

Essential oils of two *Nepeta* species inhibit growth and induce oxidative stress in ragweed (*Ambrosia artemisiifolia* L.) shoots in vitro

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Abstract In vitro shoot cultures of ragweed (*Ambrosia artemisiifolia* L.), an important weed and allergen species, were established and utilized to explore the phytotoxic effect of essential oils of *Nepeta rtanjensis* Diklić and Milojević, and *N. cataria* L. Ragweed shoots were exposed to the atmosphere enriched with volatile compounds emitted from essential oils which differ in their qualitative and quantitative nepetalactone content. Essential oil of *N. rtanjensis*, an endemic and critically endangered perennial in Serbia, was characterized by high amounts of 4 α ,7 α ,7 β -nepetalactone (*trans,cis*-nepetalactone), while essential oil of *N. cataria*, possessed high amounts of 4 α ,7 α ,7 α -nepetalactone (*cis,trans*-nepetalactone). After 2 weeks of exposure to *Nepeta* essential oil (2 and 4 %, final nepetalactone concentrations), in vitro morphogenesis of ragweed shoots was significantly altered. Reduction in fresh weight of shoots and roots and rooting inhibition was observed together with prominent discoloration of shoots. Alterations in antioxidative defense system of ragweed

shoots as a response to essential oils treatments were characterized by increased peroxidase activity and decreased catalase and superoxide dismutase activity. Generally, essential oil of *N. cataria*, which possesses *cis,trans*-stereoisomer of nepetalactone, had stronger inhibitory effect on shoot growth, catalase activity, and was more efficient in stimulating peroxidase activity. *N. rtanjensis* essential oil, and thus *trans,cis*-nepetalactone, was more efficient in inhibiting rooting and root growth, and in suppressing superoxide dismutase activity. Therefore, essential oils of *N. rtanjensis* and *N. cataria* might find another application as potential bioherbicides against highly invasive species such as ragweed.

Keywords Ragweed · Nepetalactone · Antioxidative enzymes · *Nepeta rtanjensis* · *Nepeta cataria* · Allelopathy

Abbreviations

BM	Basal medium
BSA	Bovine serum albumin
CAT	Catalase
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EO	Essential oil
GC–FID	Gas chromatography with flame ionization detector
GC–MS	Gas chromatography with mass spectrometry
NBT	Nitro blue tetrazolium chloride
NFDM	Non-fat dry milk
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PMSF	Phenylmethanesulfonyl fluoride
POX	Peroxidase
PVPP	Polyvinylpyrrolidone
ROS	Reactive oxygen species

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SB	Sample buffer
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TEMED	Tetramethylethylenediamine
TPBS	Tween (0.05 %) in PBS buffer
VOC	Volatile compound

Introduction

Ambrosia artemisiifolia L. (common ragweed) from the *Asteraceae* family is the most widespread weed in North America, which has been accidentally introduced in Europe in XIX century and spreads across the continent. Some bioactive compounds (allelochemicals) released by ragweed, competitiveness with native flora, and herbicide resistance, were possibly a reason for quick spreading and ecological success of this invasive species, as well as for its dramatic effects on agricultural production. On the other hand, ragweed pollen is notorious for causing allergenic reaction in humans, specifically allergic rhinitis (Gyoffry et al. 1995; Tamarcaz et al. 2005). Adequate control of ragweed is, therefore, necessary to prevent crop yield loss and reduction of pasture productivity, but also to minimize negative effects on human health. Due to the fact that available mechanical and chemical ragweed control methods are not efficient enough, there is a need for new classes of herbicides with alternative mechanisms of action and target sites not previously exploited. Eco-friendly trends in weed management highlight natural compounds as new environmentally safe herbicides, so-called “bioherbicides”, which are based on compounds produced by living organisms. Allelochemicals, secondary metabolites of plants belonging to terpenoids, phenolic compounds, long chain fatty acids, organic cyanides, alkaloids, coumarins, tannins, steroids and others (Oleszek and Stochmal 2002; Li et al. 2010), are recognized as potent bioherbicides. Lately, there has been a growing interest for using essential oils (EOs) with bioherbicide potential, and some of them have already been commercialized and successfully launched in organic agriculture: GreenMatch EX which consists of lemongrass (*Cymbopogon* sp.) oils, InterceptorTM with pine (*Pinus sylvestris* L.) oil (Dayan et al. 2009; Soltys et al. 2013) and cynmethylin which is 2-benzyl ether-substituted analog of the monoterpene 1,4-cineole (Grayson et al. 1987; Duke 2010; Soltys et al. 2013). Cinch and Argold are the trade names of cynmethylin outside of the United States of America (Soltys et al. 2013). Constituents of EOs, such as monoterpenoids cineol and citronellol (Romagni et al. 2000; Singh et al.

2002, 2004), citronellal (Singh et al. 2006a), camphor and menthol (Schulz et al. 2007), camphor, 1,8-cineole and β -pinene (Nishida et al. 2005), α -pinene (Singh et al. 2006b), and nepetalactone (Mutlu et al. 2011), are reported to be particularly active in phytotoxic interactions.

Phytotoxic effects of some *Nepeta* species EOs on agricultural and weed species have been previously demonstrated, and are usually attributed to iridoid monoterpenoids nepetalactones (Kobaisy et al. 2005; Mutlu et al. 2011; Nestorović Živković 2013). Essential oils of *N. pannonica* containing 4 α ,7 α ,7 α -nepetalactone (*cis,trans*-nepetalactone) and 1,8-cineole as the main components completely inhibited the germination of lettuce seeds (Kobaisy et al. 2005), while EOs of *N. nuda* ssp. *albiflora* and *N. curviflora* inhibited radical elongation of *Lepidium sativum* and *Raphanus sativus* seedlings (Mancini et al. 2009). Also, it has been shown that EO of *Nepeta meyeri* had phytotoxic effect on seeds of several weed species, such as *Amaranthus retroflexus* L., *Bromus danthoniae* Trin., *Bromus intermedius* Guss., *Chenopodium album* L., *Cynodon dactylon* L., *Lactuca seriola* L., and *Portulaca oleracea* L. (Mutlu et al. 2011). Nestorović Živković (2013) showed a high phytotoxic potential of *N. rtanjensis* EO against some agricultural crops (*Lactuca sativa* L. var. “Majska kraljica”, *Lotus corniculatus* L., *Lepidium sativum* L.) and weed species (*Stellaria media* (L.) Vill. and *Arabidopsis thaliana* L. Heynh ecotype Columbia).

The aim of the present study was to examine the possibility of using EOs of *Nepeta* species as bioherbicides against ragweed, and to investigate their mode of action. To provide fundamental information on phytotoxicity, which is the basis and a prerequisite for the successful weed control in agricultural practice, an in vitro culture experimental system of ragweed shoots has been established. Some literature data explaining differential biological activities of nepetalactone stereoisomers on animals and bacteria (Bates and Sigel 1963; Kalpoutzakis et al. 2001; Peterson and Coats 2001; Peterson et al. 2002; Gkinis et al. 2003) encouraged us to look for differential phytotoxic effect of 4 α ,7 α ,7 α -nepetalactone (*trans,cis*-nepetalactone) and 4 α ,7 α ,7 β -nepetalactone (*cis,trans*-nepetalactone), by tracing ragweed shoot growth and antioxidant enzymes activities. EOs of two *Nepeta* species, differing in their qualitative nepetalactone content, were used in experiments. *Nepeta rtanjensis* Diklić and Milojević, an endemic and critically endangered perennial in Serbia, is characterized by high amounts of *trans,cis*-nepetalactone, and the presence of *cis,trans*-nepetalactone in significantly lower amounts (Chalchat et al. 2000; Stojanović et al. 2005; Nestorović et al. 2010; Mišić et al. 2014). *N. cataria* L. possess high amounts of *cis,trans*-nepetalactone (Birkett et al. 2011; Mišić et al. 2014) in EO.

Materials and methods

Plant material

Seeds of common ragweed (*Ambrosia artemisiifolia* L.) were collected in September 2012 in Bački Monoštor (Bačka, Vojvodina, Serbia). Seeds were kept in paper bags at room temperature until use. Species was authenticated by the authors and the corresponding voucher specimens have been deposited at the Department of Plant Physiology, Institute for Biological Research “Siniša Stanković”, Belgrade, Serbia.

Nepeta rтанjenensis was collected in 2009 on experimental fields of the Institute for Biological Research “Siniša Stanković”, Belgrade, Serbia. Plant material was air-dried at 30 °C and stored in paper bags at room temperature until essential oils extraction. Voucher specimens are deposited at the Herbarium of the Institute of Botany and Botanical Garden “Jevremovac”, Faculty of Biology, Belgrade, Serbia (16064 BEOU).

Plant culture conditions

The ragweed seeds were surface sterilized in 70 % ethanol for 1 min, subsequently treated with 80 % solution of commercial bleach (3.2 % active chlorine) for 15 min, and with 0.1 % aqueous mercury chloride solution for 20 min. Seeds were washed six times in sterile deionized water. Ragweed seeds were aseptically placed in Petri dishes on a double layer of filter paper soaked with 10 ml of water. The Petri dishes with seeds were stratified at 4 °C for 30 days in dark and subsequently transferred to a growth chamber and kept at 25 ± 2 °C, with 16/8 h light/dark regime (irradiance of $70 \mu\text{mol m}^{-2}\text{s}^{-1}$). After 7 days, seedlings were transferred to Erlenmeyer flasks (250 ml) with 100 ml of solid culture medium, named basal medium (BM), containing a modified MS medium (Murashige and Skoog 1962) with one half-strength macro elements, and supplemented with 20 g l^{-1} sucrose and 7 g l^{-1} agar. Culture medium was brought to pH 5.8 before sterilizing by autoclaving at 114 °C for 25 min. Cultures were maintained at the same growth conditions as described above.

Ragweed multiplication

Axillary buds, which were used as explants, were subcultured every 4 weeks in the corresponding BM to establish continuous clone propagation. Twelve-month-old ragweed cultures were used in experiments.

Preparation of essential oils

N. rтанjenensis EO was isolated from air-dried aerial parts (flowering stage), by hydrodistillation for 2 h in a

Clevenger type apparatus, as previously described (Ljalević Grbić et al. 2008). The essential oil of *N. cataria* was the gift from Dr. M. Birkett (Biological Chemistry and Crop Protection Department, Rothamsted Research, Harpenden, Herts., Great Britain), and is prepared by steam distillation using cyclohexane as the cosolvent as described previously (Birkett and Pickett 2003).

Gas chromatography (GC) of *N. rтанjenensis* and *N. cataria* essential oils

HP-5890 Series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany) was equipped with split-splitless injector and automatic liquid sampler (ALS) and coupled with flame ionization detector (FID). Chromatographic separations were performed on HP-5 column ($25 \text{ m} \times 0.32 \text{ mm}$, $0.53 \mu\text{m}$ film thickness). The FID conditions were as follows: carrier gas flow rate (H_2) 1 ml min^{-1} , injector temperature 250 °C, detector temperature 300 °C, column temperature was linearly heated from 40 to 260 °C (at rate of 4 °C min^{-1}) and held isothermally at 260 °C for 10 min. Solutions of EO samples in ethanol ($\sim 1 \%$) were consecutively injected by ALS (1 μl , split mode, 1:30). Area percent reports, obtained as a result of standard processing of chromatograms, were used as base for the quantification purposes.

HP G 1800C Series II GCD system MS detector (Hewlett-Packard, Palo Alto, CA, USA) attached to a HP-5MS column ($30 \text{ m} \times 0.25 \text{ mm}$, $0.25 \mu\text{m}$ film thickness) was operated using helium as a carrier gas, and with transfer line heated at 260 °C. Mass spectra were acquired in EI mode (70 eV) in m/z range 40–450. The constituents were identified by comparison of their mass spectra to those from Wiley275 and NIST/NBS libraries, using different search engines. Determination of experimental values for retention indices was performed using calibrated Automated Mass Spectral Deconvolution and Identification System Software (AMDIS ver. 2.1, National Institute of Standards and Technology (NIST), Standard Reference Data Program, Gaithersburg, MD, USA), and by comparison with the available literature (Adams 2007).

Proton transfer reaction mass spectrometry (PTR-MS) analysis of nepetalactones in the ragweed surrounding atmosphere of the glass vessels

Concentrations of volatile compounds (VOCs) in the atmosphere of glass vessels were measured by the use of Proton Transfer Reaction Mass Spectrometer (Standard PTR-quad-MS, Ionicon Analytik, GmbH, Austria). Measurements were performed in triplicate, at the beginning of experiments (after 24 h), and at the end of experiments (after 2 weeks).

The identification and quantification of VOCs by PTR-MS is based on protonated parent ion masses (molecular mass plus one atomic mass unit, $[M + 1]^+$), yielded in proton transfer reaction with H_3O^+ ions ($H_3O^+ + R \rightarrow RH^+ + H_2O$, $R = VOC$), and relative abundance of ions obtained by the use of quadrupole mass spectrometer and secondary electron multiplier. A detailed description of the method is given elsewhere (Lindinger et al. 1998).

Twelve masses, including monoterpenoids with $[M + 1]^+$ at m/z 137 and m/z 153, nepetalactones with $[M + 1]^+$ at m/z 167, and sesquiterpenoids with $[M + 1]^+$ at m/z 205 (0.1 s dwell time), and data of five control parameters (m/z 21, m/z 25, m/z 30, m/z 32, and m/z 37) were obtained in 1.8 s cycles. Drift tube parameters included: pressure 2.11–2.13 mbar; temperature 60 °C; voltage 600 V; E/N parameter 145 Td; and reaction time 90 μ s. The count rate of H_3O^+/H_2O was 2.1–14.1 % of the count rate of H_3O^+ ions, which was in the range 2.1×10^6 – 6.1×10^6 counts s^{-1} . The calibration was done according to Taipale et al. (2008), using TO-15 Supelco gas mixture (m/z 57, m/z 79, m/z 93, m/z 107, and m/z 121), diluted with ASGU 370-p HORIBA system zero air to five concentrations ranging from 0.5 to 100 ppb. For the calculation of transmission coefficients above m/z 170, the logarithmic extrapolation was used. Calculated normalized sensitivities were 2.23, 1.77, 1.52, and 1.33 npcs ppb^{-1} for m/z 137, 153, 167, and 205, respectively.

In vitro bioassay

Phytotoxic potential of *Nepeta rtanjenensis* Diklić and Milojević and *Nepeta cataria* L. EOs against ragweed shoots, (about 1 cm high) were investigated. EO of *N. rtanjenensis* contained 88 % of nepetalactone (72 % of *trans,cis*-nepetalactone and 16 % of *cis,trans*-nepetalactone), while *N. cataria* EO was composed of 77 % *cis,trans*-nepetalactone, as revealed by GC–MS and GC–FID analyses. EOs of both *Nepeta* species were diluted in methanol (AppliChem, Cheshire, SAD) to obtain final nepetalactone concentrations of 2 and 4 %.

Five axillary buds were placed in each 350-ml glass jar containing 75-ml BM. Two layers of filter paper (2.5×2.5 cm) were rolled up, placed on a sterilized metal holder that was stuck into the culture medium, and moistened with 50 μ l of diluted *N. rtanjenensis* or *N. cataria* EOs containing 2 or 4 % nepetalactone (Fig. 1). To test whether methanol also influences the growth of ragweed shoots, two groups of control plants were introduced into experimental setup: (1) those treated with the same maximal applied volume of methanol; (2) non-treated plants. All glass jars were closed with transparent polycarbonate caps, wrapped with parafilm (Bemis Flexible Packaging, Neenah, WI) and placed in growth chamber at 25 ± 2 °C,

under 16/8 h light/dark regime (irradiance of $70 \mu\text{mol m}^{-2}\text{s}^{-1}$). Fourteen days later shoot and root fresh weight (FW), rooting and FW increment of seedlings (weight of the shoots at the end of the experiment/corresponding initial values—10 mg) were measured. The percentage of inhibition of shoot and root FW was calculated using the formula: $\% = (1 - T/C) \times 100$, where T is the parameter of treated variants and C is the parameter of the control variants. The experiments were repeated three times, using 20 explants each.

Protein extraction

To study the effect of *N. rtanjenensis* and *N. cataria* EOs on activities of antioxidant enzymes, total soluble proteins were extracted from ragweed shoots grown in vitro for 14 days. Seedlings grown in the atmosphere enriched with methanol or without it were used as positive and negative control, respectively. Protein extraction buffer contained 50 mM Tris (pH 8.1), ethylenediaminetetraacetic acid (EDTA), 30 % glycerol, 1.5 % (w/v) polyvinylpyrrolidone (PVPP), 10 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF). About 750 mg of plant tissues were ground with liquid nitrogen and 2.5 ml of cold extraction buffer was added. Plant homogenate was centrifuged at 12,000g for 15 min at 4 °C. The supernatants were collected, stored at -70 °C and used for soluble protein determination and enzyme activity assays. The soluble protein content was determined according to Bradford (1976) with bovine serum albumin (BSA) used as protein standard.

Polyacrylamide gel electrophoresis

Native polyacrylamide gel electrophoresis (native PAGE) was performed in discontinuous non-denaturing gels using the Mini-Protein II system (Bio-Rad, Richmond, CA). Total proteins were separated on gels with final acrylamide concentration of 7 % for catalase (CAT) and 10 % in the case of peroxidase (POX) and superoxide dismutase (SOD). The concentration of stacking gels was 5 %, while Tris–glycine system (25 mM Tris, 192 mM glycine, pH 8.3) was used as a running buffer for the electrophoresis performed at 120 V and 4 °C. The amount of total proteins applied to each well was: 5 μ g for CAT and 20 μ g for either POX or SOD. Gels were subsequently stained for CAT, SOD or POX activities.

The final gels with developed enzyme activities were recorded by Gel-Doc™ EQ System (Life Science Research, Bio-Rad Co.) and the intensity of the CAT, POX and SOD bands was estimated by determining band volume with ImageJ 1.32j software (W. Rasband, National Institute of Health, USA) and presented as a percentage of the

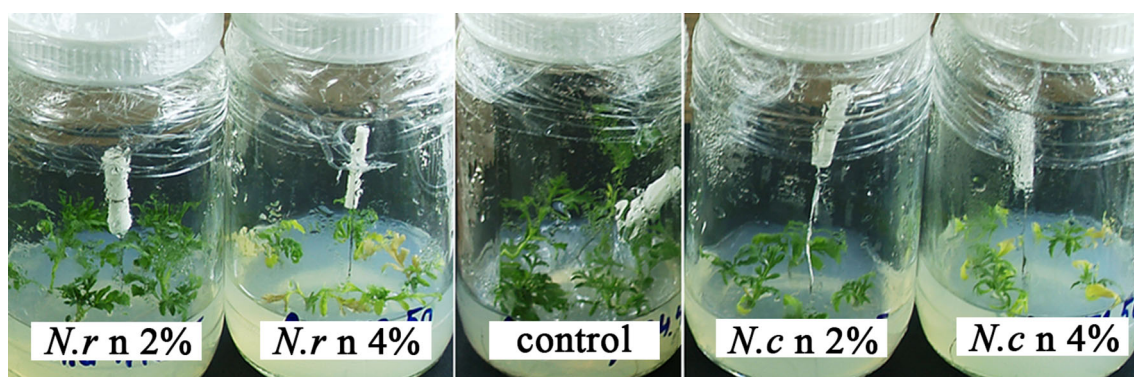


Fig. 1 Ragweed explants after 2 weeks of growing in glass jars on BM in the absence or in the presence of 50 µl diluted *N. ratanjensis* and *N. cataria* essential oils (2 and 4 % concentration of nepetalactones, both) on filter paper placed on metal holder

control values. The assays of gels were performed in triplicates.

Detection, characterization and quantification of CAT activity

The CAT activity was visualized after gel preincubation in 0.01 % H_2O_2 for 25 min, followed by a brief wash and incubation in a mixture of 2 % potassium ferricyanide (III) (K_3FeCN_6) and 2 % FeCl_3 for 10 min at 25 °C, as described by Woodbury et al. (1971). The principle involves the reaction of H_2O_2 with potassium ferricyanide (III) by reducing it to ferrocyanide (II). The peroxide is oxidized to molecular O_2 . FeCl_3 further reacts with ferrocyanide (II) to form stable, insoluble Prussian blue pigment. CAT signaled its location by scavenging H_2O_2 and stopping described reaction, causing the appearance of transparent bands on the gel.

Total CAT activity was measured spectrophotometrically by detecting the decrease in the H_2O_2 concentration at 240 nm due to the decomposition of H_2O_2 (Aebi 1984). The reaction mixture in a total volume of 1.5 ml consisted of 10 µl crude protein extract and 0.2 % H_2O_2 in 50 mM Na–K-phosphate buffer, pH 7. The activity was determined by measuring the rate of disappearance of H_2O_2 every 20 s over 3 min at 240 nm, at 25 °C (Agilent 8453 spectrophotometer, Life Sciences, USA). The results were expressed as specific activity units (U mg^{-1}), i.e. as mmol H_2O_2 ($\epsilon_{240} = 0.0436 \text{ mM}^{-1} \text{ cm}^{-1}$) decomposed in 1 min, per mg of soluble proteins and corresponded to the mean $\pm$ SE of the values obtained in three separated measurements.

Detection and quantification of POX activity

To observe separated POX isozymes, native PAGE gels were incubated with peroxidase substrates guaiacol. The

staining solutions contained guaiacol and H_2O_2 , each in concentrations of 10 mM, in 100 mM citrate buffer, pH 4 (Rothe 2002). Red–brown bands of tetrahydroguaiacol appeared on gels after 15 min staining at 25 °C.

Total POX activity has also been determined spectrophotometrically and reaction mixture contained 10 µl of crude protein extract, 20 mM pyrogallol ($\epsilon_{430} = 2.47 \text{ mM}^{-1} \text{ cm}^{-1}$), 10 mM H_2O_2 in a 1.5-ml volume of K-phosphate buffer (pH 6.5). The increase in absorbance was measured at 430 nm at 25 °C, due to oxidation and polymerization of pyrogallol to red purpurogallin, which catalyzes peroxidases. POX activity is expressed in specific activity units (U mg^{-1}), i.e. as mmol of produced purpurogallin in min per mg of total soluble proteins and corresponded to the mean $\pm$ SE of the values obtained in three separated experiments.

Detection, characterization and quantification of SOD activity

The active SOD isozymes were localized on gels by nitroblue tetrazolium–riboflavin reaction according to Beauchamp and Fridovich (1971). After electrophoresis, gels were soaked in 50 ml of 0.1 M K-phosphate buffer (pH 7.8) containing 0.1 M EDTA, 0.098 mM NBT, 0.03 mM riboflavin and 2 mM TEMED for 30 min. The gels were then rinsed in sterile water and illuminated with 36 W fluorescent lamps for 15 min to initiate the photochemical reaction. To determine different isozymes, specific inhibitors with known effects on the different SOD metal cofactors (cyanide and H_2O_2) were employed. KCN in concentration of 1 mM was applied in reaction buffer for inhibition of CuZn-SOD, 5 mM H_2O_2 was used for simultaneous inhibition of both CuZn-SOD and Fe-SOD forms, while Mn-SOD is insensitive to these inhibitors (Michalski 1996).

Additionally, a total SOD activity was assayed following the method of Beyer and Fridovich (1987), by

measuring ability of the protein extract to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) to blue formazan. The reaction mixture (1 ml) contained 100 mM K-phosphate buffer, pH 7.8, 0.1 mM EDTA, 12 mM L-methionine, 75 μ M NBT, 2 μ M riboflavin and 0–25 μ l of crude protein extract. Reaction mixture was exposed to illumination of a 36 W fluorescent lamp for 15 min at 25 °C. Absorbance was recorded at 540 nm using an ELISA microplate reader (LKB Vertriebs GmbH, Austria). Maximal coloration was obtained in irradiated reaction mixture without enzyme. One SOD unit was defined as the amount of enzyme required to inhibit the reduction of NBT by 50 %, in comparison with the mixture lacking the enzyme and expressed as units of enzyme activity (U) per mg of total soluble proteins.

SDS-PAGE electrophoresis and immunoblotting

Samples for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were dissolved in the equal volume of sample buffer (SB) containing 62.5 mM Tris–HCl (pH 6.8), 2.5 % bromphenol blue, 2 % SDS, 10 % glycerol and 0.5 % β -mercaptoethanol (Laemmli 1970), and heated at 95 °C for 5 min. After that, samples were cooled to 4 °C and centrifuged at 12,000g for 3 min. Separation of proteins was performed at room temperature using denaturing SDS-PAGE electrophoresis in Mini-Protein II system (Bio-Rad, Richmond, CA) for 120 min at 200 V. Final acrylamide concentration in SDS gels was 12 % (w/v) for separating gel and 5 % (w/v) for stacking gel. The amount of soluble proteins loaded in each well was 20 μ g. For detecting molecular weight of separated proteins, colored molecular weight markers 10–260 kDa (Spectra™ Multicolor Broad Range Protein Ladder, Fermentas GmbH, Germany) were used.

Proteins from SDS gels were electrotransferred at 60 V for 1.5 h onto nitrocellulose membranes (Bio-Rad, SAD) in transfer buffer containing 25 mM Tris–HCl (pH 8.3) and 192 mM glycine in apparatus Mini Trans-Blot Module (Bio-Rad, SAD). Membranes were rinsed in phosphate-buffered saline (PBS) consisting of 80 g l^{−1} NaCl, 2 g l^{−1} KCl, 37.2 g l^{−1} Na₂HPO₄ × 12H₂O and 2.4 g l^{−1} KH₂PO₄.

The blotted membranes were pre-incubated overnight at 4 °C, in a solution of 10 % non-fat dry milk (NFDM; Nestle, USA), in a blocking TPBS buffer (PBS buffer + 0.05 % Tween-20). Membranes were unblocked by rinsing in methanol for 10 s and were cut into two equal parts. Membranes which were incubated in Ponceau S staining solution according to the manufacturer's procedure (Sigma Prod. No. P7170) were used as control for the total proteins detection (Romero-Calvo et al. 2010). Other halves of membranes were incubated for 2 h with primary

CSD2 antibodies (Anti Rabbit chloroplastic CuZn-Superoxide Dismutase, AS06170; Agrisera Antibodies, Sweden) or with Anti Rabbit Mn-Superoxide Dismutase antibodies (AS09524, Agrisera Antibodies, Sweden). Antibodies were diluted 1:10,000 (v:v) in TPBS containing 5 % NFDM, separately. After intensive washing with TPBS for a period of 30 min, membranes were incubated in TBPS containing 5 % NFDM and goat anti-rabbit IgG-HRP secondary antibodies (Ao545, Sigma Aldrich, St. Louis, USA) conjugated to horseradish peroxidase at 1:20,000 dilution for 1 h, at room temperature on shaker. They were then washed three times and the proteins were finally visualized using an enhanced chemiluminescence detection system (ECL) and densitometrically quantified using ImageJ 1.32j software (W. Rasband, National Institute of Health, USA). After detection of CuZn-SOD and Mn-SOD abundance, membranes were unblocked rinsing in methanol for 10 s and blot stripped by incubating at 50 °C for 30 min, 3 times washing with TPBS for 30 min, and blocking in 10 % NFDM in TPBS over night. Then, to assessed equal loading in Western blots, membranes were incubated for 2 h in primary Actin-11 antibodies (Anti Mouse monoclonal IgG2b lyophilized, AS10702; Agrisera Antibodies, Sweden) diluted to 1:1000 (v:v) and Goat Anti Mouse IgG horse radish peroxidase conjugated secondary antibodies (AS11 1772, Agrisera Antibodies, Sweden) diluted to 1:50,000 (v:v) using procedure as described above.

The obtained signal intensities for CuZn-SOD and Mn-SOD were normalized to the control and actin values, and obtained results were presented as the abundance of CuZn-SOD and Mn-SOD in comparison to the control (%).

Statistical analyses

Statistical analyses were performed using STATGRAPHICS software, version 4.2 (STSC Inc. and Statistical Graphics Corporation, 1985–1989, USA). The data were subjected to one-way analysis of variance (ANOVA). Differences between means were evaluated by Fisher's LSD test calculated at the confidence level of $P < 0.05$. The percentage data were *arcsin* transformed before statistical analysis and inverse transformed for presentation.

Results

Establishment of in vitro ragweed shoots culture

In vitro culture of ragweed was established on BM medium from germinated seeds. Ragweed was micropropagated using axillary buds as explants. Axillary buds were excised from the mother plant and subcultured every 4 weeks on fresh BM. Although several ragweed genotypes were

available, all the experiments were performed with genotype 10, which showed the best growth under experimental conditions: the highest percentage of explants forming axillary buds, the highest number of axillary buds per explant, as well as the highest shoot FW (data not presented).

Effects of essential oils of *N. ratanjensis* and *N. cataria* on growth and development of ragweed shoots

Reduction in ragweed shoots growth and their discoloration, in the presence of *N. ratanjensis* and *N. cataria* EOs was observed after 2 weeks of exposure (Figs. 1, 2). FW of ragweed shoots was lowered by 19 and 64 % in the presence of 2 and 4 % nepetalactone of the *N. ratanjensis* EO, respectively. *N. cataria* EO, when applied in the concentration of 2 % nepetalactone reduced ragweed shoot FW by 38 %, while in the presence of 4 % nepetalactone shoot FW was lowered by 65 % in comparison to the control values (Fig. 2a). Regardless the concentration of nepetalactone, *N. ratanjensis* EO showed stronger inhibitory effect on the rooting of ragweed shoots than *N. cataria* oil, and the total inhibition occurred on treatments with 4 % nepetalactone in EO (Fig. 2b). The decrease of root FW was detected in the presence of both EOs. The inhibitory

effect significantly increased with increasing concentration of EOs, and the inhibition was more pronounced in shoots treated with *N. cataria* EO (Fig. 2c). The increment of FW in ragweed shoots grown in the presence of *N. ratanjensis* and *N. cataria* EOs was significantly lower when compared to the control plants, especially when the higher concentrations of EOs were applied (Fig. 2d). Two control groups of plants were not statistically different for all investigated parameters of growth.

Quantification of VOCs in essential oils and in the atmosphere of glass vessels

GC–MS and GC–FID analysis revealed the presence of a variety of terpenoids in *N. ratanjensis* and *N. cataria* EOs, with monoterpenoids being the most abundant (Table 1). The number of identified compounds was 11 and 19, respectively. Nepetalactones were the most dominant compounds in both EOs, however, their qualitative content was different. *Cis,trans*-nepetalactone (16.31 %) and *trans,cis*-nepetalactone (72.03 %) diastereoisomers were found in EO of *N. ratanjensis*, while only *cis,trans*-nepetalactone (76.85 %) was present in *N. cataria* EO. Other identified monoterpenoids and sesquiterpenoids were much less

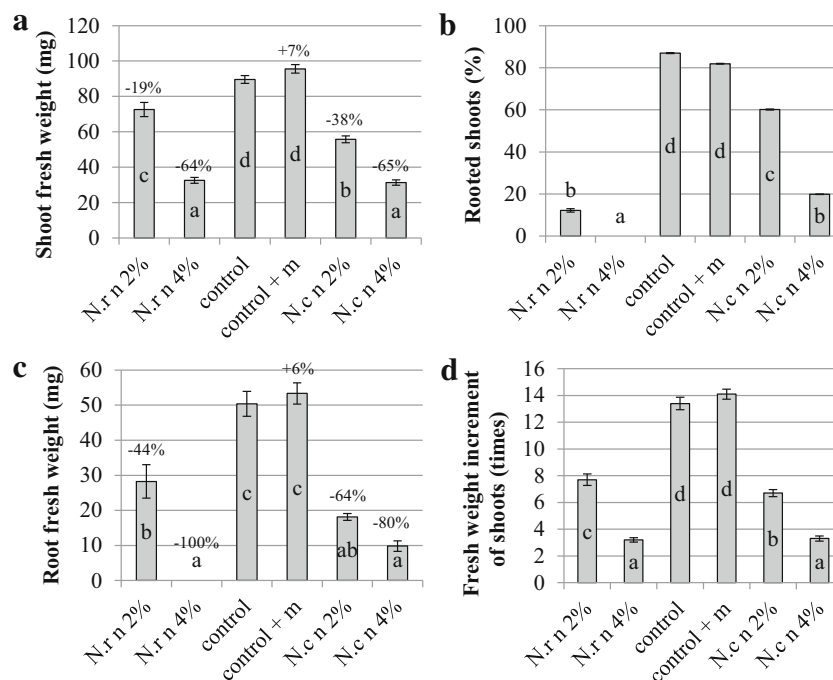


Fig. 2 Growth parameters of ragweed shoots after 2 weeks of in vitro culturing, in the absence (control) or in the presence of 50 μ l of diluted *N. ratanjensis* and *N. cataria* essential oils (2 and 4 % nepetalactones concentration): **a** shoot fresh weight (mg); **b** rooting (%); **c** roots fresh weight (mg); **d** fresh weight increment of shoots (times). The percentage data were *arcsin* transformed before statistical analysis and inverse transformed for presentation (**b**). The results

represent the mean $\pm$ SE of 3 repetitions, using 20 explants each. The values with the same letter belong to statistically homogenous groups ($P \leq 0.05$), according to Fisher's LSD test, while numbers above the bars indicate the percent of inhibition or induction relative to the control. Methanol (m) was used as positive control

Table 1 Percentage composition of terpenoids in *N. rtanjensis* and *N. cataria* essential oils, as revealed by GC–MS and GC–FID analysis

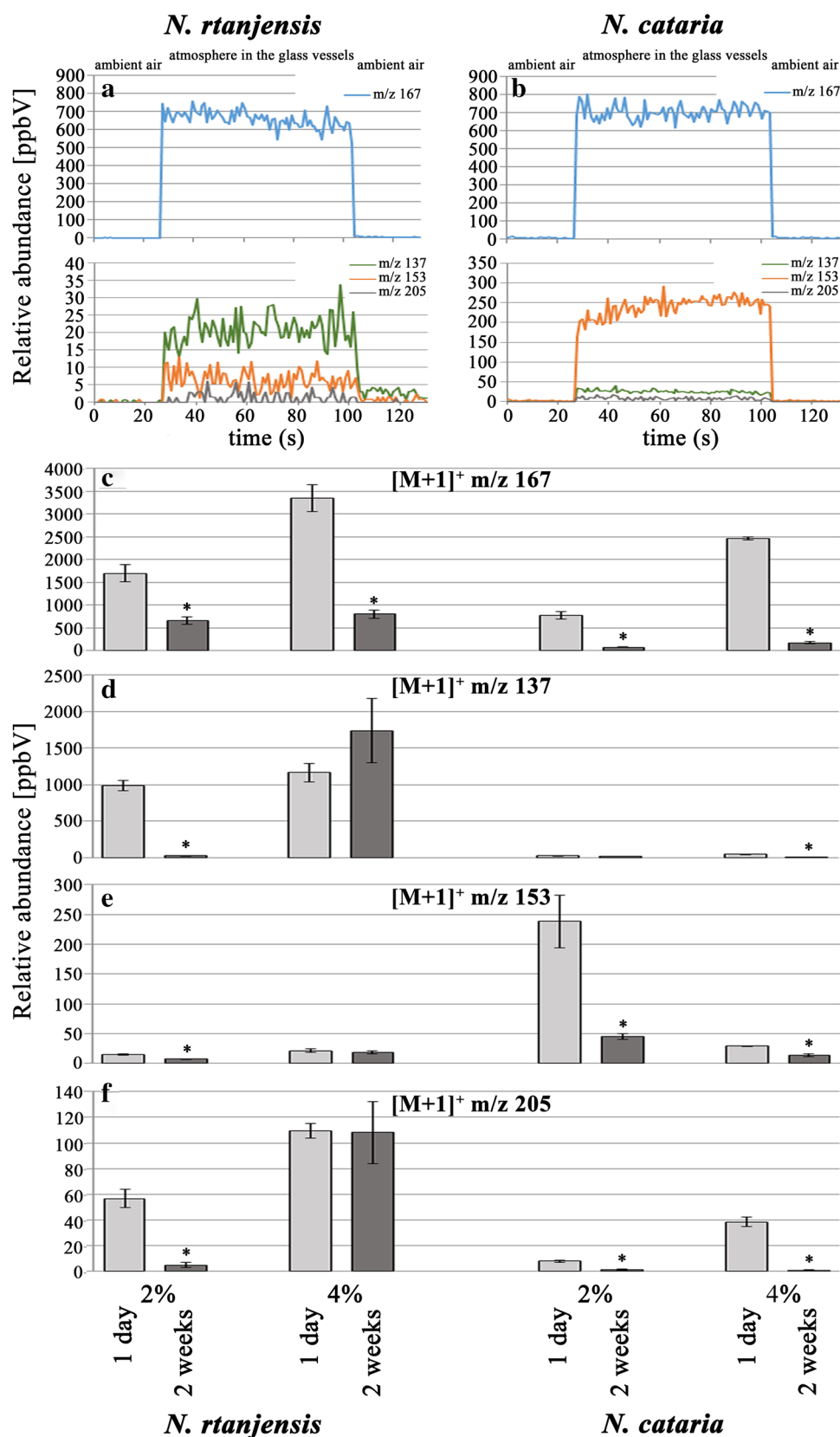
	No.	Constituents	KIE	KIL	<i>N. rtanjensis</i> % m/m	<i>N. cataria</i> % m/m
	1	α -Pinene ^a	931.5	932	2.99	
	2	β -Pinene ^a	974.7	974	0.40	0.07
	3	1,8-Cineole ^a	1030.3	1026	0.26	
	4	Linalool ^a	1095.0	1095		0.13
	5	α -Campholenal ^a	1127.9	1122	0.21	
	6	Neral ^a	1242.5	1235		0.09
	7	Geranial ^a	1266.7	1264		0.37
	8	Thymol acetate ^a	1342.8	1349		0.14
	9	4aa,7a,7aa-Nepetalactone ^a	1354.7	1357	16.31	76.85
	10	α -Copaene ^b	1374.7	1374	0.86	
	11	4a-a,7-b,7a-a-Nepetalactone ^a	1400.3	1391	72.03	
	12	cis-Caryophyllene ^b	1412.3	1408		0.50
	13	trans-Caryophyllene ^b	1416.8	1417		0.86
	14	Dihydronepetalactone ^a	1423.7	1414		0.08
	15	α -Humulene ^b	1451.8	1452		0.18
	16	Citronellol isobutanoate ^a	1478.5	1482		0.80
	17	γ -Cadinene ^b	1513.9	1513	0.15	
	18	δ -Cadinene ^b	1523.5	1522	0.59	
	19	α -Calacorene ^b	1543.8	1544	0.12	
	20	Spathulenol ^b	1571.0	1572		4.26
	21	Caryophyllene oxide ^b	1582.1	1582		4.80
	22	Humulene epoxide I ^b	1594.8	1593		0.11
	23	Humulene epoxide II ^b	1606.5	1606		0.36
KIE-Kovats (retention) index experimentally determined (AMDIS, uncorrected)	24	α -Cadinol ^b	1657.7	1652	0.13	
	25	α -Bisabolol oxide B ^b	1655.9	1656		0.12
KIL-Kovats (retention) index—literature data (Adams 2007)	26	Occidentalol acetate ^b	1681.0	1681		0.08
^a Monoterpenoids	27	α -Bisabolone oxide A ^b	1683.8	1684		0.11
^b Sesquiterpenoids	28	Neophytadiene, Isomer III ^c	1848.7	1849		1.89
^c Diterpenoids	Total				94.05	91.80

abundant. *N. rtanjensis* essential oil was characterized by the presence of significant amounts of monoterpene α -pinene (2.99 %), and relatively abundant sesquiterpenes were α -copaene (0.86 %) and δ -cadinene (0.59 %). On the other hand, sesquiterpenes significantly contributed to the total amount of VOCs in *N. cataria* EO, especially spathulenol (4.26 %) and caryophyllene oxide (4.80 %).

Organic volatiles present in the atmosphere of the glass vessels, released from the surface of filter paper moistened with EOs, were quantified using PTR-MS. Since different VOCs with the same mass cannot be separately measured, we traced the total amount of monoterpenoids with the protonated masses at m/z 137 and m/z 153, and total amount of nepetalactones (*cis,trans*- + *trans,cis*-nepetalactone) with $[M + 1]^+$ at m/z 167 (Fig. 3a–e). $[M + 1]^+$ at m/z 137 corresponds to protonated masses of monoterpenoids such as α - and β -pinene, while α -campholenal, neral and geranial show $[M + 1]^+$ at m/z 153. Another targeted protonated mass in PTR analysis was $[M + 1]^+$ at m/z 205, corresponding to sesquiterpenoids such as γ -

cadinene, δ -cadinene, *cis*- and *trans*- caryophyllene, and α -humulene (Fig. 3a, b, f). All these compounds were previously identified in *N. rtanjensis* and *N. cataria* EOs by GC–MS and GC–FID. PTR-MS analysis revealed that nepetalactones were the most abundant in the atmosphere of glass jars enriched both with *N. rtanjensis* and *N. cataria* EOs (Fig. 3c). Taking into account the previous GC–MS characterization of EOs, it could be presumed that on *N. rtanjensis* EO treatments, the major component present in the ragweed surrounding atmosphere was *trans,cis*-nepetalactone, while in *N. cataria* EO treatments *cis,trans*-nepetalactone predominates. Other monoterpenoids and sesquiterpenoids were much less abundant (Fig. 3d–f). Although the starting concentrations of nepetalactones in EOs dilutions of two *Nepeta* species were brought to 2 and 4 %, PTR-MS analysis revealed that nepetalactones were present in significantly higher concentrations in the ragweed surrounding atmosphere enriched with *N. rtanjensis* EO, than with *N. cataria* EO. Furthermore, PTR-MS measurements, performed at the beginning and at the end

Fig. 3 PTR-MS detection of targeted volatile organic compounds (VOCs) present in ragweed surrounding atmosphere of the glass vessels, and emitted from *N. rtanjensis* and *N. cataria* essential oils (EOs). PTR-MS profiles of targeted VOCs emission in the atmosphere of the glass vessels revealed nepetalactones to be the major compounds on treatments with both **a** *N. rtanjensis* (2 % of nepetalactones) and **b** *N. cataria* (2 % of nepetalactones) EOs. Total amounts of **c** nepetalactones (*cis,trans*- + *trans,cis*-nepetalactone) showing $[M + 1]^+$ at m/z 167; **d** monoterpenoids with the protonated masses at m/z 137 and **e** m/z 153; and **f** sesquiterpenoids with $[M + 1]^+$ at m/z 205 were recorded either 1 day or 2 weeks after the onset of experiments. Applied nepetalactones concentration in EOs was 2 or 4 %. The results represent the mean $\pm$ SE of three measurements. Asterisks indicate statistically significant differences in targeted VOCs concentration between measurements in the beginning and at the end of experiments, at $P \leq 0.05$ significance level based on Fisher's LSD test



of experiments, revealed that concentration of VOCs present in the atmosphere of glass jars decreased during time. Two weeks after the onset of experiments, the concentration of nepetalactones decreased by 61–76 and 90–93 % in ragweed shoots surrounding atmosphere enriched with *N. rtanjensis* and *N. cataria* EOs, respectively (Fig. 3c). Similar trend was observed for other targeted compounds.

Effects of *N. rtanjensis* and *N. cataria* essential oils on the activities of antioxidant enzymes in ragweed shoots

Catalases (CAT, E.C. 1.11.1.6) activity

In ragweed shoots treated with *N. rtanjensis* and *N. cataria* EOs for 2 weeks, the decrease of CAT activity in shoots

was confirmed by in-gel assay (Fig. 4a). Statistically significant decrease of CAT activity occurred in shoots grown on 4 % of *N. rtanjensis* oils (75 % of control) and 2 % *N. cataria* oils (77 % of control). Spectrophotometric assay (Fig. 4b) confirmed that only *N. rtanjensis* EO applied in 2 % nepetalactone concentration caused no significant change of the total CAT activity (Fig. 4b) when compared to the control values.

Peroxidases (POX) activity

Regardless the treatment, in-gel assay of peroxidase activity in ragweed shoots revealed seven POX isoforms in all samples (Fig. 4c). Relative POX activity was significantly increased in response to *N. rtanjensis* and *N. cataria* EOs treatments. The highest relative POX activity

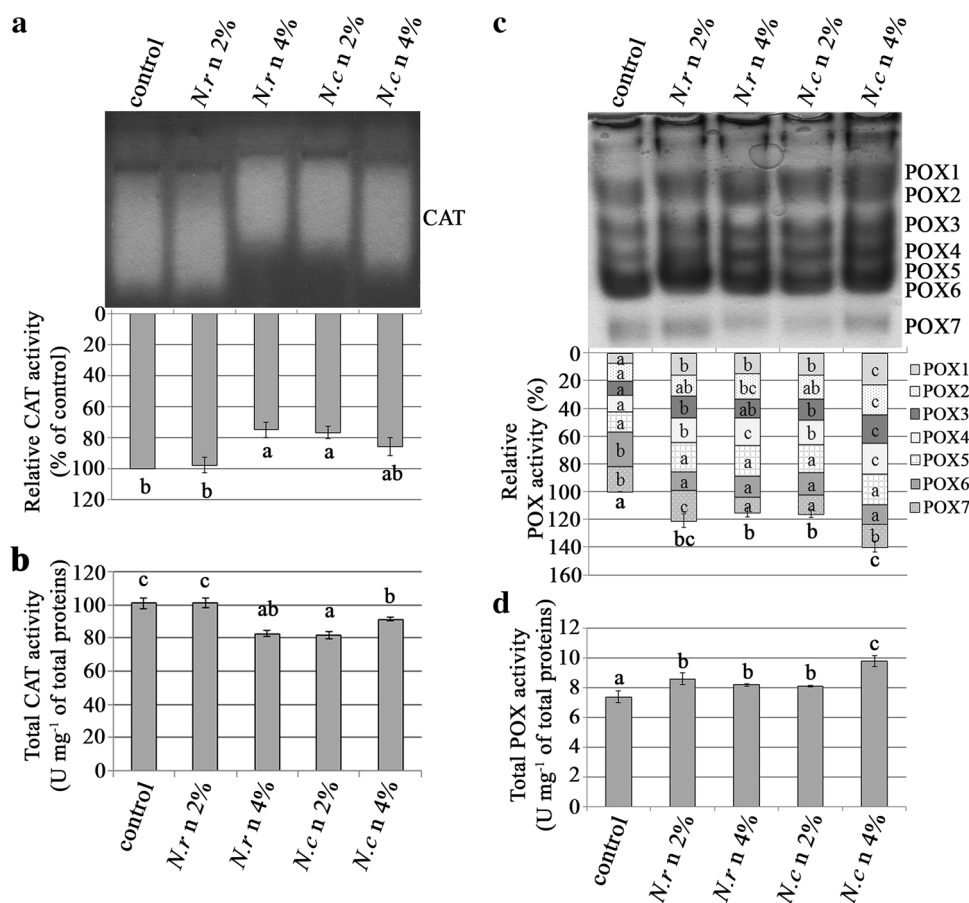


Fig. 4 Catalases (CAT) and peroxidases (POX) activity of in vitro cultured ragweed shoots exposed to diluted *N. rtanjensis* and *N. cataria* essential oils (2 and 4 % nepetalactones concentration), for 2 weeks. Total soluble proteins were extracted from whole seedlings and used for determination of the CAT and POX activity: **a** proteins from control and *N. rtanjensis* and *N. cataria* essential oil-treated ragweed shoots were loaded (5 µg per lane) on 7 % gel, separated by native-PAGE and assayed for CAT activity. The detected activities were measured densitometrically and presented as means of 3 replicates; **b** total CAT activity from the same samples, determined

spectrometrically; **c** proteins from control and *N. rtanjensis* and *N. cataria* essential oils -treated ragweed shoots were loaded (20 µg per lane) on 10 % gel, separated by native-PAGE and assayed for POX activity. The detected activities were measured densitometrically and presented as means of 3 replicates; **d** total POX activity from the same samples, determined spectrometrically. The results represent the mean ± SE of 3 replicates. The values with the same letter belong to statistically homogenous groups ($P \leq 0.05$), according to Fisher's LSD test

(139 %) was detected in the presence of 4 % nepetalactone from the *N. cataria* EO (Fig. 4c). Total POX activity assayed spectrophotometrically was significantly increased in all treated samples, and amounted from 8.1 to 9.8 U mg⁻¹ of total proteins, both recorded for *N. cataria* treatments (2 and 4 % nepetalactone, respectively) (Fig. 4d).

Superoxide dismutase (SOD, E.C. 1.15.1.1) activity

Staining native gels for SOD activity revealed four SOD isoforms in ragweed shoots grown in the presence of *N. rtanjensis* and *N. cataria* EOs, as well as in non-treated ragweed shoots (Fig. 5a), that were identified as Mn-, Fe- and CuZn-SOD isoforms, based on their differential

sensitivity to corresponding inhibitors (Fig. 5b). In treated shoots, we demonstrated the presence of Mn-SOD A isoforms, while in non-treated shoots there was Mn-SOD B isoform. Mn-SOD C isoform was present in all experimental groups. In-gel assay showed that relative SOD activities were significantly decreased in all treatments, when compared to the control; the lowest SOD activities (57 and 54 %) were achieved on treatments with 4 % nepetalactone of *N. rtanjensis* and *N. cataria* EOs, respectively. In the non-treated ragweed shoots, the highest portion in the relative SOD activity represents Mn-SOD activity, followed by Fe-SOD and CuZn-SOD activities. When exposed to EOs for 2 weeks, ragweed shoots demonstrated the reduction in Mn-SOD activity, and

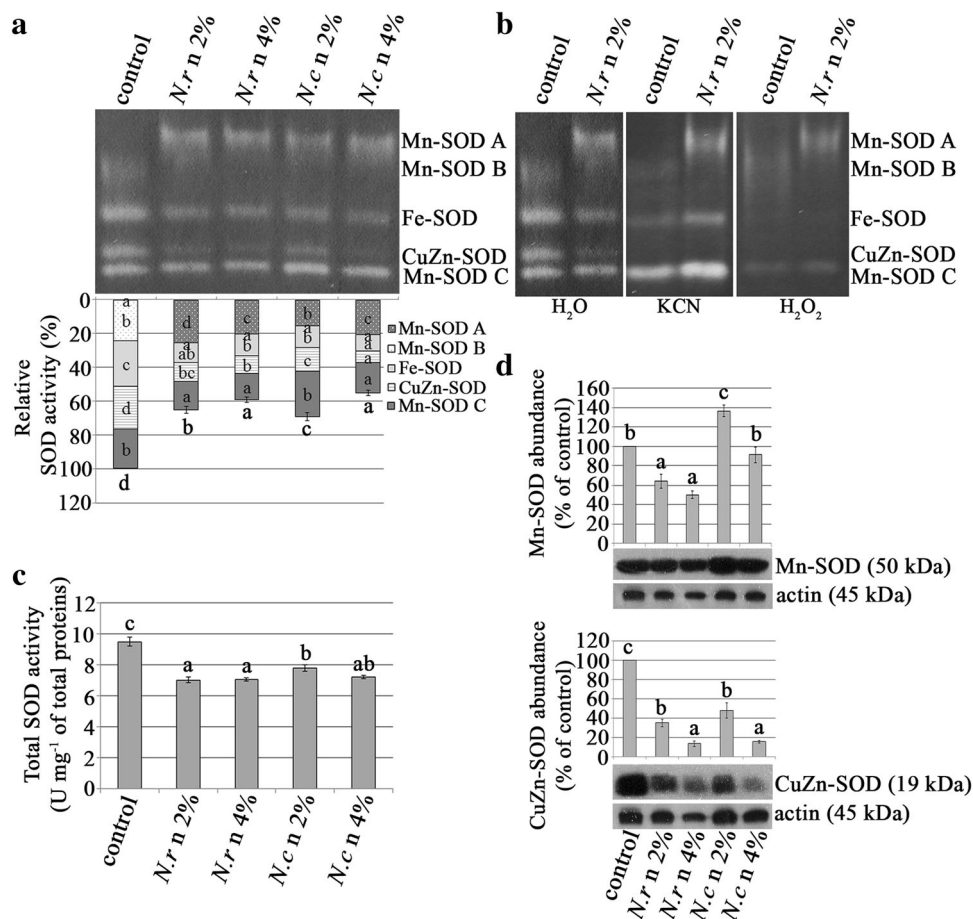


Fig. 5 Superoxide dismutase (SOD) activity in ragweed shoots exposed to diluted *N. rtanjensis* and *N. cataria* essential oils (2 and 4 % nepetalactones concentration), after 2 weeks. Total soluble proteins were extracted from whole seedlings and used for determination of the SOD activity: **a** proteins from control and *N. rtanjensis* and *N. cataria* essential oil-treated seedlings of ragweed were loaded (20 µg per lane) on 10 % gel, separated by native-PAGE and assayed for SOD activity. The detected activities were measured densitometrically and presented as means of 3 replicates; **b** identification of SOD isoforms based on differential sensitivity to hydrogen peroxide and potassium cyanide; **c** total SOD activity from the same samples,

determined spectrometrically. The results represent the mean $\pm$ SE of 3 replicates; **d** protein gel blot analysis of Mn-SOD and CuZn-SOD from the same samples were loaded on 10 % gel, transferred to nitrocellulose membrane and immunoblotted using primary antibodies Anti Rabbit Mn-Superoxide Dismutase or CSD2 (Anti Rabbit chloroplastic CuZn-Superoxide Dismutase). Bar diagrams show densitometric values expressed as a percentage of the value of control. The results represent the mean $\pm$ SE of 3 replicates. The values with the same letter belong to statistically homogenous groups ($P \leq 0.05$), according to Fisher's LSD test

especially in Fe-SOD and Cu-SOD activities. The spectrophotometric assay for the total SOD activity confirmed the results obtained by in-gel assay. In EOs-treated ragweed shoots, total SOD activities were significantly decreased in comparison with the control (Fig. 5c), especially on treatments with *N. rtanjensis* EO.

The results of the immunodetection assay revealed that significant amounts of Mn-SOD were present in control and EOs-treated ragweed shoots. Significant decrease of Mn-SOD abundance was detected in the presence of *N. rtanjensis* oil (2 and 4 % nepetalactone); while Mn-SOD abundance was even increased on treatments with 2 % *N. cataria* oil (Fig. 5d). The highest amount of CuZn-SOD was detected in control ragweed shoots. The application of EOs resulted in the significant decrease of CuZn-SOD amounts, especially on treatments with 4 % *N. rtanjensis* and *N. cataria* EOs (Fig. 5d).

Discussion

In vitro micropropagation offers the possibility to obtain a lot of genetically uniform and disease-free plant material under nutritionally and environmentally controlled laboratory conditions, and for a relatively short period of time (Thorpe 2007; Akin-Idowu et al. 2009). Further advantages of establishing in vitro culture experimental system in the current study were: (1) the possibility to specify and describe the direct effects of targeted allelochemicals under controlled laboratory conditions; (2) exclusion of the influence of other VOCs from the ambient air or, on the other hand, spreading the targeted compounds, as is the case under field conditions; (3) the possibility to control qualitative and quantitative VOCs content in the atmosphere of the tissue culture vessels and in the target plants surrounding, and to quantify them in real time by the use of PTR-MS; (4) collecting the data on VOCs mode of action on target plants within a short period of time. In vitro propagation of ragweed plants has been achieved on phytohormone-free MS culture media. To exclude the effect of genotype on phenotype variations in our experiments, all the experiments were performed using uniform plant material, i.e. genotype 10, which showed the satisfactory shoot regeneration capacity. The literature data on ragweed tissue culture in vitro are rather scarce. Hairy root cultures of ragweed have been previously established with the aim of producing secondary metabolites of interest (Gomez-Barriou et al. 1992; Bhagwath and Hjortsø 2000). To our knowledge, this is the first attempt to establish ragweed shoot cultures and further utilize them in exploring the phytotoxic effect of VOCs by direct exposure of plants to the atmosphere enriched with bioactive principles present in EOs. The aim of the present study was to investigate the

possibility of utilizing *N. rtanjensis* and *N. cataria* EOs as bioherbicides against ragweed, by applying them during vegetative growth phase of weed, thus providing valuable information for future investigations and in-field weed control.

It is well-known that EOs of some *Nepeta* species have a great phytotoxic potential against many agricultural and weed species inhibiting seed germination and seedlings growth by inducing changes in antioxidant system of target plants (Kobaisy et al. 2005; Mancini et al. 2009; Hyun Eom et al. 2006; Mutlu et al. 2011; Nestorović Živković 2013). EOs are complex mixtures of VOCs belonging to various groups of secondary metabolites, mostly different classes of terpenoids (monoterpenoids, diterpenoids, sesquiterpenoids, etc.), all showing specific volatility under given environmental conditions. As confirmed in the present study, qualitative and quantitative content of EOs does not necessarily reflect the content of VOCs in the target plants surrounding atmosphere, and it should be determined by applying sophisticated analytical methods such as head-space GC-MS, PTR-MS, or other adequate analytical methodologies. PTR-MS in our experiments enabled rapid and simultaneous quantification of VOCs in the atmosphere of the glass vessels, without the need for the pre-concentration or chromatography, but with the presence of isobaric interferences. However, GC-MS characterization of EOs, prior to PTR-MS analysis of VOCs in the atmosphere of glass jars, enabled us to define masses of interest, to assume which compounds present in EOs share the same masses, and what is their ratio. Based on these assumptions, *trans,cis*-nepetalactone represented the most abundant diastereoisomer of this monoterpenoid in the surrounding of ragweed plants treated with *N. rtanjensis* EOs. In the case when shoots were exposed to the atmosphere enriched with the components of *N. cataria* EO, *cis,trans*-nepetalactone was the dominant bioactive principle present. Monoterpenoids α -pinene and β -pinene are the second most abundant group of VOCs recorded on treatments with *N. cataria* EO. Although sesquiterpenoids showing $[M + 1]^+$ at m/z 205 represent the significant contributors to the total VOCs amount in EO of *N. cataria*, as revealed by GC-MS analysis, these compounds are minor components in the ragweed atmosphere enriched with EOs of this species. Taking into account the lower volatility of sesquiterpenoids in comparison with monoterpenoids, these findings are not surprising. The concentration of VOCs in the ragweed surrounding atmosphere, originating from EOs, decreased during 2 weeks of exposure. It could be presumed that changes in nepetalactone concentration might be a result of its transitions between liquid and gas phases, degradation, transformation, precipitation, or could arise from the compound leaking from the glass vessels to the ambient air. Although

the initial concentrations of nepetalactones in EOs were equalized (2 and 4 %) by diluting the EOs with methanol, the concentration of nepetalactones in ragweed surrounding atmosphere was higher on treatments with *N. rtanjensis* EO. These surprising findings might be the result of different physico-chemical properties of two nepetalactone diastereoisomers (melting point, boiling point, volatility, etc.), and/or two EOs (viscosity, density, composition, etc.). Therefore, the experimental setup established in the present study should be treated as a dynamic system (partially controlled), which, however, offers the possibility to trace the amounts of targeted VOCs in the glass vessels and their changes during time.

Allelochemicals (mostly nepetalactones) present in EOs of *N. rtanjensis* and *N. cataria* induced inhibition of growth in ragweed shoots and roots (Fig. 2), as well as discoloration of shoots, i.e. pigment disappearance. This phenomenon is pronounced on treatments with higher concentrations of *N. rtanjensis* and *N. cataria* EOs, indicating possible chlorophyll degradation or its reduced synthesis. This is in accordance with previous findings (Singh et al. 2002), which lead to the conclusion that volatile monoterpenes can induce adverse effects on photosynthesis in bill goat weed. Prominent negative influence of EOs on rooting of ragweed shoots and roots growth was especially expressed on treatments with *N. rtanjensis* EO, where high concentration of oils completely inhibited rooting (Fig. 2b, c). These findings are in accordance with the researches of Singh et al. (2006b), Nishida et al. (2005) and Romagni et al. (2000), showing that EOs and individual monoterpenoids can inhibit cell division and induce structural disruption and decomposition in roots. Considering the high nepetalactone content in EOs of *N. rtanjensis* and *N. cataria*, and the ratio of the two stereoisomers in both type of oils, it could be presumed that, *cis,trans*- and *trans,cis*- nepetalactone exhibit similar inhibitory effect on shoot growth (Fig. 2a, d), while *trans,cis*-stereoisomer has more pronounced inhibitory action on rooting and root growth (Fig. 2b, c). However, the co-influence of the minor EOs components could not be excluded. α -Pinene and β -pinene, common monoterpenoids present in EOs of aromatic plants, are known for their inhibitory effect on root growth (Singh et al. 2006b; Chohan et al. 2011, 2013).

Monoterpenoids from EOs of some phytotoxic plants inhibited plant growth by generating reactive oxygen species (ROS), particularly H_2O_2 , and thus inducing oxidative stress (Singh et al. 2006b; Mutlu et al. 2011). It is known that high amount of H_2O_2 can be removed from plant cells by antioxidant enzymes such as CAT and POX (Noctor and Foyer 1998; Singh et al. 2009). Singh et al. (2006b) observed that the phytotoxic substance α -pinene, when applied in appropriate concentrations (1–5 mM), causes

increase in POX activity in *Cassia occidentalis*. Since CAT activity in ragweed shoots showed no significant increase in response to *N. rtanjensis* or *N. cataria* EOs treatments, it could be presumed that POX mostly contributes to the H_2O_2 elimination. We might assume that *N. rtanjensis* and *N. cataria* nepetalactones from the EOs caused the increase of ROS in ragweed shoots, which further resulted in elevated POX activity (up to 1.4 times for 4 % *N. cataria* EO; Fig. 4a). The mechanism of the signal transduction could not be suggested based on the presented results. However, phytotoxic mechanism of monoterpenoids action can be partially explained by the disruption of cell membranes and membrane enzymes (Singh et al. 2009; Mutlu et al. 2011).

The ability of plants to overcome oxidative stress is only partially dependent on the induction of SOD activity, in view of the fact that many factors can regulate the availability of the substrates for SOD. SOD activity was decreased in ragweed shoots treated with *N. rtanjensis* or *N. cataria* EOs, which is in agreement with some previous investigations showing that monoterpenes can cause the inhibition of SOD activity (Dhindsa et al. 1981; Singh et al. 2006b; Ding et al. 2007; Hong et al. 2008; Mutlu et al. 2011). This decrease may be the result of decreased enzyme abundance, as revealed by immunodetection of Mn-SOD and Cu/Zn-SOD isoforms. Therefore, it could be presumed that allelochemicals present in *N. rtanjensis* and *N. cataria* EOs might cause lowered expression of *Mn-SOD* and *CuZn-SOD* genes, and thus lead to the decrease in the abundance of corresponding enzymes and their activity. However, these assumptions need experimental confirmation. The exception is the treatment with *N. cataria* EO, where no reduction in Mn-SOD abundance was observed. However, the trend of reducing the abundance of Mn-SOD isoform with increasing the concentrations of EOs was evident. Taking into account the fact that Cu-SOD isoform is localized in the chloroplast of plant cells, and an observation that ragweed shoots treated with EOs suffer from pronounced discoloration, it may be suggested that decrease in Cu-SOD abundance and activity is associated with altered chloroplasts function and/or reduced photosynthetic activity. Further studies are needed to confirm these assumptions.

The present study reveals that EOs of *N. rtanjensis* and *N. cataria* inhibited growth of ragweed shoots and induced oxidative stress, which led to the response of treated plants through the activation or inactivation of antioxidant defense system enzymes. When comparing the effect of *N. rtanjensis* and *N. cataria* EOs, it can be concluded that inhibition of CAT activity, as well as stimulation of the POX activity in ragweed shoots, was more pronounced on treatments with *N. cataria* EO, while suppression of SOD activity was more evident in the presence of *N. rtanjensis*

EO (Figs. 4, 5). These effects of EOs could be ascribed to nepetalactones (*trans,cis*- for *N. ratanjensis* and *cis, trans*- for *N. cataria*) which are by far the most abundant constituents of EOs (72 and 77 %, respectively). However, the synergism of major and minor EOs constituents probably represents a significant factor of the phytotoxic effects, as demonstrated in the case of *Calamintha nepeta* EOs (Araniti et al. 2013). The presented results highlighted the possibility of using *N. ratanjensis* and *N. cataria* EOs as bioherbicides. However, it is necessary to further explore the mode of their action, to examine possible toxic effects on non-target organisms (particularly on humans), to explore whether phytotoxicity exists in field conditions, and finally to calculate the feasibility of commercial production. These are the courses of our further work.

Author contribution statement SD was the main contributor to the performed work, and responsible for the establishment and maintenance of in vitro cultures, for designing and supervision of the experiments, as well as for writing of the manuscript. JNŽ was involved in collecting the data on in vitro cultures growth parameters, while SŽ, JB and NA participated in antioxidant enzyme assays. MP and AS performed PTR-MS measurements and processed the obtained data, while MR performed GC-MS and GC-FID analyses. DM participated in designing the experiments, in data interpretation and supervision of manuscript writing.

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