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Morpho-histological and bioherbicidal evaluation of wild-type and transformed hairy roots of goosefoot

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ABSTRACT

Chenopodium murale L. wild-type roots (WR) and hairy roots (HR) exudates have been previously shown to exert allelopathic activity against test plant species lettuce, wheat and *Arabidopsis*. To further investigate their allelopathic nature, a comparative morpho-histological characterization and total phenolic content (TPC) evaluation were performed. Furthermore, the phytotoxic activity of WR and HR clone R5 against three weed species and their antioxidant responses were also assessed. Except for the higher degree of branching and root hair incidence in HR clones, both WR and HR showed similar anatomical features, typical for higher plants. No significant difference in total phenolic content between WR and HRs, nor their exudates was found. Root exudates of WR and R5, applied as growth medium (GM) wherein these *C. murale* roots were cultured, displayed selective phytotoxic activity depending on the target weed species, ranging from suppression of germination to inhibition of seedling growth, that were linked with alterations in antioxidant enzyme activities. R5 greatly inhibited germination and seedling growth of *Capsella bursa-pastoris*, while WR inhibited those of *Vicia angustifolia* and *Amaranthus retroflexus*. Such phytotoxic properties (of WR and R5) qualify them as a promising natural resource in the management of weeds. Differences in allelopathic activity between HR and WR should be searched for in their allelochemical profile and the content of each allelopathic substance. The lack of anatomical abnormalities in hairy roots renders them an efficient tool for functional-genomic studies of *C. murale* root genes.
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1. Introduction

To elude the negative impacts of chemical herbicides on the environment, the overuse of which can lead to development of herbicide-resistant weed biotypes (Duke and Powles, 2009; Duke, 2012), numerous studies have recently attempted to exploit allelopathy of plants for weed control. A number of plants have been demonstrated to produce allelochemicals with mode of action similar to that of the synthetic herbicides. Consequently, these compounds are considered as an alternative in weed management (reviewed by Soltys et al., 2013). Secondary metabolites with allelopathic potential are produced in different plant tissues including leaves, stems, roots and seeds (Weston and Duke, 2003; Parvez et al., 2004). In contrast to an extensive progress in studying allelopathic plant–plant interactions that occur in the aboveground plant organs such as leaves and stems, very little research has focused on root–root interactions that play an important role in the establishment and maintenance of plant communities in the rhizosphere. These communities have important implications for agriculture since their effect may be beneficial, as is the case in natural weed control, or detrimental, when allelochemicals produced by weeds affect the of crop plants (Callaway and Aschehoug, 2000).

Goosefoot (*Chenopodium murale* L.) is a fast-growing annual weed plant native to Europe, Asia and northern Africa (Holm et al., 1997). It provides a good example of plant exerting allelopathic effects on different, particularly cultivated plant species (El-Khatib et al., 2004; Batish et al., 2007a,b) by releasing into the soil phenolics as putative allelochemicals, produced just by the roots (Batish et al., 2007a,b). Phenolic compounds are known as plant allelochemicals that may affect different metabolic and morphogenic processes in plants (Blum, 2011) by inducing generation of reactive oxygen species (ROS) thus leading to oxidative stress (Li et al., 2010) as one of the principal ways of phytotoxic action (Weir et al., 2004). The affected plants respond to this condition by increasing antioxidant defense that includes enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX).

Abbreviations: GM, growth medium; HR, transformed hairy roots; WR, non-transformed wild-type roots

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To facilitate research on allelopathic effects of *C. murale* root exudates released into the soil, we have previously established in vitro liquid culture system of *C. murale* transgenic hairy roots as an alternative strategy for allelopathic assays (Mitić et al., 2012). The hairy root cultures have already been used for the investigation of herbicidal activity of secondary metabolite compounds in *Fagopirum tataricum* (Kim et al., 2009; Uddin et al., 2011). The advantage of this model manifests in high growth rate of transformed roots, genetic stability of the cells and the ability of root growth in hormone-free medium. Moreover, this model eliminates the effects of competitive interference from the experimental system of allelopathy research. Bioassays revealed that *C. murale* hairy roots synthesize certain bioactive substances with inhibitory effect on seed germination and seedlings growth of crop plants, wheat and lettuce (Mitić et al., 2012) and the model plant *Arabidopsis* (Dmitrović et al., in press). However, in the forementioned studies differences in root growth capacity, phenotypic characteristics and particularly inhibitory activity were observed between wild-type and hairy root clones. The objectives of this study were to compare the physiological and morpho-histological features of wild-type and transgenic hairy roots and to test their allelopathic potential and herbicidal effects against common weed species *Capsella bursa-pastoris*, *Amaranthus retroflexus* and *Vicia angustifolia* distributed worldwide. Results of this investigation suggest that *C. murale* could be taken into consideration as yet another natural selective herbicide pool.

2. Materials and methods

2.1. *C. murale* root cultures

The genetic transformation, regeneration of hairy roots and their growth were carried out as previously described (Mitić et al., 2012). Briefly, transgenic hairy root clones were induced by *Agrobacterium rhizogenes* A4M70GUS from roots, cotyledons, leaves, and internodes of *C. murale* seedlings. Transformed roots were detached from the explants and grown further in 100-mL Erlenmeyer flasks containing 50 mL of liquid MS (Murashige and Skoog, 1962) medium, on a rotary shaker (70 rpm) for four weeks. Cultures were transferred on fresh MS medium in 4-week intervals. Wild-type (untransformed) roots (WR) were obtained from *C. murale* seedlings grown in MS liquid medium for four weeks and cultured further in the same manner as the transformed ones.

2.2. Morpho-histological analysis

Hairy root (HR) clones initially obtained from root (R1, R3 and R5) or cotyledon (C9 and C10) explants, as well as wild-type roots (WR), cultured in liquid MS medium for four weeks were morphologically and histologically analyzed. For morphological studies, the average hair density (number of hairs mm^{-2}), counted along 10 mm of the root tip, and the average root hair length (mm) were determined for each clone (three root samples per clone). Root morphology was observed and photographed using an Aristoplan stereomicroscope (Leiz, Vidovdale, Canada).

For histological analysis, root tips (10 mm long) were excised and fixed for 24 h in 3% (*v*/*v*) glutaraldehyde in 100 mM phosphate buffer, pH 7.2, at 4 °C. After a wash in the phosphate buffer (6 changes over 2 h), the material was post-fixed (24 h) in 1% osmium tetroxide in phosphate buffer, at 4 °C. The samples were dehydrated in ethanol and embedded in Araldite resin CY 212 (Agar Scientific Ltd. England) according to Glauert and Glauert (1958). Cross sections (1.0–1.5 μm thick) through the zone of apical meristem or its proximity and the zone of maturation were cut on a LKB III ultramicrotome and stained with 0.1% methylene blue solution in 1% borax. Sections were photographed under a Zeiss Axiovert microscope (Carl Zeiss GmbH, Göttingen, Germany).

2.3. Determination of total phenolic content (TPC)

The amount of total phenolics in *C. murale* WR and HRs, as well as in root growth media (GM), was determined using Folin–Ciocalteau's reagent according to Singleton and Rossi (1965) method. Air-dried root tissue was powdered in liquid nitrogen and soaked in 80% methanol (root tissue: methanol = 1 g: 10 mL). Liquid growth medium was filtered using Whatman 1 filter paper. To evaluate total phenolic content, 50 μL of root extract or GM filtrate was mixed with 0.475 mL of 5% Na_2CO_3 and vortexed. After 3–5 min, 0.475 mL of Folin–Ciocalteau's reagent was added to the mixture, immediately shaken and mixed, then incubated for 1 h in the dark. Methanol or control liquid medium was used as blanks. Absorbance of each solution was determined spectrophotometrically at 724 nm. Three replicates per sample were used. TPC was expressed in mg of gallic acid equivalents (GAE) per g of root dry weight or per mL of GM.

2.4. Bioassay on weeds

Liquid MS medium wherein initial 25 mg of HR clone R5 or WR was grown for four weeks (designated as growth medium, GM) was evaluated for its potential phytotoxicity against three weed species: shepherd's-purse (*C. bursa-pastoris* (L.) Medik.), redroot pigweed (*A. retroflexus* L.) and common vetch (*V. angustifolia* L.).

Seeds of the target weed species were collected in 2010 from a broader area of Belgrade (Zemun Polje), Serbia. Seeds were surface sterilized with 50% commercial bleach (4% active chlorine) for 10 min and then washed five times with sterile distilled water. Thirty seeds of *C. bursa-pastoris* and *A. retroflexus* and twenty seeds of *V. angustifolia* were placed in 90-mm Petri dishes, on a sterile filter paper wetted with 5 mL of either R5 or WR GM. Fresh liquid MS medium without root exudates was used as a control. The bioassay was repeated three times, using three Petri dishes (90 or 60 seeds in one repetition) per treatment. Petri dishes were sealed with parafilm (Bemis Flexible Packaging, Neenah, WI) and incubated two weeks in a growth chamber under standard cool-white fluorescent tubes (16 h light/8 h dark cycle) with a photon flux density of 70 $\mu mol\ m^{-2}\ s^{-1}$, at 25 ± 2 °C. The allelopathic activity of GMs was evaluated by final germination percentage, the highest root length and seedling fresh weight of each weed species and additionally by other parameters convenient for measurements in each individual plant species.

The percentage of inhibition was calculated using the following formula:

$$\%\ \text{inhibition} = (1 - \mathrm{T/C}) \times 100,$$

where T is the parameter of treated variants and C is the parameter of control variants. Obtaining positive value was indicative of stimulation by treatment.

2.5. Protein extraction and determination of antioxidant enzyme activities

Total soluble proteins were isolated from ~750 mg of two-week GM-treated and control seedlings (grown on MS without root exudates) of *C. bursa-pastoris* and *A. retroflexus* seedlings and *V. angustifolia* shoot and root tips, by grinding the tissue in liquid nitrogen and extracting with 3 mL of cold 50 mM Tris buffer (pH 8) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 30% glycerol, 1.5% (*w*/*v*) polyvinylpolypyrrolidone (PVPP), 10 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Plant extracts were subsequently cleared by centrifugation at 12,000 *g* for 10 min at +4 °C. The obtained supernatants were used for soluble protein determination and enzyme activity assays. The soluble protein concentration was calculated with reference to the standard curve obtained with bovine serum albumin (BSA) used as standard according to Bradford (1976).

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