from Dr D. Plakhine in Newe Ya'ar Research Center, Israel. These seeds were collected in 1994 from a heavily infested tomato field in Gesher Haziv, on the Coastal Galilee, Israel. *Orobanche* seeds were handled under quarantine conditions (APHIS permit # 38 698; CDFA permit # 57-33-98).

2.2. Seed sterilization, conditioning and plating

Arabidopsis seeds were surface sterilized in 70% methanol in 15 ml disposable centrifuge tubes agitated on a gyrotory mechanical shaker for 5 min. The methanol was replaced with a solution of 1% sodium hypochlorite and 0.1% Triton X-100 detergent and agitated an additional 10 min. The sodium hypochlorite was removed and the seeds were washed three times with sterile de-ionized water. Seeds were then suspended in 0.1% noble agar and plated in 9 cm Petri dishes containing 1/4 strength MS (Murashige and Skoog Salt Mixture), 0.75% sucrose and 0.4% Phytagar. Petri dishes were sealed with Paraflim, placed for 2 days in the dark at 4°C and then transferred to a 25°C growth chamber under 18 h light of 65 $\mu Es^{-1}m^{-2}$ for 7 days.

Orobanche seeds were surface sterilized in an empty sterilized tea bag. The bags were sealed, inserted into a 50 ml disposable centrifuge tube containing 25 ml of 70% methanol, and agitated on mechanical shaker for 1 min. The methanol was replaced with 25 ml of 1% sodium hypochlorite and 0.1% Triton X-100 and agitated for 30 min. The seed-containing bag was washed 5 times with sterile de-ionized water.

For preconditioning, sterilized *Orobanche* seeds were suspended in water and transferred by vacuum onto a 9 cm GF/A glass fiber filter disc. The dry inoculated filter was then placed in a 9 cm petri dish and moistened with 2.5 ml de-ionized sterile water. Petri dishes were sealed with Parafilm, wrapped with aluminum foil and placed in a 25°C growth chamber. *O. ramosa* seeds were preconditioned for 14 days and the *O. aegyptiaca* seeds for 7.

2.3. Orobanche germination assay

Arabidopsis seedlings were screened for their ability to germinate *O. ramosa* in 96 well tissue culture plates (Falcon 3872, Becton Dickinson, New Jersey). The screening procedure is outlined in Fig. 1. Preconditioned *O. ramosa* seeds were suspended in de-ionized sterile water and 20–50 seeds added to each well with a micropipette (20 μl suspension). One hundred μl $\frac{1}{4}$ strength MS without sucrose and containing 0.6% phytagar (45°C) was added on top of the *Orobanche* seeds in each well. Seven-day-old *A. thaliana* plants were transferred from their germination plates into the 96 well plate, one plant per well. In each plate eight

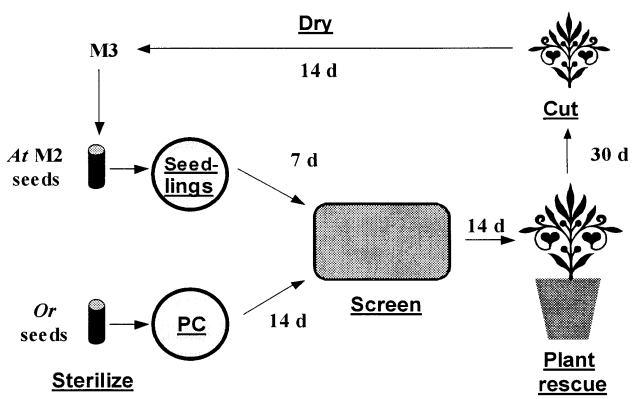

Fig. 1. *A. thaliana* mutant screen bioassay flow chart. *At*, *Arabidopsis thaliana; Or*, *Orobanche ramosa;* PC, Preconditioning. See Section 2 text for details.

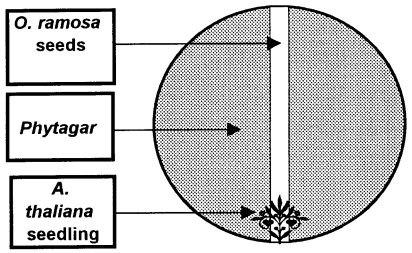

Fig. 2. Maximum Germination Distance (MGD) Petri dish bioassay. See Section 2 text for details.

wells were planted with the wild type *A. thaliana* 'Columbia' and eight wells left without host plants as controls. Plates were then sealed with micropore surgical tape and placed in clear plastic storage boxes in a 25°C growth chamber under 18 h light (65 $\mu Es^{-1}\ m^{-2}$). Germination of *Orobanche* seeds was scored 7 and 14 days later by inverting the plates and examining the seeds under the stereoscopic microscope.

After scoring, *Arabidopsis* seedlings that did not induce *Orobanche* germination were rescued from wells and transferred to 2'' square pots containing moist Sunshine soil mix # 1. The pots were placed into closed magenta boxes, which were then placed in a 23°C continuous light (90 $\mu Es^{-1}m^{-2}$) growth chamber. Lids of magenta boxes were removed after 3 days and plants were replenished periodically with nutrient solution. One month after planting, seed-bearing *Arabidopsis* plants were cut at soil level and dried at room temperature in a paper bag. Seeds were isolated two weeks later using a 500 micrometer sieve and stored in microfuge tubes at 4°C.

2.4. *O. ramosa* germination rate and maximum germination distance assay

O. ramose germination rate and the maximum germination distance of *Orobanche* seeds from host roots (MGD) were bioassayed in 50 × 11 mm Petri dishes. Six ml 0.7% Phytagar were poured into each Petri dish containing a 200 ml pipette tip positioned in the center of the Petri dish. After the agar was hard, the tip was removed leaving a 3–5 mm wide groove in the agar. Approximately 100 preconditioned *O. ramosa* seeds were added evenly to the groove in 350 μl of warm 0.6% Phytagar. A 1-day-old *A. thaliana* seedling was then placed on one side of the groove (Fig. 2). Petri dishes were sealed with parafilm, wrapped with aluminum foil and placed in a 25°C growth chamber. Seven days after planting, the number of germinated *O. ramosa* seeds and the maximum germination distance from *A. thaliana* roots were recorded under a stereoscopic microscope. Controls plates included *Orobanche* seeds without host plants or with 50 μl GR24.

3. Results and discussion

3.1. Stimulation of Orobanche seed germination by *A. thaliana* seedlings

In previous studies we revealed that *O. ramosa* and *O. aegyptiaca* seeds germinate in the presence of factors released from *A. thaliana* roots [25]. In the 96-well plate assays, *O. ramosa and O. aegyptiaca* seeds began to germinate within 3 days of plating with 7-day-old *A. thaliana* seedlings. Germination reached a maximum rate after 7–10 days (Fig. 3). By 2 weeks, germination rates *for O. ramosa and O. aegyptiaca* were typically 40–50% and 60–70%, respectively. In contrast, the spontaneous germination rate without *A. thaliana* hosts is 0.05–0.10% for *O. ramosa* and 1–2% for *O. aegyptiaca*. The synthetic germination factor

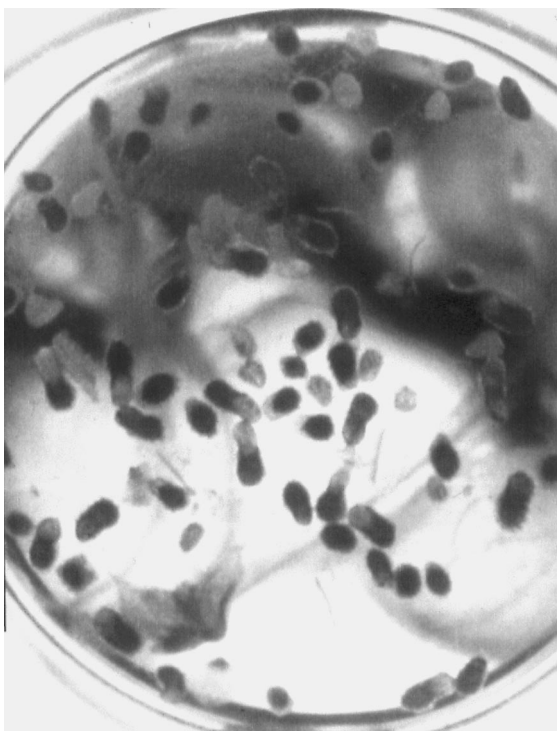

Fig. 3. *O. ramosa* seeds germinating in response to *A. thaliana* root induction in the 96-well plate bioassay, 7 DAP.

Table 1
A. thaliana ecotype screening for non-induction of *Orobanche* seed germination

ABRC[a] Number	Abbreviation	Number of plants tested	Ecotype origin
900	Aa-0	8	Germany
904	Mh-0	8	Poland
905	Ms-0	3	Moscow
911	Est	6	Germany
916	Condara	7	Tajikistan
917	Da	8	Czechoslovakia
919	Di-M	7	Moscow
921	En-T	6	Tajikistan
922	Hodla-Obi-Garm	4	Tajikistan
923	H55	8	Czechoslovakia
924	Je54	4	Czechoslovakia
925	Litva	7	Lithuania
926	Petergof	7	Russia
927	Rubezhnoe-1	6	Ukraine
928	Rubezhnoe-1	8	Ukraine
929	Shahdra	6	Tajikistan
930	Sn	8	Czechoslovakia
931	Sorbo	5	Tajikistan
3081	No-0	5	Germany
3109	Ber	8	Denmark
3110	Wei-1	3	Switzerland
3112	M7323S	6	Dr Knapp-Zinn
3179	Gr3	4	Austria
3180	Co	8	Portugal
6002	–	8	Italy
6003	–	8	Germany
6004	–	8	UK
6005	–	8	UK
6006	–	8	UK
6013	–	8	UK
6014	–	8	UK
6016	–	6	UK
6017	–	6	UK
6019	–	8	UK
6021	–	7	UK
6022	–	6	UK
6024	–	3	UK
6025	–	7	UK
6028	–	7	UK
6030	–	7	UK
6033	–	6	Netherlands
6035	–	6	France
6036	–	7	France
6037	–	7	France
6038	–	6	Germany
6039	–	6	Germany
6040	–	6	Germany
6041	–	7	Germany
6187	–	7	Seattle
6194	Bla-2	6	Spain

[a] ABRC, Arabidopsis Biological Resource Center, The Ohio State University, Columbus Ohio.

GR24 induces 50–60% and 80–90% for each under these conditions.

We assayed three to eight seedlings of fifty different *A. thaliana* ecotypes for their ability to induce germination of *O. aegyptiaca* (Table 1). All *Arabidopsis* examined induced gennination of *O. aegyptiaca* to a significant degree over background. There were no significant differences in the germination rates between the ecotypes. Similar results were obtained by Westwood and Foy [26], who screened 303 *Arabidopsis* ecotypes for their susceptibility to *O. aegyptiaca* and found them all susceptible.

3.2. Screen of M2 Arabidopsis populations for Orobanche germination

We next screened individuals of a fast neutron mutagenized *A. thaliana* M2 population for their ability to germinate *Orobanche* seeds. Over 13 000 M2 individuals, representing 1625 M1 parents were assayed in the 96 well assay for their ability to germinate *O. ramosa*. Ninety four *A. thaliana* (0.7%) did not stimulate germination in the first round of screening (Table 2). After 14 days, these *Arabidopsis* seedlings were rescued from the plate and transferred to soil. Thirty four non-germinating *A. thaliana* seedlings grew to maturity and set seeds. The M3 seeds were recovered for further analysis.

M3 families were assayed for *O. ramosa* germination capacity in the single cell plate assay. The number of *Arabidopsis* M3 seedlings that did not induce *Orobanche* germination ranged from 0 to 20% from each line. Data from the 96-well plates screening experiments was first subjected to a χ^2-test performed to examine if results were comparable from one plate to the other. The χ^2-test detected no significant plate-to-plate differences so the replications were pooled into a 2 by 2 Line contingency table. χ^2 suggested that there were significant differences in the number of sibs that germinated *Orobanche* between the different families ($\chi^2 = 65.04$, $P < 0.0001$). Pairwise comparisons between all the families revealed that the families fell into two groups: those with greater than average number of non-inducing sibs and those with less (Table 3). The five lines belonging to the non-inducing group, 26A5, 27C9, 27G3, 116B7, and 75A2 were examined further.

Table 2
Summary of *A. thaliana* mutant screening for non-induction of *O. ramosa* seed germination[a]

A. thaliana description	Number	Percent
Fast Neutron M2 Seedlings	13 000	
Non-Inducing M2	94	0.7
Non-Inducing M2 Rescued	34	36.2
Low-Inducing M3	5	14.7

[a] Fast Neutron M2 populations of *A. thaliana* Columbia with the *gl*1 marker were used for screening studies.

Table 3
Segregation of non-inducing *A. thaliana* M3 lines in the 96-well plate bioassay[a]

M3 line	Number of plants tested	% Non-inducing
3E5	161	13
8C1	162	1
26E3	24	4
26A5	52	17*
26H5	41	5
27C9	66	18*
27G3	47	17*
27G10	51	10
33C6	67	4
51F1	91	10
52C7	79	2
58A10	77	0
81F2	22	0
90A3	52	4
92B9	27	0
93B3	42	9
93E3	29	0
94C2	33	0
94F5	32	6
98E9	32	6
98E4	42	12
115G7	30	3
116B7	42	17*
116F1	26	4
6A1	10	0
17A2	10	0
17C6	10	0
21H3	10	0
37F8	10	10
71A8	10	0
75A2	10	20*
90A5	10	0
102B4	10	0
102G8	10	0

[a] Lines marked with an asterisk were significantly higher than normal in non-inducing plants.

3.3. Maximum germination distance (MGD) is decreased in low inducing lines

We measured the maximum distance from the root that *Orobanche* germinated in the presence of

each *Arabidopsis* mutant. The assay is similar to the one used by Hauck et al. (1992) to monitor *Striga* seed germination factors. Results were analyzed according to the General Linear Model Procedure using the Student–Newman–Keuls t-test, $\alpha = 0.05$.

No germination was observed in the Petri dishes without *A. thaliana* plants (the negative control), indicating that no spontaneous germination occurred. Forty nine percent germination was detected in Petri dishes treated with GR24, representing the maximum germination potential of *O. ramosa* seeds in this trial. Induction rates of all M3 lines ranged from 0 to 45 per Petri dish while the 5 selected low germination mutants that were similarly examined induced 0–27 with an average of 8.2 (Fig. 4).

MGD of all tested M3 lines ranged from 0 to 36 mm, while in the 5 selected low germination mutants that were similarly examined, the MGD ranged from 5.8 to 11.9 mm with an average of 8.7 mm (Fig. 4). The 5 selected mutants significantly induced lower germination and lower MGD than the control line 102G8, which in the 96-well screen did not yield non-inducing progeny (Fig. 4). The correlation within lines between germination rate and MGD was high and statistically significant according to Pearson's partial correlation analysis ($r = 0.689$, $P < 0.0001$) as was correlation across lines tested by Pearson's correlation analysis ($r = 0.881$, $P < 0.0001$).

The results from the MGD bioassay were consistent with the 96 well bioassay findings: M3 lines with a high percent of non-inducing plants induced low germination rates and low MGD of *O. ramosa*. These results are in agreement with the findings of Hess et al. [27], who found in a similar agar gel assay a high positive correlation (0.93) between percent germination of *Striga hermonthica* seeds induced by *Sorghum bicolor* and the distance of germination from the *S. bicolor* root. Their study also demonstrated that the capacity of *S. bicolor* cultivars to induce germination correlate with their field resistance to *Striga*. *S. bicolor* genotypes that failed to induce germination of *S. hermonthica* seeds at a distance of more than 10 mm from their root were found resistant in field trials. Voegler et al. [28] studied the inheritance of stimulates exuded by S. *hermonthica* in this system and found that low stimulant production is controlled by a single, nuclear recessive gene.

In this study, screening of *A. thaliana* mutants resulted in the identification of 5 low inducing germination mutants. None of these eliminated *Orobanche* germination completely. It seems likely that multiple compounds released by roots can act as germination factors; therefore resistance will require pyramiding multiple factors into a single

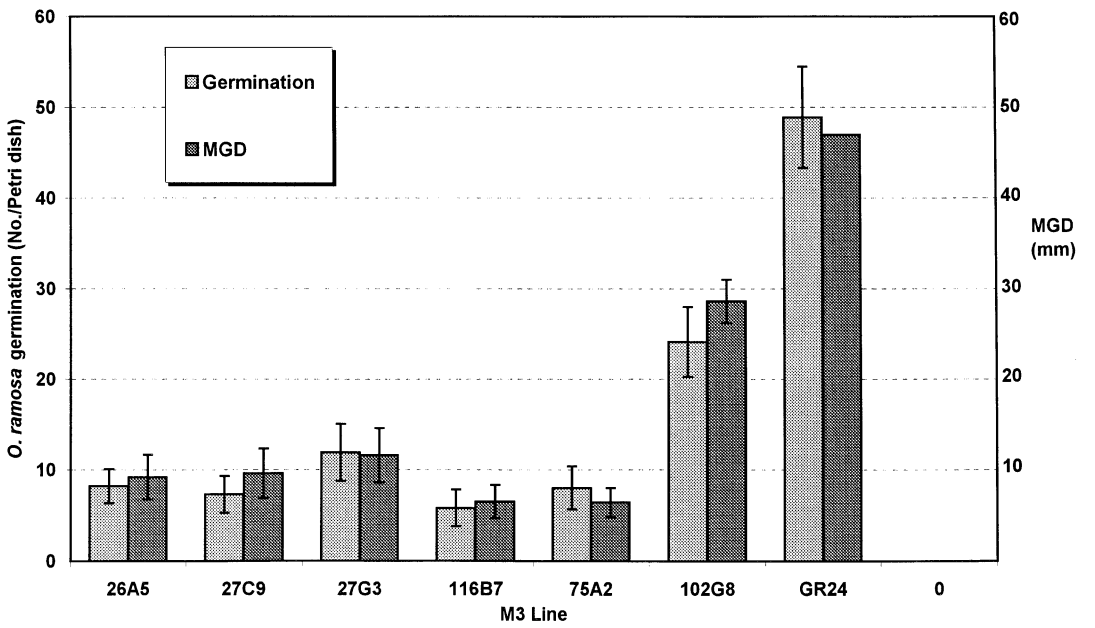

Fig. 4. Selected *A. thaliana* M3 lines induction of *O. ramosa* seed germination and maximum germination distance (MGD) from their roots in the Petri dish bioassay. Line 102G8 that in the 96-well screen did not yield non-inducing progeny served as a control. Columns are means of 10 replications and error bars represent the standard error of the means.

plant. Alternatively, *Orobanche* might use molecules critical to host plant survival as xegnosins. The correlation of low germination rates in these lines with a decrease in maximal germination distance suggests these lines might be promising for a reduction of germination in the field. Further investigation of the low inducing lines is being continued to determine how the phenotypes segregate in genetic crosses.

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