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Butenolide from plant-derived smoke functions as a strigolactone analogue: Evidence from parasitic weed seed germination

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Abstract

The main germination active compound in smoke, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (butenolide), has structural similarities with strigolactones that function as germination stimulants for root parasitic plants such as *Orobanche* spp. and *Striga* spp. (Scrophulariaceae). Consequently, we tested whether butenolide also functions as a germination stimulant for parasitic weeds. Butenolide stimulated germination of both *Orobanche minor* and *Striga hermonthica* to similar levels as the synthetic strigol analogue GR24 and was effective at similar concentrations (10^{-5} to 10^{-11} M). Both butenolide and GR24 were more effective than the synthetic strigol analogue Nijmegan-1. Across eight further *Orobanche* spp., and for species from the root parasitic genera *Cistanche*, *Conopholis* and *Lathraea*, butenolide also had a similar level of activity to GR24. These results suggest that the germination stimulatory activity of butenolide may result from analogy with strigolactones.

Keywords: Butenolide; Germination; Parasitic plants; Seed; Strigolactones

1. Introduction

Smoke from the combustion of plant material stimulates seed germination in a wide range of species (e.g. Brown et al., 2003). The main germination active compound in smoke, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one, hereafter referred to as butenolide, has recently been discovered (Flematti et al., 2004; Van Staden et al., 2004), increasing our opportunities for understanding the mode of action of smoke on germination.

The bioactivity of 'butenolides' that are structurally related to the butenolide from smoke was first identified by Pepperman and Cutler (1991) who conducted bioassays on wheat coleoptiles. These authors attributed the activity of these compounds to their structural similarities to strigolactones (e.g. strigol) which are important germination stimulants for parasitic weed species (Butler, 1995). Furthermore, while the mode of action of strigolactones on seed germination of root parasitic weeds has not yet been identified (Humphrey and Beale, 2006) the bioactiphore resides in the lactone-enol ether D-ring portion of the molecule (see Fig. 1; Mangnus and Zwanenburg, 1992; Wigchert and Zwanenburg, 1999) which is shared with butenolide (Fig. 1).

Root parasites, such as *Orobanche* spp. and *Striga* spp., rely on host plant(s) for mineral nutrition and as a carbon source. To obtain nutrients, the parasites form a connection, usually *via* a haustorium, to the host plant root. Due to this host plant dependency, the parasitic seedling can only survive, *post* germination, for a short period of time. Consequently, such parasitic plants have a requirement for germination in proximity to a host plant root, usually <20 mm (Kuiper, 1997), which is reinforced by the minute size (2–3 μg; Flynn et al., 2006) of the seed, i.e. the seedling has minimal reserves to support host independent growth. This parasite/host plant interaction is often highly specific and seeds of many parasitic plants persist in the soil seed bank until they come into contact with secondary metabolites, such as strigolactones, secreted by host plant roots, which stimulate subsequent germination (Butler, 1995).

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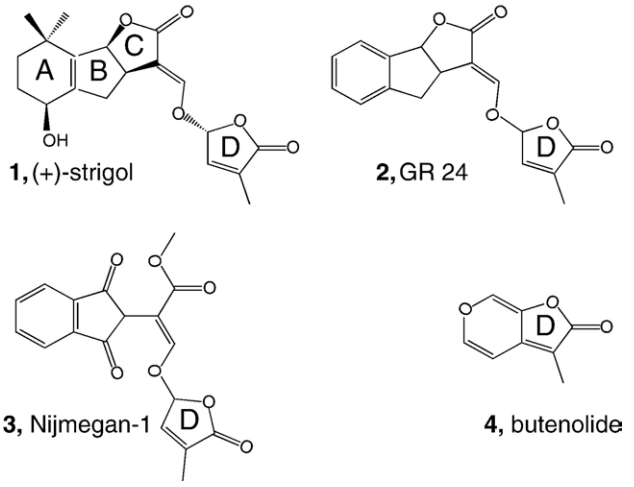

Fig. 1. The chemical structures of (**1**) the naturally occurring germination stimulant (+)-strigol (Cook et al., 1972), (**2** and **3**) the synthetic strigol analogues GR24 and Nijmegan-1 (Johnson et al., 1981; Nefkens et al., 1997) and (**4**) butenolide, the main germination active compound in plant-derived smoke (Van Staden et al., 2004). The D-ring is shared between all four germination stimulants.

We hypothesised that, given the structural similarity between butenolide and strigolactones, butenolide may also function as a germination stimulant for seeds of parasitic plants such as *Orobanche* and *Striga*. Consequently we have tested this proposition for 13 species of root parasitic plants in the family Scrophulariaceae by comparing the efficacy of butenolide with that of two synthetic strigol analogues, GR24 and Nijmegan-1 that are known to stimulate seed germination in a range of parasitic plants (Wigchert et al., 1999).

2. Materials and methods

All seedlots, except for *Striga hermonthica*, came from the Millennium Seed Bank of the Royal Botanic Gardens, Kew, U.K. after storage at −20 °C following drying to c. 15% RH (see Table 1 for seedlot details). *S. hermonthica* was obtained from Prof. A. Murdoch, University of Reading, U.K.

For the largest seeded species (*Lathraea squamaria*, 0.698 mg) a TZ test (2,3,5 triphenyl tetrazolium chloride) was used to assess viability (ISTA, 2003). Part of the seed coat was chipped off using a scalpel blade and seeds were soaked in a 1% TZ solution for 24 h at 30 °C in the dark. Seeds were then cut longitudinally and the degree of staining assessed. Seeds stained red throughout were assumed to be viable. Those that were incompletely stained or unstained were assumed to be non-viable.

Seeds of the remaining, smaller seeded species were stained using fluorescein diacetate (FDA; Pritchard, 1985). Seeds were rehydrated over water for 3 h and then gently crushed between two glass slides in order to isolate the embryo from the membranous testa. FDA solution, 0.5% (w/v) with absolute acetone, was added to the slide 1:1 (v/v) with distilled water. Slides were then left over night to allow the stain to develop. The number of embryos on the slide was then counted using a light microscope. This number was then compared to the number of fluorescent, presumably viable, embryos visible under a UV microscope.

Parasitic weed seeds, including *Orobanche* and *Striga*, require a pre-conditioning phase before treatment with a germination stimulant. Before this, all seeds were sterilized in an aqueous solution containing sodium hypochlorite (2 g/100 ml active chlorine) and Triton X-100 (1% v/v) for 5 min (Mangnus et al., 1992). Seeds (three replicates of c. 30 seeds per species per treatment) were then conditioned by post-sterilisation drying in a flow bench for 30 min followed by transfer onto one layer of filter paper, in 90 mm Petri dishes, which had been moistened with 1 ml sterile distilled water. Petri dishes were wrapped with parafilm and aluminium foil to reduce water loss and ensure darkness. *S. hermonthica* seeds were transferred to 30 °C (Wigchert et al., 1999) for 7 d. Kebreab and Murdoch (1999) found that conditioning at 20 °C for 14 d was suitable for four species of *Orobanche* (*O. aegyptiaca*, *O. cernua*, *O. crenata* and *O. minor*): we used these conditions for the nine *Orobanche* species we studied. While there are no data in the literature regarding pre-conditioning of *Cistanche* sp., *Conopholis* sp. and *Lathraea* sp., these were also pre-conditioned at 20 °C for 14 d since, like *Orobanche* species, they have a more temperate/Mediterranean distribution (Table 1) than the tropical genus *Striga*.

Table 1
Species used in comparing the germination stimulants

Species	Country of origin	Viability %*	Germination %		
			Butenolide	GR24	Nijmegan-1
Cistanche phelypaea (L.) Cout.	Saudi Arabia	72.2±5.9^{a}	51.1±4.4^{b}	45.6±4.0^{b}	18.9±2.9^{c}
Conopholis alpina Liebm.	Mexico	75.6±7.8^{a}	57.8±6.8^{ab}	58.9±7.8^{ab}	38.9±6.6^{b}
Lathraea squamaria L.	England	63.3±3.3^{a}	43.3±5.1^{b}	46.7±5.1^{ab}	21.1±2.9^{c}
Orobanche aegyptiaca Pers.	Jordan	65.6±4.4^{a}	45.6±2.9^{b}	61.1±4.8^{a}	56.7±3.84^{ab}
O. caryophyllacea Sm.	England	57.8±5.9^{a}	50.0±5.1^{ab}	47.8±2.9^{ab}	34.4±4.8^{b}
Orobanche cernua Loefl.	Jordan	61.1±4.4^{a}	47.8±4.0^{a}	53.3±1.9^{a}	42.2±2.9^{b}
O. corymbosa (Rydb.) Ferris	USA	60.0±3.3^{a}	43.3±3.3^{b}	52.2±2.9^{ab}	36.7±3.8^{c}
Orobanche minor L.	New Zealand	85.6±4.0^{a}	57.8±4.4^{b}	73.3±5.1^{ab}	23.3±3.8^{c}
O. purpurea Jacq.	England	70.0±3.8^{a}	51.1±4.8^{b}	48.8±4.0^{b}	38.9±2.9^{b}
O. ramosa L.	South Africa	58.9±4.8^{a}	24.4±6.2^{b}	42.2±2.9^{ab}	23.3±3.3^{b}
O. rapum-genistae Thuill.	Belgium	76.7±3.3^{a}	34.4±2.9^{b}	56.7±5.1^{c}	37.8±4.4^{bc}
O. uniflora L.	Canada	61.1±4.0^{a}	43.3±3.3^{ab}	50.0±3.5^{ab}	33.3±5.1^{b}
Striga hermonthica Benth.	West Africa	80.0±3.8^{a}	61.1±4.4^{b}	63.3±3.3^{ab}	26.7±3.3^{c}

Included are seedlot viability and germination (±SE) data obtained with the three germination stimulants. Germination data is with 10^{-7} M butenolide, 10^{-7} M GR24 and 10^{-6} M Nijmegan-1.

Within each row, different superscripted letters indicate a significant difference between treatments ($P<0.05$).

* Determined using FDA staining except for *Lathraea squamaria* where TZ staining was used.

For the germination treatments, test solutions were prepared by dissolving 1 mg of GR24 or 1.5 mg of Nijmegan-1 in 1 ml acetone. Subsequently, 100 ml distilled water was added to this solution. These solutions contained concentrations of 3.35×10^{-5} M GR24 and 4.39×10^{-5} M Nijmegan-1, respectively. From these stock solutions, concentrations of 10^{-5} to 10^{-11} M were prepared. These values are within the range that has previously been found to be suitable for germination of parasitic weed seeds (e.g. Wigchert et al., 1999; Humphrey and Beale, 2006). Butenolide used in these experiments was isolated, purified and identified from smoke saturated water derived from burned *Passerina vulgaris* Thoday and *Themeda triandra* L. as described by Van Staden et al. (2004). Butenolide solutions were prepared at concentrations of 10^{-5} to 10^{-11} M. For non-parasitic plants, concentrations in this range stimulate seed germination of a wide range of species (Van Staden et al., 2004; Daws et al., 2007).

Following removal from the pre-conditioning conditions, seeds of *O. minor* and *S. hermonthica* were placed on two layers of filter paper in 90 mm diameter Petri dishes and covered by two further filter papers. Approximately 30 seeds were sown per dish. To each Petri dish was added 1.2 ml of either GR24, Nijmegan-1, butenolide or sterile distilled water. All three germination stimulants were applied at concentrations from 10^{-5} to 10^{-11} M. Each treatment was replicated three times. Subsequently, Petri dishes were wrapped in parafilm and aluminium foil and then placed at either 30 °C (*S. hermonthica*, Wigchert et al., 1999) or 20 °C (*O. minor*, Kebreab and Murdoch, 1999).

Subsequently, the additional species (Table 1) were pre-conditioned and germinated as above except that, based on the results from *O. minor* and *S. hermonthica* (Fig. 2), seeds were only treated with one concentration of either GR24 (10^{-7} M), Nijmegan-1 (10^{-6} M) or butenolide (10^{-7} M). All seeds were germinated at 20 °C.

One-way ANOVA on arcsine transformed data followed by Tukey's *post hoc* test was used to determine, within each seedlot, whether there were differences in the effectiveness of the three germination treatments and the viability test.

3. Results

Seeds of *O. minor* and *S. hermonthica* failed to germinate after pre-conditioning when treated with distilled water. However, over a wide concentration range, treatment with the two synthetic strigol analogues, GR24 and Nijmegan-1, elicited germination (Fig. 2). While both compounds stimulated germination, GR24 consistently resulted in higher levels of germination than Nijmengan-1 and resulted in some germination (c. 5%) even at 10^{-11} M: Nijmegan-1 ceased to be effective below c. 10^{-7} M (Fig. 2). Butenolide also stimulated germination of both species across a wide concentration range (10^{-11} to 10^{-5} M) and resulted in germination levels similar to those achieved with GR24 (Fig. 2).

For all 11 species subsequently investigated, ≤2% germination was recorded in the distilled water control. In addition, the three growth stimulants resulted, in all cases, in germination higher than that observed in the distilled water control (Table 1). In addition, Nijmegan-1 (10^{-6} M) resulted in germination levels that were consistently lower than those observed with GR24 (10^{-7} M), while butenolide application (10^{-7} M) resulted in germination levels that were, on average, similar to those achieved with GR24 (Table 1). For the majority of species (9 of 11), maximum observed germination, in any treatment, was lower (albeit only significantly in 3 of 11 species, One-way ANOVA, $P<0.05$) than viability levels (as assessed by FDA or TZ staining).

4. Discussion

Several natural germination stimulants, including strigol (Cook et al., 1972), sorgolactone (Hauck et al., 1992) and alectrol (Müller et al., 1992), exuded by host plants, have been identified. Among parasitic weed species, there is well documented host-specific variation in germination responses. For example, seedlots of *S. hermonthica* grown on either maize or sorghum as the host plant responded differentially to the germination stimulants GR24 and sorgolactone (Wigchert et al., 1999). Furthermore, ethylene has been reported to be important in the germination of *S. hermonthica*, but not *S. forbesii*

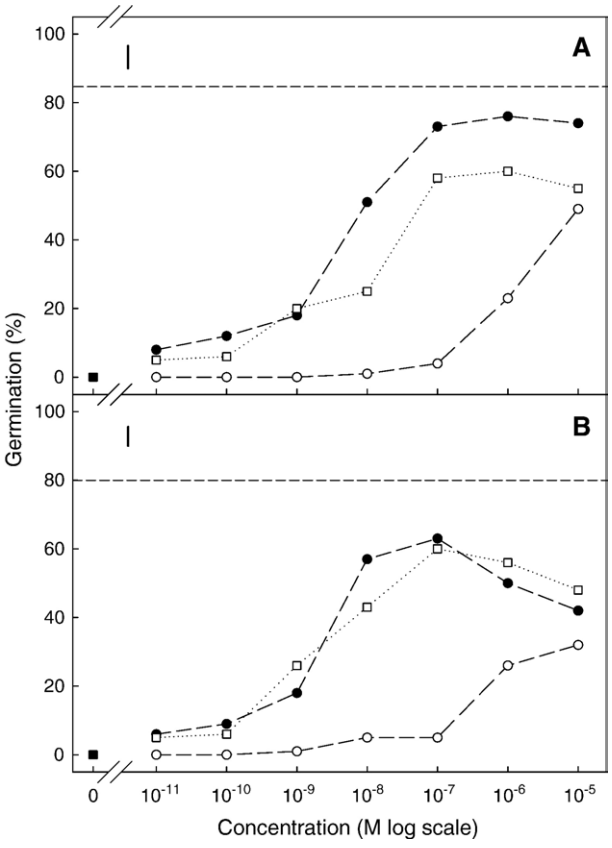

Fig. 2. Dose response curves of (A) *Orobanche minor* and (B) *Striga hermonthica* to butenolide (□), GR24 (●) and Nijmegan-1 (○). *O. minor* seeds were pre-conditioned at 20 °C in the dark for 14 d before treatment with the germination stimulants and incubation at 20 °C in the dark. *S. hermonthica* seeds were pre-conditioned at 30 °C in the dark for 7 d before treatment with the germination stimulants followed by incubation at 30 °C in the dark. The dashed horizontal lines correspond to viability levels estimated using fluorescein diacetate staining. For clarity, error bars are not shown. However, the SE never exceeded the vertical bars in the top left hand corner of each graph.

(Jackson and Parker, 1991). Thus, variation in the specific natural germination stimulant, host species dependent sensitivity to germination stimulants and potentially different modes of action of strigolactones (with respect to ethylene) may all contribute to the differing effectiveness of butenolide, GR24 and Nijmegan-1 observed across our study species.

Furthermore, although pre-conditioning treatments have been optimised for species such as *S. hermonthica* and *O. minor* (e.g. Reid and Parker, 1979; Mangnus et al., 1992; Kebreab and Murdoch, 1999) species-specific protocols have not been determined for most of the species used in this study. This may provide an explanation for why, in almost all species, the application of a single set of conditions failed to result in germination levels that matched viability (Table 1). Nonetheless our data support previous work that found GR24 to be a highly effective germination stimulant across diverse taxa and that Nijmegan-1 is only effective at higher concentrations than GR24 (Wigchert et al., 1999). While smoke has been reported to stimulate germination of *O. aegyptiaca* (Bar Nun and Mayer, 2005) our data demonstrate butenolide to be an effective germination stimulant across a wide range of parasitic species, and may therefore be considered as a strigol analogue.

Strigolactones have been shown to be important for the germination of root parasitic Scrophulariaceae in the genera *Alectra*, *Orobanche* and *Striga* (Müller et al., 1992; Butler, 1995). However, to the best of our knowledge this is not only the first report of germination of species in the genera *Lathraea*, *Conopholis* and *Cistanche* in responses to such compounds but also suggests that this mechanism of host recognition is widespread among root parasites.

Arbuscular mycorrhizal (AM) fungi form a symbiotic relationship with plant roots, in which the fungus supplies the plant host with essential nutrients, such as phosphate and obtains photosynthates from the host plant. Branching of the fungal hyphae that penetrates the host roots to allow nutrient exchange is stimulated by a 'branching factor', which is secreted from the roots of the host plant (Akiyama and Hayashi, 2006). A branching factor has recently been isolated from the root exudates of *Lotus japonicus* and identified as a strigolactone, 5-deoxy-strigol (Akiyama et al., 2005). Spores of AM fungi are able to germinate in the absence of a host, but hyphal branching and development is restricted, (Bécard and Piché, 1989). Interestingly, strigol and GR 24 are able to induce extensive hyphal branching in *Gigaspora margarita* (Akiyama and Hayashi, 2006) and 5-deoxy-strigol is effective at stimulating germination of *O. crenata* (Bergmann et al., 1993). Consequently it appears that parasitic plants find their potential hosts by detecting the same system of chemical signals that AM fungi use for host recognition and branching (Akiyama and Hayashi, 2006).

The agricultural application of strigolactones (e.g. Nijmegan-1) to soil to induce suicidal germination of parasitic weeds has been proposed (Wigchert et al., 1999). However, such application may potentially have unwanted negative effects on soil fungi. Similarly, since butenolide is a naturally occurring chemical in fire environments, it would also be of interest to investigate any potential wider role for this chemical in the rhizosphere. As strigol stimulates germination of both parasitic weeds and non-parasitic plants (e.g. *Avena sativa*; Bradow et al., 1990) and impacts on fungal growth, strigolactones appear to have a wide range of roles in the soil that remain to be explored in detail.

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