

Effects of earthworms and mycorrhizal fungi on the growth of the medicinal herb *Calendula officinalis* (Asteraceae)

J.G. Zaller, F. Saccani, T. Frank

*Institute of Zoology, Department of Integrative Biology and Biodiversity Research,
University of Natural Resources and Life Sciences Vienna, Vienna, Austria*

ABSTRACT

Both earthworms and symbiotic arbuscular mycorrhizal fungi (AMF) often co-occur in ecosystems, however very little is known on their interrelationships. Here we tested to what extent earthworms (Annelida) or AMF (Glomales) separately or in combination affect the growth of the pharmaceutical plant species, pot marigold (*Calendula officinalis*, Asteraceae). We conducted a greenhouse experiment using non-sterilized field soil where we manipulated the factors earthworms (addition/no addition of the vertical burrowing species *Lumbricus terrestris*) and AMF (addition/no addition of a mix of the four *Glomus* taxa *G. geosporum*, *G. mosseae*, *G. intraradices*, *G. claroideum*). Leaf length and flower stem length was significantly increased by earthworms but remained unaffected by additional AMF. The longest leaves and flower stems were observed in pots containing earthworms but no additional AMF. The number of flower buds was unaffected by earthworms but marginally significantly increased by AMF. Plant shoot biomass production was significantly higher when earthworms were present; AMF inoculation had no effect on biomass production. Root biomass production and total plant biomass production remained unaffected by earthworms or AMF. These results indicate that in soil already containing AMF earthworm addition primarily affects vegetative growth while additional AMF inoculation tended to affect reproductive plant parts.

Keywords: belowground-aboveground interactions; biomass production; earthworm-plant interactions; ecological engineers; *Glomus*; pot marigold

Both earthworms (Jones et al. 1997) and arbuscular mycorrhizal fungi (AMF; Cameron 2010) commonly co-occur in all terrestrial ecosystems and are both considered as ecosystem engineers because of their great effects on the structure and functioning of ecosystems. Nevertheless, very few reports exist where combined effects of earthworms and AMF on plants were studied (Tuffen et al. 2002, Wurst et al. 2004, Yu et al. 2005, Eisenhauer et al. 2009).

Earthworms are a major component of many natural and agriculturally used ecosystems usually dominating the biomass of soil invertebrates (Edwards and Bohlen 1996). In Central Europe, particularly the anecic species, *Lumbricus terrestris* L., through burrowing, casting and mixing of litter and soil (bioturbation) influences aggregate stability, soil structure, infiltration of water, microbial biomass and nutrient mineralization (Edwards

and Bohlen 1996). Arbuscular mycorrhizal fungi build a symbiosis with the majority of herbaceous plants creating hyphal networks that extend the plant root system and thereby enhancing plant nutrient uptake and growth (Smith and Read 2008).

Many potential interactions between earthworms and AMF can be envisaged including effects of nutrient cycling by annual egesting of several tons of nutrient-rich casts per hectare (Zaller and Arnone 1997) and thereby affecting the AMF-plant symbiosis as AMF are usually more effective under low nutrient conditions. Earthworms were also shown to selectively feed on fungal mycelia (Bonkowski et al. 2000) increasing AMF spore dispersal and colonization of plant roots (Gange 1993, Gormsen et al. 2004). Results from the few studies on earthworm-AMF interactions on plant performance suggest that effects are species-specific varying from increased plant nutrient uptake

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and productivity (Yu et al. 2005) to absence of interactive effects (Wurst et al. 2004, Eisenhauer et al. 2009).

Here, we wanted to test whether earthworms and/or AMF affect the growth and flower development of *Calendula officinalis* L. (Asteraceae). This yellow-orange flowering, aromatic perennial plant grows up to 80 cm tall, is grown as an ornamental or medicinal plant throughout the world and is often recommended for soil improvement and pest repellence in organic gardening (Bisset 1994). Several attempts were made to increase the production of this species including the use of synthetic growth-promoting substances and pesticides (Pal et al. 1986), however there are concerns regarding the use of these substances for medicinal plants because of possible residues in the end products. As medicinal plants are often cultivated in field soil containing both earthworms and AM fungi we wanted to test to what extent these soil organisms separately or in combination affect the growth of this plant species.

MATERIAL AND METHODS

Experimental setup. We set up a full-factorial pot experiment (4.3 L volume, 14 cm × 14 cm side length, 22 cm height) in a greenhouse of the University of Natural Resources and Life Science, Vienna (BOKU).

Before planting, pots were filled with two liters of a 2:1 mixture of field soil (Haplic Chernozem, silty loam) and quartz sand (quartz sand particle size 1.4–2.2 mm); the mixture had a pH-H₂O = 7.6, C_{org} = 22.0 g/kg, N_{tot} = 0.92 g/kg, P-CAL = 64.5 mg/kg, K-CAL = 113.6 mg/kg. The field soil was obtained from an arable field of the Experimental Farm of BOKU located near Vienna, sieved through a 1 cm sieve. This soil mixture was successfully used for other experiments including the same earthworm and AMF taxa (Heiner et al. 2011, Zaller et al. 2011). In order to prevent earthworms from escaping, (i) the drainage holes of the pots were covered with water-permeable fleece material; (ii) a 20 cm high barrier of transparent plastic coated with soft soap on the upper 2 cm was attached to the upper rim of the pots. Fleece and barriers were also installed on pots containing no earthworms to ensure similar microclimatic conditions between different treatments.

We manipulated two factors each consisting of two levels. To establish the +AMF treatment pots already filled with two liters of the substrate mix

received an additional liter of soil inoculated with 40 g of a mix of *Glomus intraradices* N.C. Schenck & G.S. Smith BEG 163, La Banque Européenne des Glomales, *G. claroideum* N.C. Schenck & G.S. Smith BEG 96, *G. mosseae* (T.H. Nicolson & Gerdemann) Gerdemann & Trappe BEG 198 and *G. geosporum* (Nicol. & Gerd.) C. Walker BEG 199 obtained from a commercial supplier (Symbio-m Ltd., Lanskrout, Czech Republic). The inoculum consisted of fragments of colonized roots, spores and mycelium of these AMF taxa. The AMF controls (treatment – AMF) received the same amount of autoclaved AMF inoculum in the additional liter of soil mixture. No rhizobial wash from the active AMF inoculum was added to the AMF controls. Afterwards one individual of seedlings of *C. officinalis* obtained from a garden near the city of Eisenstadt (Burgenland, Austria) were planted in the center of each pot. Roots of these seedlings were carefully washed free of attached soil and planted in the center of the pots. Average size of these seedlings was 15.8 ± 0.5 cm (mean ± SE) and was similar in the treatments. One week after planting, two adult specimens of the anecic earthworm species *L. terrestris* per mesocosm (average earthworm biomass per pot 9.62 ± 1.68 g) were placed on the soil surface (treatment +Ew). Earthworm control treatments (–Ew) received no earthworms. Earthworms were obtained from a fishermen bait shop in Vienna (Austria) and cultivated in plastic boxes with soil and ground oat flakes as food in a dark climate chamber (15°C) for one week for acclimation before they were added to the pots. Prior to the addition to the pots earthworms were carefully washed free of attached soil, dried on filter paper, weighed and inserted into pots. The majority of earthworms buried themselves into the soil within a few minutes; earthworms that were still on the surface the next day were replaced by new specimens cultivated in substrate in the climate chamber. Earthworms received 0.8 g/pot of ground oat flakes for extra nutrition; oat flakes were also added to –Ew treatments for consistency.

These treatments were replicated six times in a full-factorial design (totally 24 pots: 2 earthworm × 2 AMF treatments × 6 replicates). All pots were watered with a constant amount of tap water among treatments according to temperature and humidity conditions in the greenhouse. No fertilizer was applied during the experiment.

The experiment was conducted between December 2010 and January 2011. The pots were randomly arranged on a greenhouse table; to ensure optimal light conditions, a 1000-W Radium lamp

(type HRI-T100W/D, WE-EF Leuchten, Bisingen, Germany) was placed in about 1 m distance over the pots. Average day-time air temperature in the greenhouse was 18°C; average night-time temperatures was 15°C.

Measurements and harvesting. Maximum leaf length and length of flower stems was measured once a week using a ruler; number of not-yet open flower buds and number and diameter of open flowers was measured once at the end of the experiment.

Pots were harvested 8 weeks after planting. First, shoots were cut at the soil surface, measured, counted, put in paper bags and dried at 55°C for 48 h for dry mass determination. Second, pots were flipped over, the soil carefully searched for earthworms. Earthworms were counted, rinsed under tap water, dried on a paper towel and their fresh mass weighed. Third, roots in the soil were washed free of soil under tap water, weighed and cut into two parts where one half was used for dry mass determination after drying in the oven at 55°C for 48 h. The other half was used for mycorrhizal analysis. Therefore, fresh root subsamples were cleared with boiling KOH for four minutes and stained for one minute with Shaeffer® black ink (Vierheilig et al. 1998). Stained roots were observed with a dissecting microscope (100 × magnification), the percentage of colonized roots was calculated based on the gridline intersection method considering both the presence of vesicles and arbuscules (Giovanmetti and Mosse 1980).

Statistical analysis. Time courses of leaf length and length of flower stems were analyzed using two-way repeated measures analysis of variance (ANOVA) with the factors earthworms and AMF. Additionally, post-hoc comparisons after the Tukey's test were made for each measurement date to be able to determine differences

between specific treatments. The last dates from the measurements of leaf lengths and lengths of flower stems and the data on biomass production and number of flower buds were analyzed using two-way ANOVAS with the factors earthworms and AMF followed by Tukey's post-hoc mean comparisons. All statistical analyses were performed using generalized linear models in SPSS 17.0 for Apple MacIntosh at a significance level of $\alpha = 0.05$. The biomass data were log-transformed prior to analysis to meet normal distribution and homoscedasticity of variance among treatments. Values given throughout the text are means \pm 1 SE ($n = 6$).

RESULTS

During the harvest of the experiment we recovered 72% of the initial number of earthworms weighing 95% of the initial earthworm biomass. Neither earthworm numbers (–AMF: 1.67 ± 0.29 , +AMF: 1.75 ± 0.36 , $P = 0.861$) nor earthworm biomass were affected by AMF treatments (–AMF: 10.34 ± 1.36 , +AMF: 8.54 ± 1.52 , $P = 0.360$). Root AMF colonization was averaged over all four treatments $22.4 \pm 3.2\%$ but not significantly affected by earthworm ($P = 0.854$) or AMF treatment ($P = 0.607$, AMF \times Ew: $P = 0.766$).

Time course of the maximum leaf length was similar between treatments until the third week but afterwards significantly longer leaves were observed in +Ew/–AMF pots compared to the mean of the other treatments (Table 1, Figure 1). A similar pattern could be seen regarding the length of flower stems, however here the differentiation of significantly longer flower stems of +Ew/–AMF treatments began one week earlier (Table 1, Figure 1).

Table 1. ANOVA results for the effects of earthworms and arbuscular-mycorrhizal fungi inoculation (AMF) on growth and biomass production of *C. officinalis*

Variable	Earthworms		AMF		Earthworms \times AMF	
	F	P	F	P	F	P
Leaf length (cm)	9.323	0.006	1.130	0.300	6.553	0.019
Length of flower stems (cm)	6.087	0.023	1.245	0.278	1.651	0.214
Number of flower buds (1/pot)	1.089	0.314	3.678	0.076	2.021	0.177
Shoot mass (g/pot)	4.856	0.048	0.005	0.945	2.493	0.135
Root mass (g/pot)	0.477	0.501	0.009	0.926	0.007	0.984
Total plant mass (g/pot)	3.471	0.082	0.045	0.835	1.978	0.180

For leaf length and length of flower stems only the last measurement dates of the time course were included in the analyses

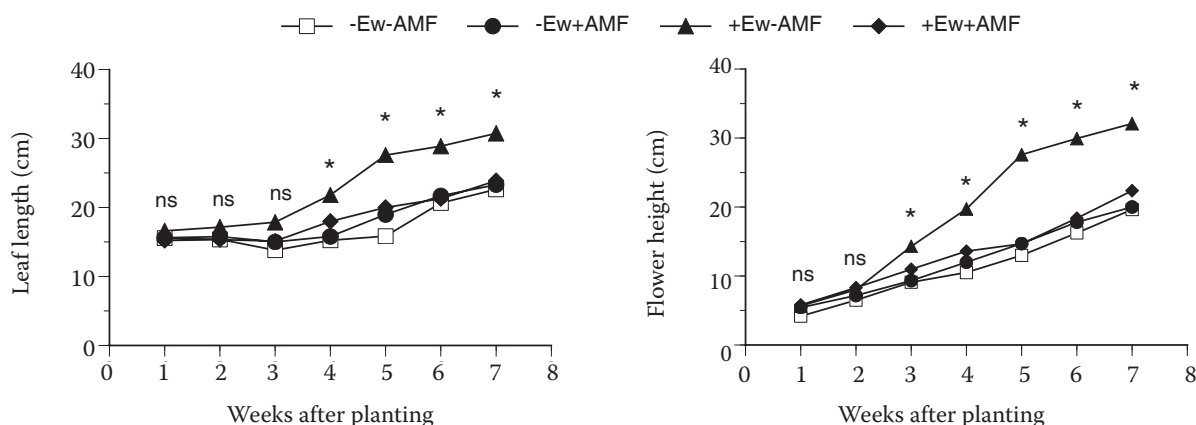


Figure 1. Time course of growth of maximum leaf length and length of flower stems of *C. officinalis* plants in response to soil inoculation with earthworms (–Ew, +Ew) and/or arbuscular mycorrhizal fungi (–AMF, +AMF). Asterisks denote statistically significant difference ($P < 0.05$, Tukey's tests) between the uppermost data points and the three data points below. Means, $n = 6$

Number of flower buds was marginally significantly affected by AMF inoculation, but not affected by earthworms (Table 1, Figure 2). Individual analysis showed that AMF inoculation only significantly increased the number of flower buds when no earthworms were present (Tukey, $P = 0.013$; Figure 2). Each plant had on average one open flower with an average diameter of 5.2 ± 0.4 cm (data not shown); however neither the number nor the diameter of flowers were affected by earthworms ($P = 0.773$ and $P = 0.532$ for numbers and diameter, respectively) or AMF treatment ($P = 0.544$ and $P = 0.329$ for numbers and diameter, respectively).

Plant shoot biomass was significantly higher in pots containing earthworms, but remained unaffected by AMF (Table 1, Figure 3). Root biomass was neither affected by earthworms nor AMF (Table 1, Figure 3). Total plant biomass was marginally significantly higher in +Ew pots (Tukey, $P = 0.077$) but unaffected by additional AMF inoculation (Tukey, $P = 0.785$).

DISCUSSION

We found a stimulation of the growth of *Calendula* leaves and flower stems by earthworms. An earthworm-induced growth stimulation was often seen in agricultural plant species (Scheu 2003), however, it was less often found in field experiments (Zaller and Arnone 1999a,b). To the best of our knowledge earthworm-induced growth stimulation has never been investigated in ornamental or medicinal plants. In our experiment, this earthworm effect on growth was only present when no additional AMF inoculation was applied, plants growing in soil containing earthworms with additional AMF inoculation showed even less growth. Several explanations are possible for this observation: (i) additional AMF inoculation added more earthworm food to pots reducing earthworm burrowing activity because there was abundant food around; (ii) both AMF and earthworms usually affect plant growth separately, when both organisms are present their relative

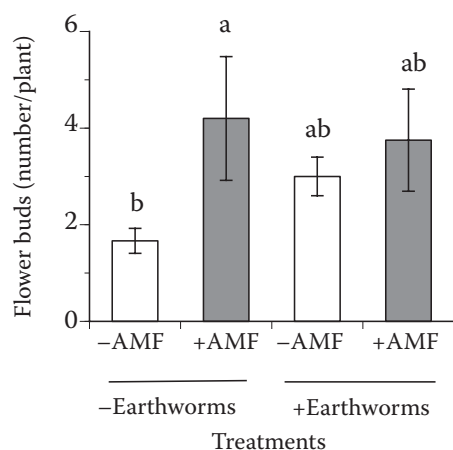


Figure 2. Number of flower buds of *C. officinalis* grown in soil inoculated without (–Earthworms) or with earthworms (+Earthworms) and/or arbuscular mycorrhizal fungi (–AMF, +AMF). Different letters above bars refer to significant differences ($P < 0.05$, Tukey's tests). Means \pm SE, $n = 6$

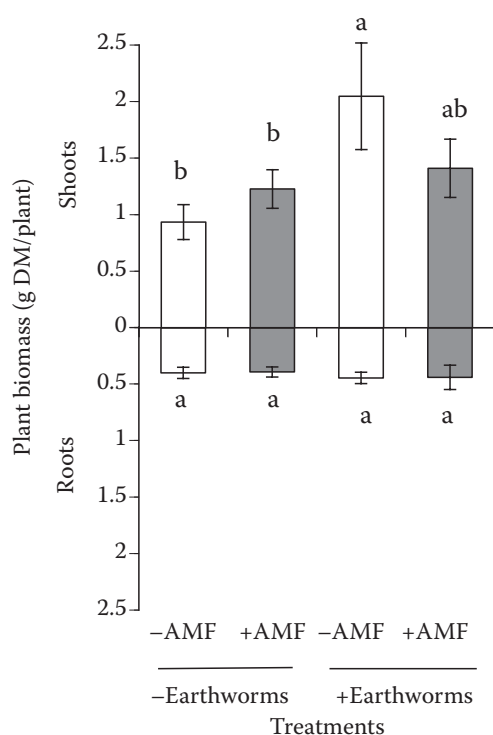


Figure 3. Shoot and root dry mass production of *C. officinalis* grown in soil inoculated without (–Earthworms) or with earthworms (+Earthworms) and/or arbuscular mycorrhizal fungi (–AMF, +AMF). Different letters above bars refer to significant differences ($P < 0.05$, Tukey's tests). Means \pm SE, $n = 6$

contribution is less clear; (iii) since we did not correct for microorganisms possibly attached to the active AMF inoculum it is possible that pathogens or rhizobacteria affecting plant growth or earthworm activity were added to the pots with the AMF inoculum, or (iv) the relatively high P content in the substrate may have weakened the AMF effects. We expected to see an altered root growth in +Ew pots as a consequence of their bioturbation and effects on nutrient availability. The lack of an earthworm-stimulated root growth might indicate some root herbivory by earthworms (Curry and Schmidt 2007) or a slow response of the root system to changes in nutrient availability and soil structure. However, this remains to be investigated in more detail. The stimulation of height growth in pots containing earthworms but no additional AMF also translated into higher shoot biomass production compared to the pots without earthworms.

Counterintuitively, AMF inoculation did not affect root AMF colonization in our experiment indicating limited effects of extra AMF in this study system for the duration of the experiment. In experiments studying AMF effects, soil is commonly sterilized before AMF inoculation in order

to deactivate all AMF in the soil (e.g. Zaller et al. 2011). In the current experiment we used a different approach and added AMF inoculum to soil already inhabiting active AMF in order to mimic a more realistic situation in the field. Indeed, in other studies it could be shown that field AMF inoculation increased plant productivity and quality even until the second year after inoculation (Ceccarelli et al. 2010, Pellegrino et al. 2011). Interestingly, although we could not find altered AMF root colonization, AMF inoculation led to significantly higher numbers of flower buds, however only in pots without earthworms. If we interpret a higher number of flower buds with better nutrition for plants, this could indicate that earthworms interfered with AMF leading to less nutrient acquisition, perhaps because earthworms destroyed AMF hyphal networks (Tuffen et al. 2002) and/or fed on fine roots (Curry and Schmidt 2007).

Growth and quality of medicinal plants were shown to be strongly affected by soil conditions and amendments of earthworm-worked compost (vermicompost) and other composts increased the growth and flower production of *Calendula* (Contreras et al. 1994, Paim et al. 2010) and other ornamental plant species (Lazcano and Dominguez 2010). It remains to be investigated whether earthworms and/or AMF also evoke changes in the chemical quality of plants as observed for vermicompost for horticultural plants (Zaller 2007).

This preliminary study showed that *C. officinalis* growth benefits from earthworm activity, however additional inoculation of soil with AMF does not necessarily amplify this effect. Further research is needed to ensure that the reported interrelationships between earthworms and AMF also affect the chemical quality of *C. officinalis* appreciated for pharmaceutical and/or cosmetic uses.

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Corresponding author:

Dr. Johann G. Zaller, University of Natural Resources and Life Sciences Vienna, Department of Integrative Biology and Biodiversity Research, Institute of Zoology, Gregor Mendel Straße 33, A-1180 Vienna, Austria
phone: + 431 476 543 205, fax: + 431 476 543 203, e-mail: johann.zaller@boku.ac.at
