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Inhibitory Potential of Naphthoquinones Leached from Leaves and Exuded from Roots of the Invasive Plant *Impatiens glandulifera*

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Abstract Exploring the effects of allelopathic plant chemicals on the growth of native vegetation is essential to understand their ecological roles and importance in exotic plant invasion. Naphthoquinones have been identified as potential growth inhibitors produced by *Impatiens glandulifera*, an exotic annual plant that recently invaded temperate forests in Europe. However, naphthoquinone release and inhibitory potential have not been examined. We quantified the naphthoquinone content in cotyledons, leaves, stems, and roots from plants of different ages of both the invasive *I. glandulifera* and native *Impatiens noli-tangere* as well as in soil extracts and rainwater rinsed from leaves of either plant species by using ultra-high pressure liquid chromatography-mass spectrometry (UHPLC-MS). We identified the compound 2-methoxy-1,4-naphthoquinone (2-MNQ) exclusively in plant organs of *I. glandulifera*, in resin bags buried into the soil of patches invaded by *I. glandulifera,* and in rainwater rinsed from its leaves. This indicates that 2-MNQ is released from the roots of *I. glandulifera* and leached from its leaves by rain. Specific bioassays using aqueous shoot and root extracts revealed a strong inhibitory effect on the germination of two native forest herbs and on the mycelium growth of three ectomycorrhiza fungi. These findings suggest that the release of 2-MNQ may contribute to the invasion success of *I. glandulifera* and support the novel weapons hypothesis.

Keywords *Impatiens glandulifera* · Chemical defence · 2-methoxy-1,4-naphthoquinone · Ectomycorrhiza · Invasion

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Introduction

Invasions of non-native plant species are a major threat for the native biodiversity in many ecosystems (Pejchar and Mooney 2009; see Weidenhamer and Callaway 2010, for a review). Various hypotheses have been proposed to explain the invasiveness of non-native plants (e.g., Bakker and Wilson 2001; Blossey and Notzold 1995; Davis et al. 2000; Keane and Crawley 2002). The novel weapons hypothesis assumes that some invasive plant species produce secondary metabolites that are novel in their non-native ranges, and that this novelty provides advantages to the invasive as it interacts with native plants, microbes, or generalist herbivores (Callaway and Ridenour 2004; Inderjit et al. 2011). An increasing number of studies provide support for the novel weapons hypothesis (Abhilasha et al. 2008; Barto et al. 2010; Callaway et al. 2004; Cantor et al. 2011; Scharfy et al. 2011).

Impatiens glandulifera, an annual plant belonging to the family *Balsaminaceae*, was first introduced from the western Himalaya to Europe as an ornamental garden plant in 1839 (Beerling and Perrins 1993). It became naturalized and invaded riparian habitats in most European countries (Hejda and Pysek 2006). In the last decade, *I. glandulifera* has increasingly invaded deciduous and coniferous forests disturbed by windthrows and/or intensive forest management (Nobis 2008). *Impatiens glandulifera* negatively affects ecosystems by competing successfully with native plants for pollinators, which may reduce native plant fitness (Chittka and Schurkens 2001). *I. glandulifera* also causes changes in the species

composition of native plant communities in riparian habitats (Hejda and Pysek 2006; Maule et al. 2000). In mixed deciduous forests, *I. glandulifera* influences the litter-dwelling gastropod community by increasing soil moisture and dampening daily soil temperature fluctuations (Ruckli et al. 2013), and suppresses arbuscular mycorrhiza symbiosis in *Acer pseudoplatanus* saplings (Ruckli et al. 2014).

Impatiens glandulifera contains secondary metabolites including flavones, caffeic acid derivatives, and naphthoquinones (oxygen-derivatives of naphthalene) (Lobstein et al. 2001; Šerá et al. 2005). Naphthoquinones have been the subject of medical and ecological studies because of their anticarcinogenic properties (Babula et al. 2009; Devi et al. 1999; Vennerstrom and Eaton 1988) and their inhibitory effects on fungal spore germination (Foote et al. 1949; Yang et al. 2001), bacteria growth (Athip and Pharkphoom 2012), seedling germination and growth (Terzi 2008), and their negative effects on larval development of insects (Lee and Lee 2008; Mitchell et al. 2007). High levels of 2-methoxy-1,4 naphthoquinone (2-MNQ) were detected in leaves of *I. glandulifera*, but not in leaves of the native *Impatiens noli-tangere* (Chapelle 1974; Mitchell et al. 2007). Furthermore, Lobstein et al. (2001) found 2-MNQ and 2-hydroxy-1,4 naphthoquinone (lawsone) in leaves and stems of *I. glandulifera* plants of different ages. However, it is not known whether these naphthoquinones also occur in the roots of *I. glandulifera*.

In general, phytotoxic substances are released from plants to the environment by: (1) exudation from roots, (2) leaching from plants by rain, or (3) decomposition of residues. Aqueous shoot extracts of flowering *I. glandulifera* inhibit germination success and root length of the native plant *Sinapis alba* (Csiszàr et al. 2012), *Leucinapis alba,* and *Brassica napis* (Vrchotová et al. 2011). However, the natural pathway for release of allelopathic substances by *I. glandulifera* and the entire spectrum of inhibitory potential have not yet been examined. In this study, we investigated the way(s) allelopathic naphthoquinones are released from *I. glandulifera* plants, and we explored the inhibitory potential of these substances on the growth of ectomycorrhiza fungi (EM) and the germination of native forest plants by using resin bags, plant extracts, leachate analysis, and bioassays on native plants and fungi. We addressed the following questions: (1) Do different plant organs of the invasive *I. glandulifera* and the native *I. noli-tangere* differ in type and content of naphthoquinones? (2) Do plants of different age (juvenile, flowering, senescent) in each species differ in naphthoquinone content? (3) Do naphthoquinones exude from roots into the soil and/or leach from the foliage by rainwater? (4) Do aqueous shoot and root extracts obtained from juvenile, flowering, and senescent *I. glandulifera* plants contain different amounts of naphthoquinones? (5) Do aqueous shoot and root extracts of the invasive *I. glandulifera* reduce the germination success of native herbaceous plant species and mycelium growth of EM fungi? (6) Does synthetic naphthoquinone affect mycelium growth of EM fungi?

Methods and Materials

Plant Sampling Invasive *I. glandulifera* and native *I. noli-tangere* were sampled in April (seedlings), May and June (juvenile plants), July (flowering plants), September (fruiting plants), October (senescent plants), and in November 2011 (partly decomposed plants) (online resource 1). Samples consisting of 3–7 individuals were collected in an area measuring 50×50 m in a semi-natural beech forest near Basel (47°26′ N, 7°33′ E). The plants were transported on ice to the laboratory, where cotyledons, leaves, stems, and roots were separated and lyophilized for 72 hr (VirTis BenchTop 2K, SP Industries; US). Dried samples were ground with a ball mill (Retsch MM200, Schieritz & Hauenstein AG, Switzerland), and 5–60 mg of the ground material were mixed with 1 ml methanol:water (4:1, v/v) and incubated at ambient temperature for 30 min before centrifuging for 15 min at 16,000 rpm and removing the supernatants for storage at −20 °C.

Sampling of Plant Exudates We used the slightly modified protocol of Ens et al. (2010) to measure the naphthoquinones released by *I. glandulifera* into the soil. Cotton fabric bags (7.5×5 cm) were filled with 10 g of Amberlite® XAD4 (Sigma-Aldrich, Switzerland), washed with distilled water, and then twice with dichloromethane (DCM, HPLC grade), dried for 24 hr at 40 °C, and stored in an air-tight glass jar prior to exposure in soil.

To assess the effectiveness of the resin bag technique, we measured the percentages of adsorption and recovery of 2-MNQ released in water by placing five resin bags in a 0.01 % 2-MNQ (Sigma-Aldrich, Switzerland) solution for 24 hr. Adsorption averaged 74.0±5.6 %, and subsequent recovery with DCM after 24 hr was 90.1±6.3 %. This indicates that the effectiveness of the resin bag technique was approximately 67 %.

Exudates released by *I. glandulifera* plants of different ages were sampled in three areas adjacent to the sampling site described above. In each forest area, three homogeneous patches invaded by *I. glandulifera* and three patches not colonized by the plant were selected. In each patch, two resin bags were buried 10–15 cm below ground surface. Bags were exposed for 20 days from 17 May to 6 June 2011 (seedling and juvenile stage), 17 July to 7 August 2011 (flowering stage), 18 October to 7 November 2011 (senescent stage), and 16 January to 6 February 2012 (partly decomposing stage; online resource 1). Afterwards, the bags were transported on ice to the lab and stored at −80 °C. Some bags were lost: in all 121 out of 144 bags were recovered.

To extract the adsorbed plant exudates, we placed 5 bags into a conical flask containing 250 ml DCM. The sealed flasks were shaken at ambient temperature for 24 hr, the solution was filtered and evaporated to dryness under reduced pressure at 40 °C. The residue was mixed with 1 ml methanol:water (4:1, v/v),incubated at ambient temperature for 30 min, and centrifuged for 15 min at 16,000 rpm. Before recovery, the supernatants were stored at −20 °C until required. The controls were five clean resin bags that had been kept in a sealed glass jar for 20 days.

Rainwater Sampling To examine whether naphthoquinones of *I. glandulifera* could be leached by rain, we collected rainwater during the flowering stage of the plant on 2 days in July 2011 (online resource 1). Five patches with *I. glandulifera* and five patches with no invasive plants were chosen. A plastic tray (21×9×10 cm) covered with a net to prevent contamination by falling foliage was placed prior to rainfall both under *I. glandulifera* (in invaded plots) and under native vegetation (in uninvaded plots). Samples of 50 ml of rainwater were obtained from each plastic tray immediately after an abundant rain, transferred to separatory funnels, and mixed with 50 ml DCM in order to collect the organic-soluble phase for analyses as described below.

Mass Spectrometry Analyses Naphthoquinones were analyzed by ultra-high pressure liquid chromatography/mass spectrometry (UHPLC/MS) using an Acquity UPLC™ system (Waters, Milford, MA, USA) coupled to a Synapt G2 MS QTOF (Waters, Milford, MA) equipped with an atmospheric pressure chemical ionization (APCI) source. An Acquity BEH C18 column (50×2.1 mm, 1.7 μm) was used under the following conditions: solvent A = water; solvent B = acetonitrile; 5–52.5 % B in 2.0 min, 52.5–100 % B in 0.5 min, holding at 100 % B for 1.0 min, re-equilibration at 5 % B for 0.9 min. The flow rate was set to 700 μl min^{-1}, and the injection volume was 2.5 μl. The temperatures of the column and of the autosampler chamber were maintained at 30 and 15 °C, respectively. Data were acquired with a scan time of 0.4 sec over an *m/z* range of 85–600 Da in the negative ion MS mode. The corona current was set to 20 μA and the cone voltage to 30 V. The source temperature was maintained at 120 °C and the APCI probe temperature at 370 °C. The desolvation gas flow was set to 800 L hr^{-1}. To quantify the different naphthoquinones in each sample, standard calibration curves obtained from different concentrations of synthetic 1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, 5-hydroxy-1,4-naphthoquinone, and 2-methoxy-1,4-naphthoquinone (Sigma-Aldrich, Switzerland) were used.

Preparation of Aqueous Extracts for Bioassays Shoot and root material of *I. glandulifera* was collected from juvenile plants in May, from flowering plants in July and senescent plants in October 2011 (online resource 1). The tissue samples (300 g fresh weight) were soaked in 1 l of distilled water at ambient temperature for 48 hr, filtered twice through cheesecloth and then under pressure through a Millipore®-membrane (0.45 μm) to prevent potential contamination by microorganisms. Three samples of 1 ml of each stock solution were stored at −20 °C.

Seed Bioassays The phytotoxic effect of aqueous shoot and root extracts of *I. glandulifera* on the germination of two native plants (*Hieracium murorum* and *Scrophularia nodosa*) growing in the same forest was tested in a seed bioassay. Stock extracts (shoot or root) of juvenile, flowering and senescent *I. glandulifera* were diluted with distilled water to obtain concentrations of 0.250, 0.125, and 0.0625 g of fresh weight of plant tissue per ml.

Twenty-five surface-sterilized seeds of *H. murorum* or *S. nodosa* were placed on a filter paper in a petri dish (90 mm, Roth AG, Switzerland) before adding 3 ml of test solutions or distilled water (control) and sealing the dishes twice with parafilm for incubation in a climate chamber at 22/15 °C with a light:dark cycle of 16:8 hr for 20 days. All dishes were checked for germinated seeds at the end of the experiment. Three replicates of each test solution and control were prepared for each plant species, resulting in a total of 126 petri dishes. Inhibition/stimulation on germination was calculated following Chung et al. (2001):

$$\text{inhibition/stimulation} = (t-c/c) \times 100(\text{in } \%)$$

where t is the percentage germination measured in dishes exposed to different extract concentrations and c represents the mean of the percentage germination of the corresponding controls.

Mycelium Growth Bioassays Potential inhibitory effects of aqueous shoot and root extracts of juvenile, flowering, and senescent *I. glandulifera* were tested on the mycelium growth of three EM fungi (*Pisolithus tinctorius, Lactarius subdulci,* and *Laccaria bicolore*) typical for beech forests. Modified Melin-Norkrans (MMN) agar (Marx 1969) containing the same concentrations of plant tissue extracts (0.250, 0.125, and 0.0625 g fresh weight per ml agar) as used in the seed bioassay was prepared, and used to fill Petri dishes with 20 ml agar. As a control, MNN agar was prepared with sterilized water. A fungal plug (diam 4 mm) was placed in the center of each petri dish before sealing the dishes twice with parafilm. Three replicates of each agar solution and control were prepared for each fungal species, resulting in a total of 189 petri dishes.

Table 1 Amount (mean ± SE) of 2-methoxy-1,4-naphthoquinone (2-MNQ) and 1,4-naphthoquinone (NQ) determined in resin bags buried for 20 days in the soil 5–10 cm from *Impatiens glandulifera* plants of different ages

Plant age	2-MNQ [μg per bag]	NQ [μg per bag]	Number of bags
Seedling/juvenile	0.701±0.273	0.483±0.289	31
Flowering	0.321±0.167	0.295±0.098	36
Senescent	1.304±0.365	0.216±0.067	36
Decomposed	0.102±0.102	not detected	18

Dishes were incubated in a climate chamber at 26 °C in darkness for 45 days. The area covered by the mycelium (mm^2) was recorded by drawing its circumference on the top of each petri dish at the end of the experiment, and its area was measured by using Image J (Rasband 1997–2012). Inhibition/stimulation of mycelium growth was calculated as described above.

Synthetic 2-Methoxy-1,4-Naphthoquinone on Mycelium Growth MMN agar solutions containing 0.1, 0.3, 1, and 3 μg of synthetic 2-MNQ (Sigma-Aldrich, Switzerland) per ml agar were prepared to assess its phytotoxic effect on the mycelium growth of the three EM fungi at concentrations similar to the levels in soils, based on the resin bag analysis (Table 1) and the levels in aqueous shoot extracts of the invasive plant (Table 2). For each EM fungi, we prepared three replicates of each concentration and control resulting in a total of 45 dishes. Inhibition was calculated as in the mycelium growth bioassay.

Statistical Analyses All analyses were carried out using R (R Development Core Team 2012, version 2.15.1). ANOVA was used to examine the effects of *I. glandulifera* plant stage (juvenile, flowering, and senescent), type of plant material (root or shoot), and concentration of extracts (0.0625, 0.125, and 0.250 g plant material/ml) on inhibition/stimulation (in %) of germination of *H. murorum* and *S. nodosa* or on mycelium growth of *L. bicolore*, *L. subdulci,* and *P. tinctorius*. Student's *t*-tests were used to examine whether the inhibition/stimulation (in %) of the different extract concentrations significantly deviated from zero. Spearman rank correlation was applied to examine whether inhibition/stimulation (%) was related to the 2-MNQ content found in aqueous shoot extracts of all plant stages of *I. glandulifera*.

Table 2 Amount (mean ± SE, *N*=5) of 2-methoxy-1,4-naphthoquinone (2-MNQ) determined in aqueous shoot extracts of *Impatiens glandulifera* of different ages (300 g fresh weight of shoot tissue extracted with 1 l of water)

Plant age	2-MNQ [μg per ml]
Seedling/juvenile	14.64±1.55
Flowering	9.03±0.07
Senescent	4.41±0.14

Results

Naphthoquinones in Plants 2-Methoxy-1,4-naphthoquinone (2-MNQ) was identified in leaves, stems, and roots of *I. glandulifera*, but not in cotyledons (Fig. 1a–c). Overall, the concentration of 2-MNQ (% dry weight) in leaves of *I. glandulifera* was up to 10–15 times higher than in roots and stems and declined with plant age (Fig. 1a–c). In contrast, 2-hydroxy-1,4-naphthoquinone (lawsone) was found only in roots of *I. glandulifera* in the senescent stage. In the native

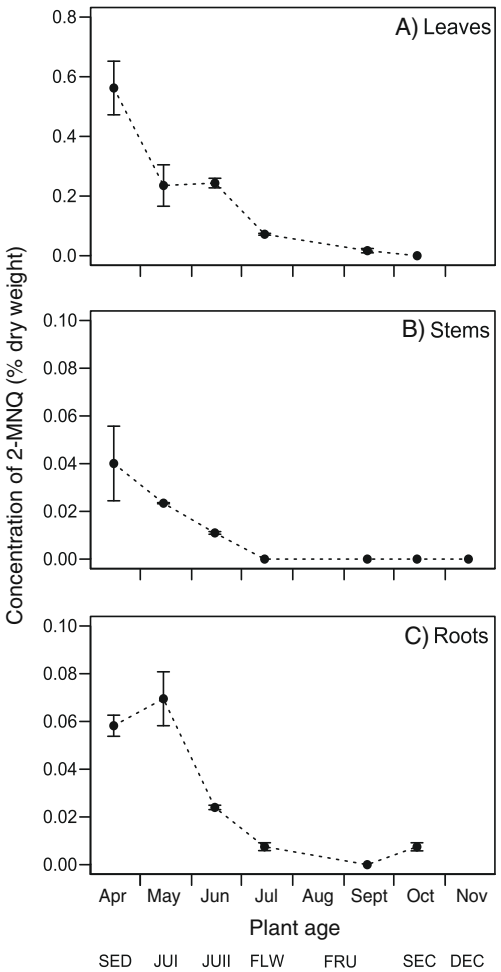

Fig. 1 Concentrations of 2-methoxy-1,4-naphthoquinone (2-MNQ) in leaves (**a**), stems (**b**), and roots (**c**) of *Impatiens glandulifera*, for plants of different ages. *SED* seedlings in April; *JUI* juveniles in May; *JUII* juveniles in June; *FLW* flowering plants in July; *FRU* fruiting plants in August; *SEC* senescent plants in October; *DEC* decomposing plants in November

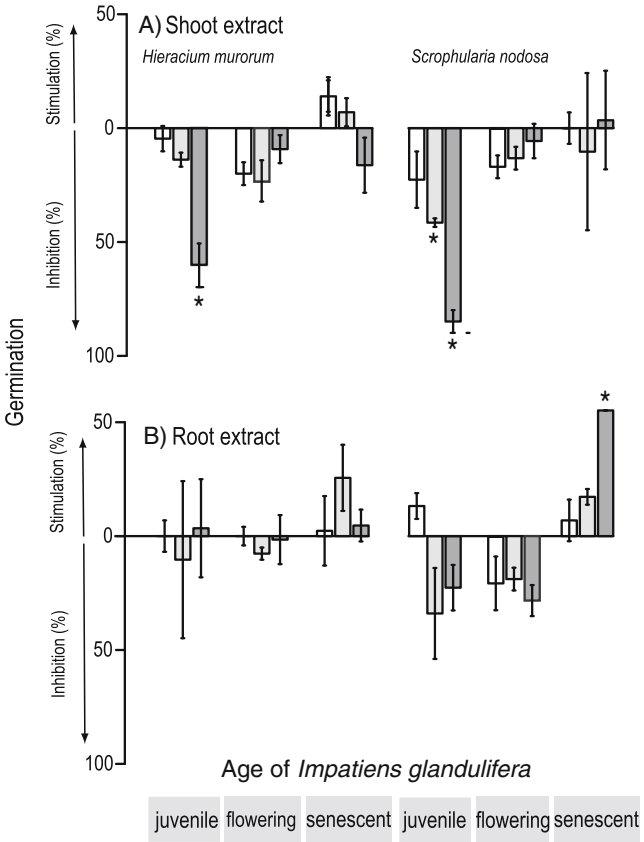

Fig. 2 Inhibition/stimulation of seed germination (%) (mean ± SE) for *Hieracium murorum* and *Scrophularia nodosa* by aqueous shoot (**a**) or root (**b**) extracts of *Impatiens glandulifera* plants of different ages. Concentrations of shoot or root tissue in different extracts were 0.0625 g/ml (*white bars*), 0.125 g/ml (*light grey bars*), and 0.250 g/ml (*dark grey bars*). Values are presented as percentage difference to the controls. Student's *t*-test was used to examine significant deviations from zero (* indicates $P<0.05$)

I. noli-tangere, lawsone was identified in leaves of flowering (mean ± SE in each case, $N=5$; 0.018±0.005 %) and fruiting (0.082±0.005 %) individuals and in roots of senescent plants (0.012±0.002 %).

Naphthoquinones in Soil Extracts, Rainwater, and Aqueous Extracts 2-MNQ and 1,4-naphthoquinone (NQ) from plant exudates of *I. glandulifera* were determined by using resin bags (Table 1). The amount of 2-MNQ determined per bag varied among plants of different ages. In contrast, the amount of NQ declined with plant age (Table 1). 2-MNQ and NQ were not detected in resin bags buried under native forest vegetation.

2-MNQ also was found in rainwater rinsed from *I. glandulifera* leaves (mean ± SE = 12.21± 3.01 μg ml^{-1}), but not in rainwater rinsed from native forest vegetation.

In aqueous extracts of *I. glandulifera* shoots, the concentration of 2-MNQ (μg/ml) declined from the juvenile to the senescent stage (Table 2). However, 2-MNQ was not detected in the aqueous root extracts.

Seed Bioassays Germination of *H. murorum* and *S. nodosa* seeds was inhibited to a different extent by aqueous extracts obtained from *I. glandulifera* plants of different ages (Fig. 2, Tukey HSD tests $P<0.05$, online resource 2). Shoot extracts of the invasive plant had a significantly higher inhibitory effect on seed germination of both native species than root extracts (Fig. 2, online resource 2). Only the most concentrated shoot extract obtained from juvenile plants significantly inhibited seed germination of *H. murorum* (Fig. 2). Similarly, seeds of *S. nodosa* were significantly inhibited by 42 and 85 % in the two most concentrated aqueous shoot extracts obtained from juvenile plants (Fig. 2). In contrast, the germination of *S. nodosa* seeds was differently affected by root extracts obtained from plants of different ages. The most concentrated root extract obtained from senescent plants resulted in a 55 % stimulation of seed germination in *S. nodosa* (Fig. 2, online resource 2). Inhibition of seed germination (%) in both species increased with increasing 2-MNQ concentrations in shoot extracts (*H. murorum*; $r_s=-0.633$, $N=18$, $P<0.001$; *S. nodosa;* $r_s=-0.503$, $N=18$, $P<0.001$; online resource 3).

Mycelium Growth Bioassays Aqueous extracts inhibited mycelium growth (%) in all three EM species (*L. bicolore*, *L. subdulci* and *P. tinctorius*) with a significant dependence on the age of the invasive plant (Tukey HSD tests $P<0.05$, online resource 4). Shoot extracts had a significantly higher inhibitory effect on mycelium growth than root extracts (Fig. 3, online resource 4). All concentrations of shoot extracts from juvenile plants inhibited mycelium growth by 100 %, while corresponding root extracts did not affect mycelium growth of the fungi examined (Fig. 3, online resource 4). Moreover, mycelium growth of *L. bicolore* and *P. tinctorius* was significantly inhibited by root extracts from flowering and senescent plants (Fig. 3), independent of their concentrations. In contrast, inhibition of mycelium growth (%) in *L. subdulci* was significantly reduced by the most concentrated root extract from flowering plants and as well as by root extracts from senescent plants (Fig. 3).

Mycelium growth decreased with increasing 2-MNQ concentrations in aqueous shoot extracts (*L. bicolore*; $r_s=-0.826$, $N=18$, $P<0.001$; *L. subdulci*; $r_s=-0.837$, $N=18$, $P<0.001$; *P. tinctorius*; $r_s=-0.845$, $N=18$, $P<0.001$; online resource 5).

Synthetic 2-MNQ Mycelium growth of all three fungal species was significantly inhibited when synthetic 2-MNQ was added to the growth medium. In all species, fungal growth decreased with increasing 2-MNQ concentration (Fig. 4, online resources 4).

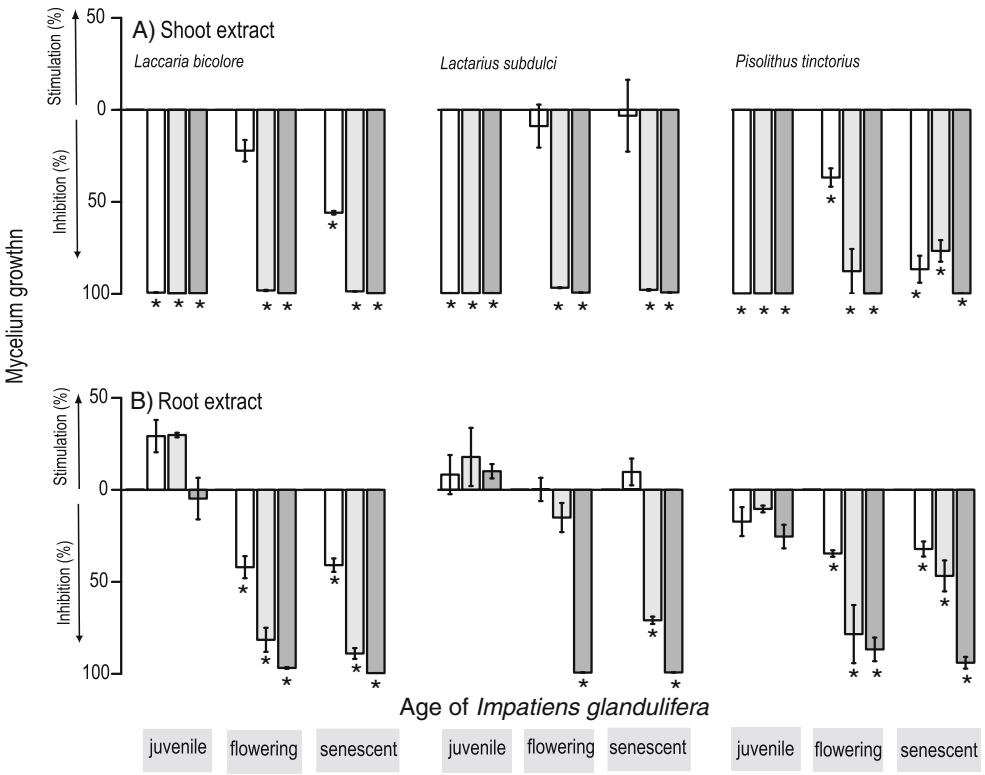

Fig. 3 Inhibition/stimulation of mycelium growth (%) (mean ± SE) for the fungi *Laccaria bicolore*, *Lactarius subdulci,* and *Pisolithus tinctorius* by aqueous shoot (**a**) or root (**b**) extracts obtained from *Impatiens glandulifera* plants of different ages. Concentrations of shoot or root tissue in extracts were 0.0625 g/ml (*white bars*), 0.125 g/ml (*light grey bars*) and 0.250 g/ml (*dark grey bars*). Values are presented as percentage difference to the controls. Student's *t*-test was used to examine significant deviations from zero (* indicates $P<0.05$)

Discussion

We observed organ-specific occurrence of 2-MNQ with the highest concentrations in leaves, confirming previous studies (Chapelle 1974; Lobstein et al. 2001) Furthermore, our study demonstrated 2-MNQ in roots of *I. glandulifera* for the first time. The 2-MNQ concentrations found in leaves of the invasive plant were 21 times higher than those detected in leaves of the native *I. noli-tangere* (Lobstein et al. 2001). The high 2-MNQ concentration in leaves could protect the invasive plant against phytophageous insects (Mitchell et al. 2007).

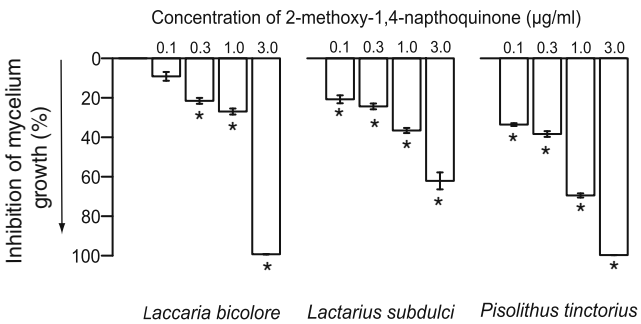

Fig. 4 Inhibition of mycelium growth (%) (mean ± SE) for the fungi *Laccaria bicolore*, *Lactarius subdulci,* and *Pisolithus tinctorius* in response to different 2-MNQ concentrations. Values are presented as percentage difference to the controls. Student's *t*-test was used to examine significant deviations from zero (* indicates $P<0.05$)

The 2-MNQ content of leaves, stems, and roots decreases with plant age, and is highest in seedlings. Thus, 2-MNQ may facilitate the establishment of young invasive plants because the germination of *I. glandulifera* occurs later in the season than the germination of most native plants in this habitat.

In the present study, lawsone was detected only in low quantities in root tissue of *I. glandulifera* in the senescent stage. Similarly, Chapelle (1974) found no lawsone in *I. glandulifera* leaves. In contrast, Lobstein et al. (2001) detected high quantities of lawsone in leaves, stems, and flowers of *I. glandulifera.* In the native species *I. noli-tangere*, lawsone was not detected in leaves in our study or by Chapelle (1974). However, our results contrast with those of Lobstein et al. (2001) who found low quantities of this naphthoquinone in flowering plants. Most probably, the differences in quantities and types of naphthoquinone reported are a result of different sample preparation methods and analytical techniques used for the determination of naphthoquinone content.

Our results show that 2-MNQ is released from the invasive plant by exuding from roots and leaching from leaves. Rainwater rinsed from *I. glandulifera* leaves contained 2-MNQ, while water rinsed from native vegetation did not contain this compound. Foliage leaching of allelopathic compounds has been demonstrated in other plants, e.g., juglone from *Juglans nigra* (Rietveld 1983) and canopy leachates from *Acacia dealbata*, an invasive tree (Lorenzo et al. 2011). Furthermore, several invasive plants exude allelopathic compounds from

roots (e.g. benzyl-isothiocyanate, Vaughn and Berhow 1999; (±)catechin, Callaway and Aschehoug 2000; 8-hydroxyquinoline, Inderjit et al. 2010). However, the present study is the first to demonstrate *in situ* the release of a potential allelopathic compound from both leaves and roots. Our data suggest that *I. glandulifera* modifies the chemical composition of the soil.

We found NQ in resin bags buried under invasive plants but could not detect it in any plant organ of *I. glandulifera*. Soil microbes modify chemical compounds that are released from plants into the soil (Inderjit 2005). Thus, soil microbes could degrade 2-MNQ to NQ by an unknown pathway. Degradation of the naphthoquinone juglone has been described (Müller and Lingens 1988; Rettenmaier and Lingens 1985). The seasonal variation of naphthoquinone amounts recorded in plant exudates of invaded soils could be explained partly by the seasonal variation in the activity of the microbial community (Kauri 1982).

Aqueous extracts frequently are used to examine the inhibitory effect of a compound on other organisms (e.g., Dorning and Cipollini 2006; Sun et al. 2006). It is essential to test different concentrations of the compound to entirely explore its inhibitory potential. In our study, the concentration of 2-MNQ found in aqueous extracts of *I. glandulifera* shoots declined in the course of the growing season. Surprisingly, we did not detect 2-MNQ in aqueous root extracts of plants of any age, although we found it in methanolic extracts of root tissues, suggesting that 2-MNQ stored in root tissues is less efficiently extracted in water than in methanol (80 %).

It previously has been shown that aqueous shoot extracts obtained from flowering *I. glandulifera* inhibit the germination of *Sinapis alba* (Csiszàr et al. 2012), *Leucinapis alba,* and *Brassica napis* (Vrchotová et al. 2011). In our study, high concentrations of shoot extracts from juvenile *I. glandulifera* had an inhibitory effect on the germination of the native forest plants *H. murorum* and *S. nodosa*. In contrast, aqueous extracts obtained from flowering *I. glandulifera* shoots and roots did not affect germination of either plant. The results correlate well with the concentrations of 2-NMQ measured in extracts used for the bioassays. Furthermore, *H. murorum* and *S. nodosa* are generalist species and may, therefore, react less sensitively to phytotoxic compounds than more specialized plant species. Our study extends the present knowledge on the inhibitory effect of 2-MNQ by showing that high concentrations of this compound inhibit germination of native forest plants.

Ectomycorrhiza fungi are sensitive to allelochemical compounds in leaf litter, plant tissue and roots (Cote and Thibault 1988; Rice 1979; Rose et al. 1983). In the invasive plant *Alliaria petiolata,* allelopathic compounds disrupt the association between EM fungi and their hosts (Wolfe et al. 2008). 2-MNQ is known for its allelopathic effect on fungal spore germination (Foote et al. 1949; Yang et al. 2001). In our study, EM fungi responded more sensitively than seeds of the plants species examined when exposed to the same concentrations of shoot and root extracts of *I. glandulifera*. Furthermore, mycelium growth of all fungi was affected to a similar extent by extracts of *I. glandulifera*. In general, shoot extracts had a stronger inhibitory effect on mycelium growth than root extracts. Extracts from roots of juvenile *I. glandulifera* were not inhibitory to mycelium growth of the EM species examined. This suggests that compounds other than 2-MNQ are active inhibitors of mycelium growth during the flowering and senescent stages of *I. glandulifera*. For example, degraded phenolic compounds could contribute to the reduction of mycelium growth.

Invasive plants including *Alliaria petiolata* and *Centaurea maculosa* produce allelopathic compounds that are novel in their non-native range and reduce the germination and growth of native plants (Callaway and Ridenour 2004; He et al. 2009). This phenomenon has been described by the novel weapons hypothesis. A similar mechanism could be responsible for the invasion success of *I. glandulifera*. The negative effect of 2-MNQ on herb germination and mycelium growth, and the absence of the compound in soils without *I. glandulifera* suggest that 2-MNQ could indeed be a "novel chemical weapon".

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