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Effects of the aqueous extracts of *Zygophyllum fabago* on the growth of *Fusarium oxyosporum f. sp. melonis* and *Pythium aphanidermatum*

ELÍAS D. DANA,[1]* JUAN GARCÍA DE LOMAS[2] and JOSÉ SÁNCHEZ[1]

[1]*Department of Plant Biology and Ecology, University of Almeria, Almeria and* [2]*Department of Biology, University of Cadiz, Puerto Real, Spain*

In this study, the effects of aqueous extracts from *Zygophyllum fabago* L. on two plant phytopathogenic fungal species (namely, *Fusarium oxyosporum* f. sp. *melonis* and *Pythium aphanidermatum*) were studied under laboratory conditions. The plant extracts (10% w/v) were prepared by using deionized water and fresh tissues. Dilutions (2, 4, 6, and 8% w/v) were obtained to test their effect on the mycelial growth of the phytopathogenic species. Additionally, the recovery of the fungi after their exposure to the *Z. fabago* extract was analyzed. The plant extracts inhibited the growth of *F. oxyosporum* and *P. aphanidermatum* (the maximum mean inhibition that was recorded with the 10% w/v extracts was 42.9% and 85.3%, respectively). A second series of experiments demonstrated the existence of residual effects in both species. The amount of residual inhibition by the 10% w/v extracts was 28.6% in *F. oxyosporum* and 53.8% in *P. aphanidermatum*. A dose–response was clearly observed in *P. aphanidermatum*, while an increase in extract concentration was not associated with a significantly greater reduction in the growth of *F. oxyosporum*. These findings give insights into the potential of *Z. fabago* as a growth inhibitor of *F. oxyosporum* and *P. aphanidermatum*, thus suggesting an interesting potential role for this common weed as a source of natural fungicides.

Keywords: *Fusarium oxyosporum*, inhibitory effect, *Pythium aphanidermatum*, *Zygophyllum fabago*.

Nowadays, the search for natural compounds and management method alternatives (or complements) to classical pesticides has become an intense and productive field of research. Legal restrictions in the use of pesticides are increasing in many countries. Besides, in many developing regions of the world, the use of commercial artificial pesticides might represent an important economic cost for local farmers. Thus, the use of artificial pesticides could be diminished by using new or complementary farming management practises, such as facilitating the use of local botanical resources as biological agents for the control of plant diseases. New sources of allelochemicals could be obtained from the widely distributed and easily cultivated plant species. This study is the continuation of a series of experiments focusing on the usefulness of common weed plants as sources of biologically active compounds (Dana & Domingo 2006).

In the present article, the effects of aqueous extracts of *Zygophyllum fabago* L. (an Asian weed species that has been introduced into different countries) on the growth of two plant pathogenic fungi (*Fusarium oxyosporum* f. sp. *melonis* and *Pythium aphanidermatum*) were studied. *Fusarium oxyosporum* causes various symptoms, such as vascular wilt, yellowing, corm rot, root rot, and damping-off. (Agrios 1988; Smith *et al.* 1988). In mature plants, the damage is usually more intense between flowering and fruit maturation (Jones *et al.* 1982). *Pythium aphanidermatum* damage includes basal stem rot, rot and crown rot, and root rot, frequently leading to the damping-off of

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**Correspondence to: Elías D. Dana, Departamento de Biología Vegetal y Ecología, Universidad de Almería, Almería E-04120, Spain.*
Email: edana@ual.es

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plants (McCarter & Littrell 1970). Both fungus species affect a wide number of crop species and varieties. Previous studies found that chemical compounds in the *Zygophyllum* species could inhibit the growth and development of cells (Ouf *et al*. 1994; Attia 1999; Rhajaoui *et al*. 2003). However, few studies have investigated the inhibitory effects of this widely distributed plant species on *Fusarium* and *Pythium* species. To our knowledge, the effective antifungal activity from *Z. fabago* extracts has been reported only by Zaidi and Crow (2005) and this was against the human pathogen, *Candida albicans*. These authors showed no inhibition of *Fusarium oxyosporum* var. *lycopersici* in methanol-soaked extracts. No previous studies tested the potential of *Z. fabago* as a biological control agent for *P. aphanidermatum*.

Our experiments included the assessment of *Z. fabago* extracts on the inhibition of fungal growth and the recovery ability of these fungi after exposure. This analysis can give insights into the duration of inhibition.

MATERIALS AND METHODS

The plant material of *Z. fabago* at the flowering and fruiting stages was collected in summer in arable fields of Almeria, south-eastern Spain. The fresh leaves were used for preparing the extracts. Previous drying of the fresh material (oven at 40°C until a constant weight was reached) showed that the equivalence of the fresh weight : dried weight material was 10:4, which means that a 10% aqueous solution of fresh material was equivalent to a 4% aqueous solution that was prepared with dried tissue. The fungi were obtained from the biological collection of the University of Almeria, Spain.

Aqueous extracts and growth media

Deionized water (sterilized by an autoclave) was used to prepare the solutions. A solution of plant material (10% fresh w/v) was prepared by soaking the fresh leaves of *Z. fabago* in dark conditions at 15–17°C (refrigerated chamber temperature) for 48 h. From this initial solution (hereafter, *C10*), 8, 6, 4, 2, and 0% (w/v) dilutions were prepared (hereafter, *C8*, *C6*, *C4*, *C2*, and *C0*, respectively) to test the "dose–response effect" of the *Z. fabago* extracts on the two fungus species. The pH of the aqueous extracts was measured, but only minor differences between them were found ($pH_{C10} = 5.70$, $pH_{C8} = 5.73$, $pH_{C6} = 5.75$, $pH_{C4} = 5.77$, $pH_{C2} = 5.80$). Then, the extracts were filtered under vacuum through a disk (pore size = 6 μm; no. 3; Whatman International, Maidstone, UK) and were refiltered through a nitro-cellulose paper (0.45 μm pore size; Albus, Cordoba, Spain) to reduce the risk of interference by microorganisms.

The nutritive substrate was prepared on potato dextrose agar (PDA), which was then sterilized (121°C for 20 min). Once cooled down (until room temperature: 20 ± 2°C), the *Z. fabago* extracts were added to the mixture, which was left to stabilize for 3 h. The pH of the PDA medium was initially 6.0 and was adjusted to 5.75 by using 1 mol L^{-1} HCl. In order to test whether the pH adjustments or possible bacterial activity could be responsible for the potential differences in the fungal growth patterns, three complementary control series (control of media adjustments, CMA) were used. The first group of control plates contained only PDA, but the pH was not manipulated as this was used as a control for statistical purposes. The second group of control plates contained only PDA and the pH of these plates was adjusted to 5.70. The third group of plates contained growth media with the PDA, streptomycine (75 mg L^{-1}), and cloranfenicol (10 mg L^{-1}).

Bioassays with the pathogenic fungi

Two experiments were conducted. In the first experiment, the effects of the *Z. fabago* extracts (at different solutions, *C0–C10*) on the mycelial growth of both *F. oxyosporum* and *P. aphanidermatum* were determined. In the second experiment, it was analyzed whether *F. oxyosporum* and *P. aphanidermatum*, previously exposed to the *Z. fabago* extracts, were capable of recovering their growth when they were transferred to the *Z. fabago*-free media. The fungal inoculums that were obtained from plates *C0*, *C2*, *C6*, and *C10*, used in the first experiment, were incubated in the *Z. fabago*-free media. If a reduction in the mycelial growth (as compared to the control plates) was observed, the existence of permanent damage on the hyphae could be inferred. The term "residual inhibition" can be understood as the inhibition observed in the fungal samples that had been subjected previously to the *Z. fabago* extracts.

For both experiments, five plates (replicates) were used for each extract concentration. The procedure was similar for both experiments 1 and 2. First, 15 mL of the mixtures were added to each plate (Petri dishes, diameter = 8 cm). The fungal inoculums of 5 mm diameter were deposited on each plate containing the nutritive substrate. Considering the faster growth of *P. aphanidermatum*, the inoculums of this species (one sample per plate) were located 1 cm from the border of the plate, whereas the inoculums of *F. oxyosporum* were deposited on the center of the Petri dish. The fungal growth was monitored by measuring the radial growth

along two scaled (mm) angle lines (45°C), set as a fixed template on the Petri-cap (external side), which gave two growth lengths for each measure. For each record, the growth was obtained as the mean number of the two recorded measures. During the incubations, the Petri dishes were kept in a growth chamber at 20°C and in dark conditions. For each plate, duplicate growth measurements were taken every 12 h for *P. aphanidermatum* and every 24 h for *F. oxyosporum* for 8 days.

Statistical analysis

Five replicates were used for each level of dilution and for the control and test conditions following a randomized block design. The experiments were repeated twice. The growth data of the two repetitions were compared by the Mann–Whitney *U*-test (Zahr 1999). No significant difference was found between the two series of repeated experiments ($P > 0.05$), suggesting that the findings were consistent. Then, the data were submitted to a one-factor ANOVA ($P < 0.05$) and Fisher's Least Significant Difference test between pairs ($P < 0.05$) (Zahr 1999). For each species, the mean growth (mm) was the dependent variable. The concentrations were considered as factors. No difference in the growth pattern was found between the CMA, which ruled out the minor differences in pH as being responsible for the differences that were found in the fungal growth. No effect of the bacteria was observed in the plates. Therefore, the effects of these potential factors having been ruled out, no further reference will be made to these plates in the text.

To estimate the inhibitory efficacy of the extracts, Abbott's index (percentage of growth inhibition, GI) (Abbott 1925; Ali *et al*. 2003) was calculated as:

$$GI = 100 \times (C0_i^{\star} - Ce_i)/C0_i^{\star},$$

where $C0_i^{\star}$ is the mean growth in the *C0* plates at a given time (i) of observation (e.g. 12, 24, 36 h) and Ce_i is the mean growth that was recorded in the plates with a given concentration (2, 4, 6, 8, 10%) at the time of observation (i).

RESULTS AND DISCUSSION

Experiment 1: Effects of the plant extracts on fungal growth

The extracts that were obtained from *Z. fabago* caused a net growth inhibition in both *F. oxyosporum* and *P. aphanidermatum*. The level of inhibition that was caused by the *Z. fabago* extracts was significantly higher ($P < 0.001$) for *P. aphanidermatum* than for *F. oxyosporum*. Tables 1 and 2 summarize the mean growth values that were recorded and the multiple range comparisons of the first experiment. *Fusarium oxyosporum* showed a very different growth pattern, as compared to *P. aphanidermatum*. Significant differences ($P < 0.05$) in the growth of *F. oxyosporum* were found between the

Table 1. Effect of the *Zygophyllum fabago* extracts on the growth of *Fusarium oxyosporum* f. sp. *melonis*

Time (h)	*Zygophyllum fabago* concentration (%) (w/v)					
	Control	2	4	6	8	10
	Average fungus growth ± SD (mm)					
24	6.7 ± 0.2a	4.7 ± 0.2b	5.0 ± 0.1a	5.0 ± 0.2a	5.1 ± 0.2a	4.6 ± 0.1a
48	10.0 ± 0.2a	7.5 ± 0.2b	7.4 ± 0.2b	7.3 ± 0.1b	7.4 ± 0.2b	7.3 ± 0.1b
72	13.5 ± 0.3a	9.2 ± 0.2b	9.7 ± 0.2b	9.7 ± 0.2b	9.7 ± 0.2b	9.2 ± 0.2b
96	17.0 ± 0.4a	11.0 ± 0.3b	12.0 ± 0.3b	12.0 ± 0.3b	12.0 ± 0.3b	11.0 ± 0.4b
120	22.0 ± 0.5a	15.0 ± 0.4b	15.0 ± 0.4b	15.0 ± 0.4b	15.0 ± 0.4b	14.0 ± 0.4b
144	28.0 ± 0.5a	18.0 ± 0.5b	18.0 ± 0.5b	18.0 ± 0.6b	18.0 ± 0.2b	16.0 ± 0.5b
168	33.0 ± 1.5a	20.0 ± 1.5b	20.0 ± 1.5b	20.0 ± 2.0b	20.0 ± 0.2b	18.0 ± 1.5b
192	35.0 ± 1.5a	21.5 ± 1.5b	21.5 ± 1.0b	22.0 ± 1.0b	22.0 ± 0.2b	20.0 ± 1.0b
Abbott's mean growth inhibition (%) at the end of the experiment		38.6a	38.6a	37.2a	37.2a	42.9a

Values with a different letter within a row are statistically different according to Fisher's Least Significant Difference test at $P < 0.05$.

Table 2. Effect of the *Zygophyllum fabago* extracts on the growth of *Pythium aphanidermatum*

Time (h)	*Zygophyllum fabago* concentration (%) (w/v)					
	Control	2	4	6	8	10
	Average fungus growth ± SD (mm)					
12	9.0 ± 0.5a	8.3 ± 0.7a	8.3 ± 0.3a	8.2 ± 0.3a	8.5 ± 0.5a	7.9 ± 0.3a
24	25.0 ± 0.5a	14.0 ± 1.7b	12.0 ± 0.6b	12.0 ± 0.5b	12.0 ± 0.5b	10.0 ± 0.1c
36	40.0 ± 0.1a	20.0 ± 2.0a	16.0 ± 2.0c	16.0 ± 1.0c	16.0 ± 1.0c	10.0 ± 0.2d
48	52.0 ± 1.0a	22.0 ± 2.0b	18.0 ± 1.0b	18.0 ± 1.0b	16.0 ± 1.0c	10.0 ± 0.3d
60	68.0 ± 0.5a	23.0 ± 1.5b	20.0 ± 1.0b	19.0 ± 0.7c	18.0 ± 0.1c	10.0 ± 0.3d
Abbott's mean growth inhibition (%) at the end of the experiment		66.2a	70.6b	72.1b	73.5b	85.3c

Values with a different letter within a row are statistically different according to Fisher's Least Significant Difference test at $P < 0.05$.

control (no extract) and *C2–C10* (the mean growth in the control plates was 35.0 mm, compared to 20–22 mm in the plates with the extracts). However, no significant relationship between the growth values and the extract concentration increases was recorded, as the growth in all the plates with the extracts was ~22 mm (Table 1). Therefore, no significant dose–response was observed for these concentrations in *F. oxyosporum*. This result was confirmed by the Abbott's index value, which varied between 37.2 and 42.9%.

A strong inhibitory effect on *P. aphanidermatum* (68 mm in the control plates, compared to 10 mm in the *C10* plates; Table 2) was found. Also, this effect was dose–dependent as the growth was increasingly inhibited with an increasing concentration. The values given by Abbott's index were considerably greater (the mean percentage inhibition was 66.2, 70.6–73.5, and 85.3 in the 2, 4–6–8, and 10% extract concentration, respectively).

Experiment 2: Residual damage by the plant extracts

The fungal inoculums that were obtained from the plates containing the *Z. fabago* extracts (*C0*, *C2*, *C6*, and *C10*), used in experiment 1, were transferred to plates containing only nutritive media. The fungal inoculums were obtained at the end of the first experiment (i.e. after 200 h for *F. oxyosporum* and after 100 h for *P. aphanidermatum*). The tested fungi showed a different response to the initial damage. As revealed by the calculations of residual inhibition, *F. oxyosporum* showed residual damage in the mycelia even after 192 h (Table 3). However, as in the first experiment, the percentage of residual inhibition did not increase with the extract concentration (no significant differences between *C2* [25.7%], *C6* [25.7%], and *C10* [28.6%]). In contrast, a clear difference in the residual inhibition of *P. aphanidermatum*, depending on the extract concentration that was used, was observed. Those inoculums coming from the 2% *Z. fabago* extracts (*C2*) showed no residual inhibition (3.6%). However, a strong residual inhibition in the *C6* (32.7%) and *C10* (53.8%) plates was found at the end of experiment 2 (Table 4).

Our results partially differ from those that were obtained by Zaidi and Crow (2005), who found no inhibition by the methanol extracts (200 $\mu g\ mL^{-1}$) for *F. oxyosporum*. However, the fast-growing *P. aphanidermatum* underwent an effective inhibition (>50%), which was reversible only in the inoculums that were previously incubated at the lowest extract concentrations (*C2*: 2% w/v solution). This particular response could be related to the different biology of each species (e.g. growth rate, physiology, and anatomy).

The underlying biochemical mechanisms have not been deeply investigated in previous studies and are not fully understood. Different studies on other *Zygophyllum* species found the existence of considerable amounts of chemical compounds with inhibitory effects on the growth and development of cells. Attia (1999) reported two triterpenoidal saponins from the aerial parts of *Z. fabago*. Strong antifungal activity (95% inhibition at 200 $\mu g\ mL^{-1}$) was reported by Zaidi and Crow (2005) for *Z. fabago* (whole plant) against the human pathogen *C. albicans*. Rhajaoui *et al.* (2003) studied the properties of *Zygophyllum gaetulum* and found moderate-to-strong inhibitory effects (≥65%, chloroformic extract) on the growth of different species belonging to the genera of *Aspergillus*, *Penicillium*, and *Candida*. These inhibitory

Table 3. Growth and % mean residual inhibition of *Fusarium oxyosporum* f. sp. *melonis* under optimal growth conditions after exposure to *Zygophyllum fabago* extracts

Time (h)	*Zygophyllum fabago* concentration (%) (w/v)			
	Control	2	6	10
	Average fungus growth ± SD (mm)			
24	6.7 ± 0.2a	5.3 ± 0.4b	5.6 ± 0.4b	5.5 ± 0.3b
48	10.0 ± 0.2a	8.0 ± 0.3b	8.5 ± 0.3b	8.5 ± 0.2b
72	13.5 ± 0.3a	11.0 ± 0.2b	11.0 ± 0.4b	11.5 ± 0.2b
96	17.0 ± 0.4a	14.0 ± 0.2b	14.0 ± 0.3b	14.0 ± 0.3b
120	22.0 ± 0.5a	17.0 ± 0.2b	17.0 ± 0.2b	17.0 ± 0.2b
144	28.0 ± 0.5a	20.0 ± 0.2b	20.0 ± 0.2b	20.0 ± 0.2b
168	33.0 ± 1.5a	26.0 ± 0.4b	26.0 ± 0.4b	25.0 ± 0.4b
192	35.0 ± 1.5a	26.0 ± 0.4b	26.0 ± 0.4b	25.0 ± 0.4b
Abbott's mean growth inhibition (%) at the end of the experiment		25.7a	25.7a	28.6a

Values with a different letter within a row are statistically different according to Fisher's Least Significant Difference test at $P < 0.05$.

Table 4. Growth of *Pythium aphanidermatum* under optimal growth conditions after exposure to *Zygophyllum fabago* extracts

Time (h)	*Zygophyllum fabago* concentration (%) (w/v)			
	Control	2	6	10
	Average fungus growth ± SD (mm)			
24	26.0 ± 2.0a	27.0 ± 2.1a	16.0 ± 1.6b	11.0 ± 1.3c
48	52.0 ± 4.0a	50.0 ± 5.0a	35.0 ± 2.8b	24.0 ± 2.0c
Abbott's mean growth inhibition (%) at the end of the experiment		3.6a	32.7b	53.8c

Values with a different letter within a row are statistically different according to Fisher's Least Significant Difference test at $P < 0.05$.

properties were attributed to the presence of sterolic substances, flavonoids, saponins, polyphenols, and tannins. Ouf *et al*. (1994) showed that the leaf and root extracts of various species of *Zygophyllum* (*Zygophyllum coccineum* L., *Zygophyllum album* L., and *Zygophyllum dumosum* Boiss) exhibited a strong fungistatic effect on *Verticellium albo-atrum* and *F. oxysporum* f. sp. *lycopersici*. Kaempferol and six saponins were isolated from the *Zygophyllum* spp. extracts, with quinovic acid being the dominant aglycone. These studies provided an effective control of pathogenic fungi under greenhouse conditions. Among the Zygophyllaceae, other species, such as *Peganum harmala*, have shown compounds with insecticidal activity (Jbilou *et al*. 2006); *P. harmala* is also a rich source of β-carboline alkaloids, such as harmol, harmine, and harmaline (Li 1996; Kartal *et al*. 2003). Corresponding bioassays under field conditions should be conducted to identify the responsible molecules of growth inhibition that were observed in *F. oxyosporum* and *P. aphanidermatum*.

The results of this study give insights into the potential of *Z. fabago* as a growth inhibitor of *F. oxyosporum* and *P. aphanidermatum*, the latter being more sensitive to this plant species. Further experiments at the greenhouse scale will give additional support to the potential production of *Z. fabago*-based alellochemicals for controlling plant diseases that are provoked by *Fusarium* and *Pythium* spp.

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