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

S. Dmitrović [a,*], N. Mitić [a], S. Budimir [a], D. Janošević [b], S. Živković [a], M. Skorić [a], S. Ninković [a]

[a] Institute for Biological Research "Siniša Stanković", University of Belgrade, Despot Stefan Blvd. 142, 11060 Belgrade, Serbia
[b] Faculty of Biology, University of Belgrade, Studentski trg 3, 11000 Belgrade, Serbia

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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.

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

* Corresponding author. Tel.: +381 11 207 8388.
E-mail addresses: smile@ibiss.bg.ac.rs (S. Dmitrović), mitic.nevena@ibiss.bg.ac.rs (N. Mitić), budimir@ibiss.bg.ac.rs (S. Budimir), dusicaj@ibiss.bg.ac.rs (D. Janošević), suzy@ibiss.bg.ac.rs (S. Živković), mdevic@ibiss.bg.ac.rs (M. Skorić), slavica@ibiss.bg.ac.rs (S. Ninković).

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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 μmol 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:

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

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).

Total catalase (CAT, EC 1.11.1.6) activity was assayed spectrophotometrically by measuring the decrease in the H_2O_2 concentration at 240 nm (Aebi, 1984). The reaction mixture contained 10 μL of crude protein extract and 0.2% H_2O_2 in 50 mM Na–K–phosphate buffer pH 7, in a total volume of 1.5 mL. Decrease in absorbance at 240 nm, caused by addition of H_2O_2, was recorded every 20 s over 3 min, at 25 °C (Agilent 8453 spectrophotometer, Life Sciences, USA). The results were expressed as specific activity (U mg^{-1}), i.e. as mmol H_2O_2 ($\varepsilon_{240} = 0.0436\ mM^{-1}\ cm^{-1}$) decomposed per min per mg of soluble proteins and presented as means ± SE of the values obtained in three separate measurements.

The reaction mixture for spectrophotometrical determination of total peroxidase (POX, EC 1.11.1.7) activity contained 10 μL of crude protein extract, 20 mM pyrogallol, 10 mM H_2O_2 and 50 mM K-phosphate buffer pH 6.5 in a 1 mL volume. The increase in absorbance due to formation of red purpurogallin ($\varepsilon_{430} = 2.47\ mM^{-1}\ cm^{-1}$) resulting from oxidation and polymerization of pyrogallol, catalyzed by peroxidases, was recorded at 430 nm at 25 °C. POX activity was expressed as specific activity (U mg^{-1}), i.e. as mmol of produced purpurogallin per min per mg of total soluble proteins and presented as means ± SE of the values obtained in three separate experiments.

Additionally, total superoxide dismutase (SOD, EC 1.15.11) activity was determined by measuring the capacity of the enzyme extract to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) to blue formazan according to Beyer and Fridovich (1987). One milliliter of the reaction mixture contained 100 mM K-phosphate buffer pH 7.8, 0.1 mM EDTA, 12 mM L-methionine, 75 μM NBT, 2 μM riboflavin and 0–25 μL of crude protein extract. The 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 color was obtained in irradiated reaction mixture without enzyme. One SOD unit was defined as the amount of enzyme which reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzymes and expressed as units of enzyme activity per mg of total soluble proteins (U mg^{-1}).

2.6. Statistical analysis

The experiment was carried out three times using a completely randomized design with three replicates per treatment. The percentage data were arcsin transformed before statistical analysis and inverse-transformed for presentation. 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 $P < 0.05$. Statistical analysis was performed using STAT-GRAPHICS software, version 4.2 (STSC Inc. and Statistical Graphics Corporation, 1985–1989, USA).

3. Results

3.1. Comparative morpho-histological characterization of nontransformed wild-type and transformed hairy roots of C. murale

Hairy root clones of *C. murale* initiated on hormone-free MS medium, showed complete hormone autotrophy, typical of transformed roots; accordingly, their growth potential was much greater compared to nontransformed WR roots. In addition, hairy root clones formed a great number of lateral roots (Fig. 1A) whereas WR cultured under the same conditions showed slight elongation with less branching (Fig. 1B). Detailed stereomicroscopic examination of root clones cultured in liquid MS medium revealed the variations in root hair density and length between WR and hairy root clones, as well as among different hairy root clones (Fig. 1C). Wild type roots were thicker, with small number of tenuous root hairs. Numerous root hairs that characterized HR clones varied in density and length (Fig. 1C).

Histological analysis did not reveal substantial differences between HR and WR. In the region of apical meristem and its proximity, the cells of both WR and HR clones were meristematic, tightly packed, with dense cytoplasm and large nuclei, having high mitotic activity (Fig. 2A–F). Closely packed epidermal cells were elongated in the radial direction. Ellipsoidal cortical cells were elongated in the tangential direction and arranged in concentric layers. Despite similar histological organization, C9 (Fig. 2E) and C10 (Fig. 2F) clones were slightly different from both non-transformed WR roots and R1, R3, and R5 HR clones. The cells of all layers of C9 and C10 clones were plasmolyzed with distinct dark content in the vacuoles and the cytoplasm.

In the zone of maturation, epidermal cells of wild-type and hairy roots were densely packed and highly vacuolated (Fig. 2G–L). Root hairs were also present in non-transformed WR roots but were primarily characteristic for hairy root clones. The cortex cells of all examined roots were vacuolated, loosely organized with intercellular spaces between cells, and of different shape and size. Central stele of vascular tissue was closely connected to surrounding cortical tissue. However,

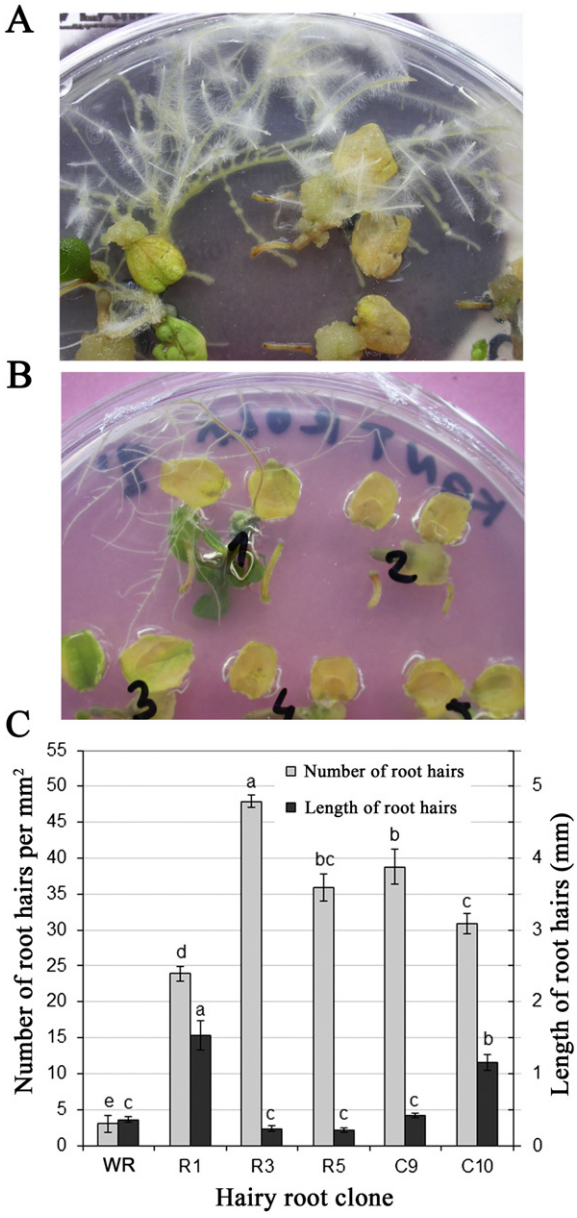

Fig. 1. Phenotypes of goosefoot (A) hairy roots with a great number of lateral roots and (B) wild type roots (WR) after 40 days of induction on MS medium. (C) Number and length of root hairs in WR and hairy root clones (R1, R3, R5, C9 and C10). Values are means of three replicates ± S.E. Different letters indicate significant differences assessed by Fisher's LSD test ($P < 0.05$).

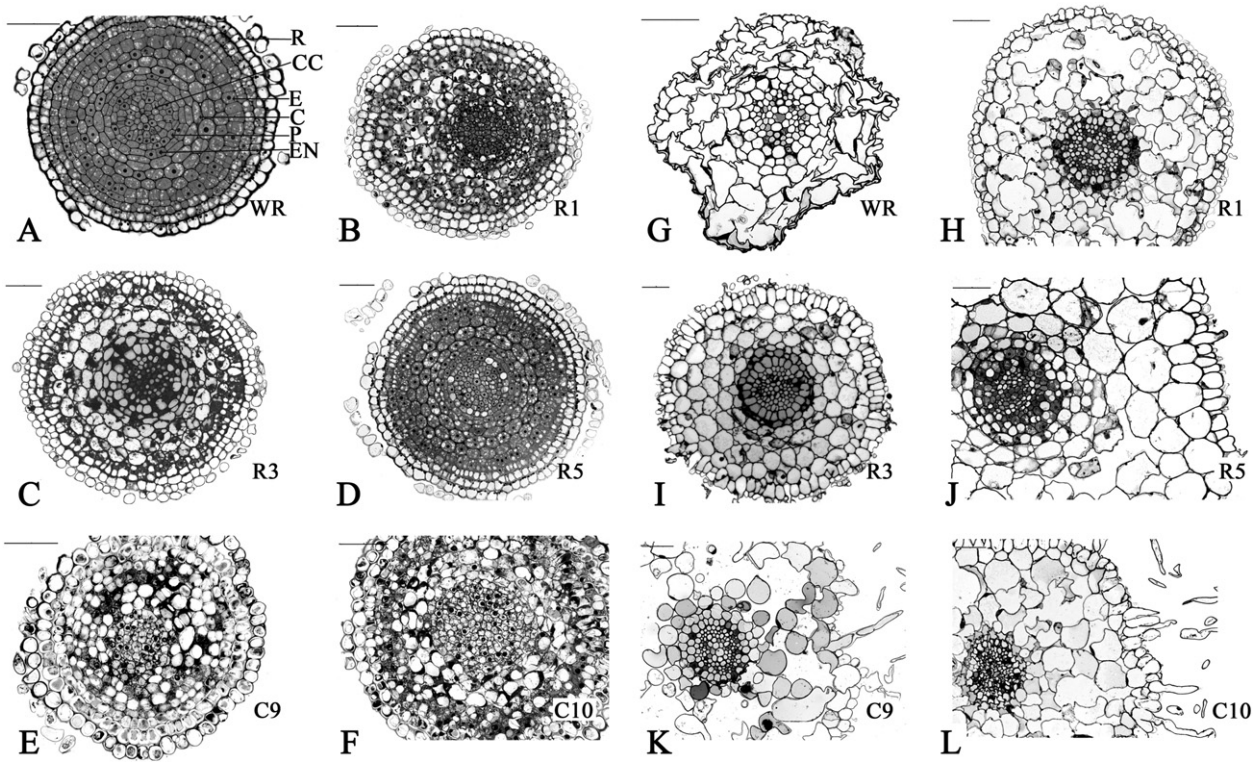

Fig. 2. Histological characteristics of goosefoot wild type (WR) and hairy root clones (R1, R3, R5, C9 and C10) in the zone of apical meristem (A–F) and in the zone of maturation (G–L). C—cortex, CC—central cylinder, E—epidermis, EN—endodermis, P—pericycle, and R—root cap. *Bar* = 50 μm.

along the root axis, the regions with ruptured epidermis, extended intercellular spaces, and central stele loosely connected to cortical cells were frequently observed (Fig. 2K).

3.2. Total phenolics content (TPC)

The quantification of TPC in the methanol extracts of different *C. murale* root clones and in their growth medium (GM), expressed as gallic acid equivalents, is shown in Table 1. Comparative analysis of total phenolics showed no difference between WR and HR tissues. Both WR and HR tissues contained far greater amount of total phenolics (4.5–5.1 mg GAE g^{-1} dry weight) than the corresponding GMs, which had remarkably lower TPC ranging from 0.093 to 0.250 mg GAE mL^{-1} (Table 1). Unlike the root tissue, where no significant differences in TPC were found among different HR clones, GM of R5 displayed significantly higher TPC (about 0.250 mg GAE mL^{-1}) than GMs from all other HR clones (<0.180 mg GAE mL^{-1}, Table 1).

Table 1
Total phenolic content (TPC) in wild type (WR) and hairy root clones (R4-R6, R8, C9 and C11) of *C. murale* and their respective growth media (GM). TPC was expressed in mg of gallic acid equivalents (GAE) g^{-1} dry weight (DW) of root tissue or in mg GAE mL^{-1} of growth medium.

Root clone	TPC	
	Hairy root tissue, mg GAE g^{-1} DW	GM, μg GAE mL^{-1}
WR	4.7 ± 0.1a	205.5 ± 16.0ab
R4	5.1 ± 0.1a	92.5 ± 5.3b
R5	4.5 ± 0.1a	249.7 ± 21.6a
R6	4.5 ± 0.02a	175.7 ± 8.9ab
R8	4.7 ± 0.004a	143.1 ± 16.7b
C9	4.5 ± 0.4a	137.9 ± 5.4b
C11	4.9 ± 0.5a	141.7 ± 8.6b

Data represent the mean of three repetition ± SE. Different letters indicate significant differences assessed by Fisher's LSD test ($P < 0.05$) after performing ANOVA analysis.

3.3. Bioassay on weeds

Since GM of HR clone R5 had significantly higher TPC than other HR clones and since it exhibited prominent phytotoxic effect on previously tested species wheat, lettuce (Mitić et al., 2012) and *Arabidopsis* (Dmitrović et al., in press), it was selected for the comparative study of the herbicidal properties of WR and HR.

The allelopathic effect of WR and R5 of *C. murale* on seed germination and seedling growth was tested in three weed species; *C. bursa-pastoris*, *A. retroflexus* and *V. angustifolia*. Results revealed WR and R5 differed in allelopathic potential depending on the weed species (Figs. 3, 4). WR exudates exerted the strongest inhibitory effect on A. *retroflexus* germination indicated by total (100%) suppression of germination (Fig. 4G). In *V. angustifolia* seed germination was not significantly affected, while the seedling growth was strongly inhibited (Fig. 4M–R). By contrast, WR was not toxic for *C. bursa-pastoris* and even significantly stimulated its seedlings growth compared to control grown on MS medium (Fig. 4A–F). However, *C. bursa-pastoris* was sensitive to R5 exudates, which significantly suppressed germination (48%) (Fig. 4A) as well as further seedling growth (Fig. 4B–F). The roots of *C. bursa-pastoris* seedlings were more sensitive than shoots, since R5 exudates almost completely inhibited root growth (Fig. 4E,F). Two other weed species were differentially affected by R5 exudates. *A. retroflexus* germinated in the presence of R5 exudates (Fig. 4G) but the hypocotyl length of the seedlings was reduced (Fig. 4J), and they displayed reduced seedling fresh weight and root growth (by about 50%) (Fig. 4K,L). The yellowing of the seedlings was also observed (Fig. 3B). In *V. angustifolia*, R5 exudates diminished the final seed germination by 17%; on the contrary, the growth of the seedlings, except for the lateral roots, was promoted by R5 exudates (Fig. 4M–R).

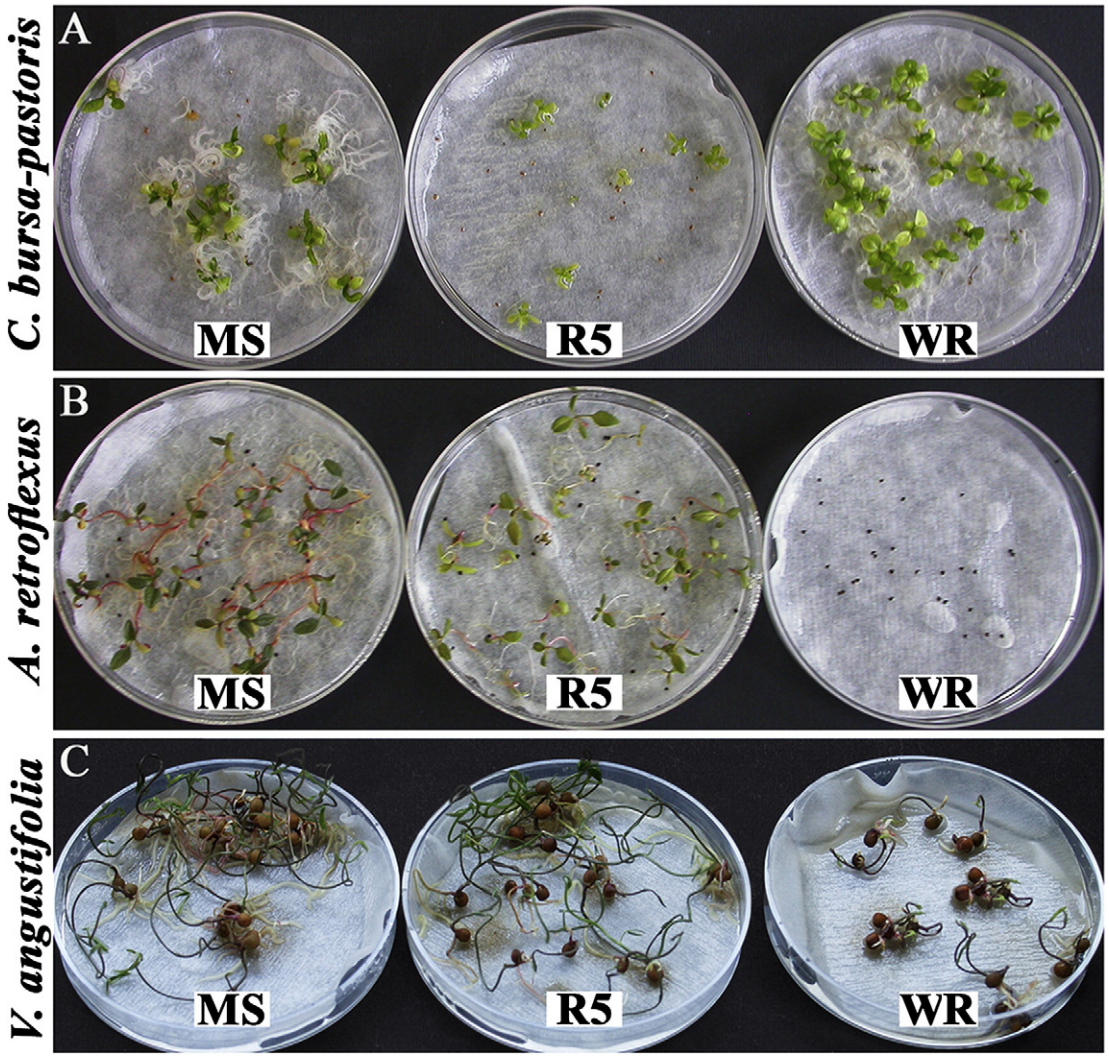

Fig. 3. Bioherbicidal potential of growth medium (GM) of *C. murale* wild-type roots (WR) and hairy root clone R5 on seed germination and seedling development of weed species: (A) *C. bursa-pastoris*, (B) *A. retroflexus* and (C) *V. angustifolia*.

3.4. Effects of WR and HR exudates of C. murale on antioxidative enzyme activity in weeds

To explore whether WR and R5 exudates induce changes in antioxidative enzyme activity in a response to allelopathic stress, the activities of CAT, POX and SOD in two-week-old seedlings of three tested weed species were determined. Since WR totally suppressed germination in *A. retroflexus*, there was no available plant material for antioxidative enzyme survey of this treatment. When treated with WR exudates, seedlings of *C. bursa-pastoris* responded by a significant increase in SOD and CAT activity (Fig. 5A,B). *V. angustifolia* shoots reacted by increasing SOD and decreasing CAT activity (Fig. 5G,H), while on the contrary, its roots showed increased CAT and decreased SOD activity (Fig. 5K,J) compared to respective controls. POX activity in these weed species was mainly reduced or not affected (as in *V. angustifolia* roots) by WR (Fig. 5C,F,I,L). When exposed to R5, seedlings of all three weeds responded by increasing both CAT and SOD activity. POX activity was increased only in *V. angustifolia* roots (Fig. 5L), while in two other species it was decreased or not affected by R5 treatment (Fig. 5C,F), similarly to the treatment with WR. Among weed species tested, the highest change in antioxidative enzyme activity was observed in *V. angustifolia* shoots, where 1.4-fold and 2.6-fold increase in SOD activity was recorded after WR and R5 treatments, respectively (Fig. 5G).

4. Discussion

Previous studies described the allelopathic effect of *C. murale* residue-amended soils against several test plant species (El-Khatib et al., 2004; Batish et al., 2007a,b). The inhibitory activity of *C. murale* hairy roots, exerted by exuded allelochemicals affecting the tested plant species wheat and lettuce in the absence of interference by soil microflora (Mitić et al., 2012), confirmed the allelopathic feature of *C. murale* roots. This indicated hairy root system as a suitable tool for further investigation of the nature of root-mediated allelopathic interference of *C. murale* and its potential usage. However, differential sensitivity of a single test plant species to exudates of various HR clones or WR, which reflected in differences in growth rate and phenotype pattern, has also been observed (Mitić et al., 2012). Therefore, for their usage in further research of allelopathic potential and functional analysis of genes involved in allelopathic interactions it would be important to perform morpho-histological characterization of HRs and to compare them with that of WR. In addition, comparative morphological and histological studies were conducted in search for explanation whether the different allelopathic mode of action of these root types may be associated with potential structural alterations. The present study revealed that HR clones differed from WR in lateral branching, root hair length and density. Although a high degree of lateral branching has often

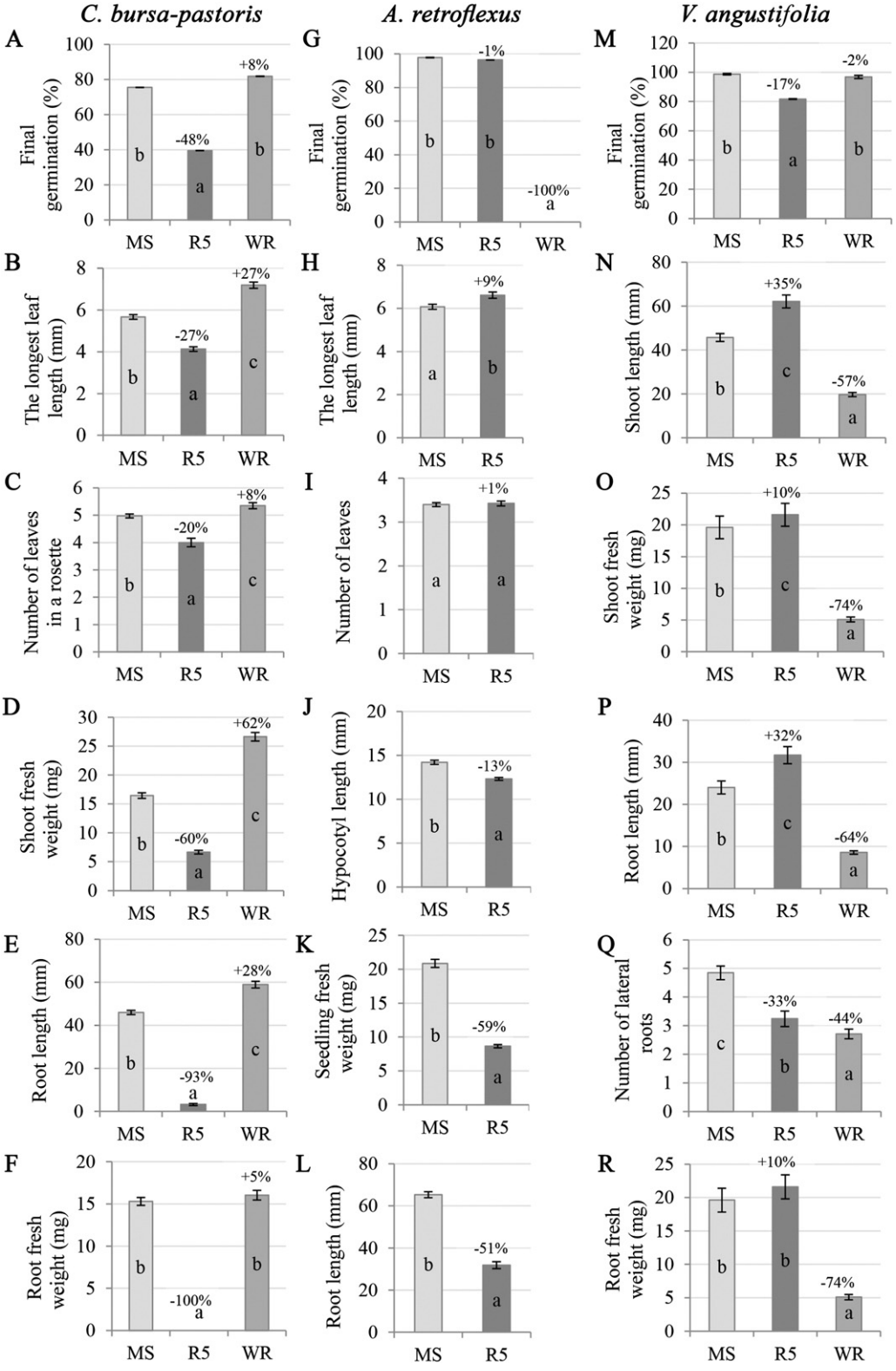

Fig. 4. The effect of 4-week-old growth medium (GM) of *C. murale* wild-type (WR) and hairy root clone R5 on final seed germination and seedling development of three weed species *C. bursa-pastoris*, *A. retroflexus* and *V. angustifolia*. Percentage of germinated seeds and seedling growth characteristics were determined two weeks after the treatment. Control treatment contained fresh MS instead of GM. The bars on each column show standard errors. Values represent the means ± S.E. from three independent experiments. Different letters show significant differences ($P < 0.05$) according to LSD test. Percentage of inhibition (−)/stimulation (+) over control was calculated as indicated in Material and methods.

been mentioned as one of the most typical traits of hairy roots (Tepfer, 1984; Spanò et al., 1988; Guivarc'h et al., 1999), Alpizar et al. (2008) observed high intraclonal variability for the branching variable in coffee root clones, which led to root branching being discarded as an efficient discrimination tool between root clones. The greatest difference between WR and HR clones of *C. murale* concerned the density and the length of root hairs, since WRs were found to have fewer thin and fragile root hairs compared to HRs. Similarly, SEM analysis of the surface of non-transformed and transformed roots of *Eurycoma longifolia* showed that control roots were essentially hairless (Danial et al.,

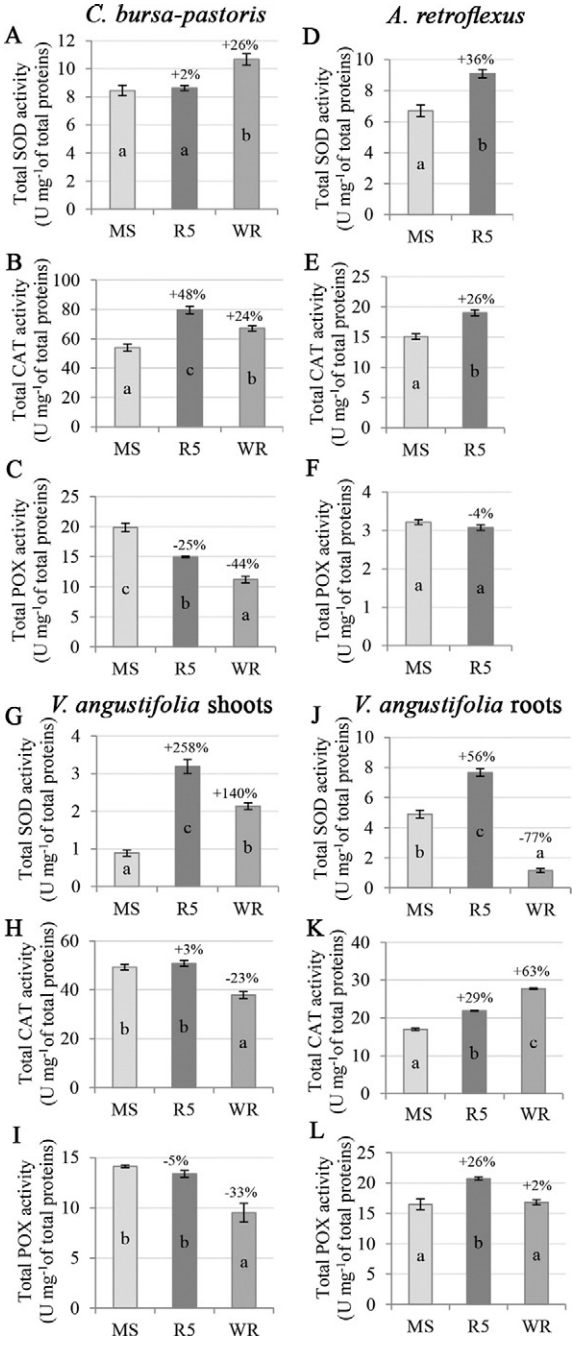

Fig. 5. Superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) activities in seedlings of *C. bursa-pastoris*, *A. retroflexus* and *V. angustifolia* exposed to growth medium (GM) of *C. murale* wild-type roots (WR) and hairy roots clone R5 for 2 weeks. Control treatment contained MS instead of GM. Values represent the means ± S.E. from three independent experiments. Different letters show significant differences ($P < 0.05$) according to LSD test. Percentage of activity decrease (−)/increase (+) over control was calculated as indicated in Material and methods.

2012). As current evidence suggests that biosynthesis of some allelopathic substances occurs exclusively in the root hair cells (Walker et al., 2003; Cook et al., 2010), our further analyses should focus on spatial localization of exudates secretion from root hairs of *C. murale* HRs.

Despite morphological differences, all root clones of examined *C. murale* root cultures were histo-anatomically similar, with only small structural differences between hairy roots and WR. This finding contradicts to some reports on the altered structure of transformed roots (Ottaviani et al., 1990; Webb et al., 1990; Budimir et al., 1998). However, it is in accordance with results of Alpizar et al. (2008) who found limited morphological variability of coffee hairy roots when compared with non-transformed root clones. Additionally, Park and Facchini (2000) observed nearly identical histology of the wild type and transformed roots of opium poppy and California poppy with the exception of epidermal cells of the transformed roots that were more loosely organized and gave rise to a large number of root hairs, compared to wild type roots.

Among different secondary metabolites in *C. murale*, phenolic compounds are most commonly occurring phytotoxins (Singh et al., 2003; Chon et al., 2005) and have been reported as putative inhibitors of seed germination and seedling development in wheat, chickpea and pea (Batish et al., 2007a,b). Contrary to findings of Kim et al. (2009) that the concentration of all phenolic compounds in hairy roots of *Fagopyrum tataricum* was several times higher than in wild type roots of the same species, we found no differences in total phenolic content among root tissues of various *C. murale* HR clones and WR. A slightly higher amount of total phenolics was found only in GM of HR clone R5 compared to other HR clones. Clone R5 exhibited prominent phytotoxic effect on previously tested species wheat, lettuce (Mitić et al., 2012) and *Arabidopsis* (Dmitrović et al., in press); hence it was suitable for use in comparative study of the herbicidal potential of WR and hairy roots.

Results of this study clearly revealed the allelophatic effect of both R5 and WR on three weed species *C. bursa-pastoris*, *A. retroflexus* and *V. angustifolia.* However, some selectivity in their allelopathic activity was also observed considering differential response of the various target species to the same allelopathic source. This was clearly demonstrated with WR, which totally inhibited germination in *A. retroflexus*, did not interfere with seed germination in *V. angustifolia* that resulted in seedlings that were impaired in growth and even slightly promoted germination and seedling growth in *C. bursa-pastoris*. On the other hand, R5 displayed the highest inhibitory potential on germination and seedling growth in *C. bursa-pastoris*. Differential sensitivity to the allelopathic source, which can even vary within the species (Prati and Bossdorf, 2004), has been reported in several other studies (Amoo et al., 2008; Tigre et al., 2012; Ghebrehiwot et al., 2013). This may be due to differential reaction of each physiological process to the given dose of a specific allelochemical (Cruz-Ortega et al., 1998; Reigosa et al., 1999). Moreover, the provoked effect may be a consequence of additive or synergistic action of different allelochemicals (and other compounds) present in the root exudate (Tigre et al., 2012). Thus, the stimulatory allelopathic effect of WR observed in *C. bursa-pastoris* could be attributed to the synergistic action of different compounds at their distinct concentrations. This inhibitory/stimulatory effect of the same allelopathic source is not surprising since it was observed earlier in other plant species (Tigre et al., 2012; Sunmonu and Van Staden, 2014). The allelopathic effect of WR and R5 reflected more on the seedling growth than the final germination, which is more dependent of the seed reserves and less susceptible to exogenous factors (Tigre et al., 2012). General observation regarding seedling growth inhibition indicated that shoots and roots of *C. bursa-pastoris* and *V. angustifolia* were mainly equally inhibited by R5 and WR, respectively, whereas *A. retroflexus* roots were more affected by R5. Most allelopathic studies indicated root growth to be more sensitive compared to shoots (Haouala et al., 2008; Omezzine et al., 2011; Ladhari et al., 2013), although there are opposite findings as well (Kundu et al., 2013). This is not surprising, considering that the roots are in the direct contact with allelochemicals that cause the inhibition in the meristematic and elongation zone (Romero-Romero et al., 2005). These visible inhibitory effects can be accounted for by certain alterations at the cellular and molecular levels, although the specific mechanisms of action are still largely unknown. The recent study indicated that the mode of allelopathic action of *C. murale* WR and HR exudates on wheat and *Arabidopsis* was based on down-regulation of the core cell cycle genes, accompanied by generation of oxidative stress in both shoots and roots of affected plants (Dmitrović et al., in press).

Elevated ROS levels are common for the stressed tissues, including those exposed to allelochemicals (Weir et al., 2004). To prevent ROS

production, various ROS-scavenging enzymes, including CAT, SOD and POX were triggered (Apel and Hirt, 2004). The activity of ROS-scavening enzymes can increase (Sunmonu and Van Staden, 2014), but some of them were also found to be unaffected or even exhibit a decrease in activity (Mutlu et al., 2011). In response to allelopathic stress caused by WR and R5, all three tested weed species had mainly increased their CAT and SOD activity. This is in agreement with other studies that reported increased activity of CAT and SOD in various plant species as a response to different allelopathic stresses, indicating their regulatory role in tolerance and defense activity (Romero-Romero et al., 2005; Singh et al., 2009; Sunmonu and Van Staden, 2014). SOD is known to play a central role as the major scavenger of ROS in the process accompanied by formation of H_2O_2 that can be eliminated by CAT or POX. This could explain the tendency of SOD activity to coincide with that of CAT in most responses to WR and R5. The resulting elevation of CAT activity supports the view of CAT playing a regulatory role in tolerance to allelopathic stress (Singh et al., 2006; Hong et al., 2008; Mutlu et al., 2011). On the other hand results of reduced or unaffected POX activity, except those in *V. angusfolia* roots treated with R5, are consistent with those of Mutlu et al. (2011), who reported either increased, decreased, or unchanged POX activity in various weed species in response to essential oils of catmint. This is all in accordance with the fact that the plant response to unfavorable or damaging conditions is mainly related to SOD activity, while POX could hamper peroxidation and thus reduce the injury of cell membranes. (Sunmonu and Van Staden, 2014). An interesting phenomenon has been observed in *V. angustifolia* shoots and roots where CAT and SOD activity was inconsistently and mutually inversely affected by WR. Chorianopoulou et al. (2012) reported differential early fluctuation in CAT and SOD activities in the response of young maize organs to S-deprivation, which were found to be opposed to one another and organ-specific. Inconsistent changes in activity of CAT enzymes with SOD and POX were also found in cucumber seedlings under short-term salt stress by Du et al. (2010), who underlined that enzyme responses may vary according to the intensity and duration of the stress, plant part affected by the stress, and induction of new isozyme(s).

Stimulatory allelopathic effect of WR and R5 in *C. bursa-pastoris* and *V. angustifolia*, respectively, followed by the increased activity of SOD and CAT may suggest that these plants reacted to specific alleloinfluence by activating a very potent defense response(s) that overcame oxidative damage. However, where seedling growth was reduced, the induced stimulation of the SOD and CAT was not sufficient to cope with the enhanced ROS production. Thus, WR can be recommended as a potential bio-herbicide in *V. angustifolia* while R5 in *C. bursa-pastoris* and vice versa. Additionaly, WR could be applied as a pre-emergence bio-herbicide to suppress the germination of *A. retroflexus* seeds.

Although the presence of plasmolyzed cells in all cell layers of *C. murale* HR clones C9 and C10 suggests the possibility that the inhibitory effects of their GMs (Mitić et al., 2012) could result from increased osmotic potential, as previously observed in some plant species (Anderson and Loucks, 1966; Wardle et al., 1992), the ability of WR and HR exudates to affect antioxidant system in tested weed species indicated that inhibitory effect was mostly due to their phytotoxicity.

Taken together, the obtained results demonstrated selective allelopathic action of WR and HR exudates of *C. murale* against three weed species, ranging from the suppression of germination to seedling growth inhibition, coupled with alterations in antioxidant enzyme activity. This qualifies them for consideration as a promising natural selective herbicide pool, where R5 could be recommended for *C. bursa-pastoris* and WR for *V. angustifolia* and *A. retroflexus* suppression. Since no significant differences in TPC or anatomical features between WR and hairy roots were found, reasons for their selective action should be further sought in qualitative and quantitative composition of their exudates while not excluding the potential role of phenolic compounds. Although the comparative HPLC analysis of WR and R5 GMs indicated certain qualitative differences between their phenolic profiles (since phenolic acids dominated in WR and flavonoids in R5, data not shown), further studies are required to evaluate the allelochemicals that singly or in combination contribute to certain allelopathic effect, by assaying them on greater number of different weed species. The identical anatomical features of WR and hairy roots render them useful as an efficient tool for functional-genomic studies of *C. murale* root genes.

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