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ORIGINAL ARTICLE

Physiological and cell ultrastructure disturbances in wheat seedlings generated by *Chenopodium murale* hairy root exudate

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Abstract

Chenopodium murale L. is an invasive weed species significantly interfering with wheat crop. However, the complete nature of its allelopathic influence on crops is not yet fully understood. In the present study, the focus is made on establishing the relation between plant morphophysiological changes and oxidative stress, induced by allelopathic extract. Phytotoxic medium of *C. murale* hairy root clone R5 reduced the germination rate (24% less than control value) of wheat cv. Nataša seeds, as well as seedling growth, diminishing shoot and root length significantly, decreased total chlorophyll content, and induced abnormal root gravitropism. The R5 treatment caused cellular structural abnormalities, reflecting on the root and leaf cell shape and organization. These abnormalities mostly included the increased number of mitochondria and reorganization of the vacuolar compartment, changes in nucleus shape, and chloroplast organization and distribution. The most significant structural changes were observed in cell wall in the form of amoeboid protrusions and folds leading to its irregular shape. These structural alterations were accompanied by an oxidative stress in tissues of treated wheat seedlings, reflected as increased level of H_2O_2 and other ROS molecules, an increase of radical scavenging capacity and total phenolic content. Accordingly, the retardation of wheat seedling growth by *C. murale* allelochemicals may represent a consequence of complex activity involving both cell structure alteration and physiological processes.

Keywords *Chenopodium murale* · Wheat · Allelopathy · Hairy root exudate · Morphophysiological changes · Cytological changes

Introduction

Under natural conditions, various stress factors affect growth and productivity of the crop plants. Many of these stresses usually act jointly, endangering the crop plants seriously. Due to extremely successful adaptation to wide range of environments, weeds are very important biotic stress factors on the crop fields, causing the considerable yield reduction (Burleigh et al. 1988; Holt and Orcutt 1991).

Apart from the competition with crop plants for resources, including water, mineral nutrients, light, and space, many weeds produce the allelochemicals with prospective harmful effects on crop plants — "allelochemical stress" (Pedrol et al. 2006; Khaliq et al. 2013, 2014a, b). When the weeds express an allelopathic effect, the qualitative and quantitative damage of the crop could be even severe. Hence, it would be very beneficial if the allelopathic effect of specific weed species on particular crop could be known, enabling weed management to become economically more effective.

The major bottleneck of achieving the higher productivity of wheat (*Triticum aestivum*) is just weed infestation in wheat fields, that can lead to the yield reduction of over 20% (Oerke 2006; Oad et al. 2007). Among these weeds, *Chenopodium murale*, commonly known as nettle leaf goosefoot, stands out significantly. Although growth inhibition by allelopathic

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impact of *C. murale* has been well documented (El-Khatib et al. 2004a, b; Batish et al. 2007a, b; Islam et al. 2014), these visible morphological effects are secondary manifestations of primary events occurring at the cellular, biochemical, or molecular level in the target plants.

Unfortunately, the mode of action for the majority of allelopathic compounds has not yet been investigated sufficiently and a lot of additional information is required, especially for the each allelopathic agent. One of the most important difficulties that limit the attempts to learn more about *C. murale* allelochemicals is the complexity of isolation and identification of metabolites exuded from roots into the soil and accordingly the lack of composed quantities, in order to study the effects on growth processes. An in vitro liquid culture system of *C. murale* transgenic hairy roots has previously been established, as an alternative strategy for allelopathic assays (Mitić et al. 2012), to facilitate research on *C. murale* allelopathic compounds. Bioassays revealed that *C. murale* hairy roots synthesize certain bioactive substances with inhibitory effect on seed germination and seedlings growth of crop plants, such as wheat and lettuce (Mitić et al. 2012), collard greens (Mitić et al. 2015), the model plant *Arabidopsis thaliana* (Dmitrović et al. 2015a), and some weed species (Dmitrović et al. 2015b).

Although allelopathic influence of *C. murale* roots was mostly attributed to the phenolic acids as the important allelochemicals (Batish et al. 2007a), our preliminary experiment demonstrated that unknown yellow-colored substance isolated recently from methanol extract of R5 phytotoxic medium using semi-preparative HPLC, displayed allelopathic influence against test plant (data not shown, research in progress).

The target plant response to allelopathic compounds may have different bases (Weir et al. 2004). The previous study, where *C. murale* root exudates, applied as phytotoxic media (PMs) were tested on *A. thaliana* and wheat, revealed that the phytotoxicity of root exudates is, at least partially, based on downregulation of the cell cycle regulators and on the generation of oxidative stress in the affected plants (Dmitrović et al. 2015a). However, besides enhanced activity of antioxidant enzymes and alterations in CDK (cyclin-dependent kinase) gene expression, inhibition generated by allelopathic compounds may also be the result of significant lipid peroxidation (Ding et al. 2016; Hussain et al. 2017), as well as the change of the content of the main phytohormones, e.g., abscisic acid, indole-3-acetic acid, and zeatin riboside (Yang et al. 2008), modifications at cell ultrastructure level (Gniazdowska and Bogatek 2005; Kaur et al. 2005; Romero-Romero et al. 2005) and by biochemical and physiological processes (reviewed by Cheng and Cheng 2015).

Since the information regarding the mechanism underlying the effect of the PMs of *C. murale* transformed roots in target plants are limited to previously mentioned CDK gene expression and antioxidant enzyme activity modification, more comprehensive study on PMs phytotoxicity was performed with the aims to compare their effects on physiological and ultrastructural changes in wheat.

Some physiological parameters such as ROS accumulation, the lipid peroxidation, H_2O_2 level, total phenolic content, and radical scavenging capacity were evaluated in the function of oxidative stress, as the common reaction of plants exposed to allelopathic impact. Whereas the physiological changes influencing the impaired growth were usually closely associated with disruption at the cell level, e.g., organelles and cell wall alterations, cell ultrastructure changes in wheat were also investigated. The present study provides deeper view inside harmful effects of this weed species on crop plants, what is important for better understanding its invasive strategy in crop fields that would be of great interest to agronomy.

Materials and methods

Plant material, preparation of phytotoxic medium, and germination bioassay

Seeds of commercial spring wheat cv. Nataša, selected at the Institute of field and vegetable crops, Novi Sad, Republic of Serbia, were used in the present study. The seeds were surface sterilized in commercial bleach (4–6% NaOCl) for 60 min, followed by 0.1% aqueous mercuric chloride solution for 10 min and then rinsed five times with sterile distilled water. The seeds were treated with either sterile liquid MS (Murashige and Skoog 1962) nutrition medium containing salts and vitamins and 30 g l^{-1} sucrose (control), or phytotoxic R5 medium prepared by putting initial 25 mg of hairy roots of clone R5 into 100-ml Erlenmeyer flasks containing 50 ml of sterile liquid MS and culturing on a rotary shaker (70 rpm) at 25 ± 2 °C under 16-h light regime (irradiance of 50 μmol m^{-2} s^{-1}) for 4 weeks. R5 hairy root clone was induced by *Agrobacterium rhizogenes* A4M70GUS from roots of *C. murale* seedlings, as described by Mitić et al. (2012). Established R5 hairy root cultures were maintained for 4 years, subculturing monthly 25 mg of the fresh weight of hairy roots into fresh liquid MS medium. Sterilized wheat seeds were first imbibed for 6 h in either control MS or in the R5 phytotoxic media. Then, the imbibed seeds were placed in 9-cm Petri dishes (20 seeds per dish), on a double layer of filter paper wetted with 10 ml of MS or R5. For each treatment, four replicates (Petri dishes) were maintained in a randomized block design and the experiment was repeated twice. All Petri dishes were wrapped with parafilm (Pechiney Plastic Packaging, Chicago, IL, USA) and were kept at 25 ± 2 °C in white fluorescent light, with an irradiance of 50 μmol m^{-2} s^{-1}, with a 16-h photoperiod. Seven days later, the germination percentage, seedling shoot length, shoot fresh weight, the number of roots per seedling, the longest root length, and root fresh weight were scored.

Total chlorophyll content

For total chlorophyll analysis, 0.5 g of fresh 7-day-old control and treated wheat shoots were ground in liquid nitrogen and extracted with methanol in 1:10 (*w*/*v*) ratio for 2 h. The extract was centrifuged at 15,000×*g* for 10 min, and the absorbance of supernatant was recorded at 632, 652, 665, and 696 nm. Total chlorophyll content was calculated according to Ritchie (2008) and expressed as mg g^{-1} of fresh weight (**FW**).

Transmission electron microscopy (TEM)

For ultrastructural examination, the root and leaves of 7-day-old control and treated wheat seedlings were cut into pieces of approximately 3 mm^2, fixed in a solution containing 3% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.2) for 4 h at 4 °C. Subsequently, they were rinsed with the same buffer and postfixed in 2% OsO_4 for 2 h at 4 °C, followed by dehydration in an ethanol series. Samples were embedded in araldite, sectioned with UC6 ultramicrotome (Leica, Wetzlar, Germany), stained with uranyl acetate and lead citrate. The cell ultrastructure was analyzed on Philips/FEI CM12 (FEI, Eindhoven, The Netherlands) transmission electron microscope.

Histochemical staining of reactive oxygen species (ROS)

Histochemical staining of ROS was performed using cell-permeant dye 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA). After treatment, roots and leaves were incubated in 25 μM H_2DCF-DA solution for 30 min, at room temperature, in the dark and then rinsed with fresh distilled water to remove excess dye (Allan and Fluhr 1997). After a brief washing, the roots and leaves were observed using an Olympus epifluorescence microscope (Leica) with a GFP filter set (excitation 480/40 nm, emission 527/30 nm).

Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) content was determined according to the method of Heath and Packer (1968) with modifications of Du and Bramlage (1992). Plant tissue samples were homogenized with liquid nitrogen and 0.3 g of tissue was weighed in micro centrifuge tubes and 3 ml of 20% trichloroacetic acid (TCA) was added to each sample. Mixture was vortexed and centrifuged at 3500×*g* for 20 min. Obtained supernatant (1 ml) was mixed with 1 ml of 20% TCA containing 0.5% thiobarbituric acid (TBA) and 0.1 ml of 4% (*w*/*v*) butylated hydroxytoluene (BHT) solution in ethanol. The mixture was then heated in water bath at 95 °C for 30 min and rapidly cooled on ice, prior to centrifugation at 10,000×*g* for 15 min. Absorbance of obtained supernatant was then recorded at 600, 532, and 440 nm. TBARS content was calculated as follows:

$$\mathrm{TBARS\ (nmol/ml)} = [(A_{532nm}-A_{600nm})-(A_{440nm}-A_{600nm}) \times 0.0571] / 157\,000 \times 10^6$$

Hydrogen peroxide (H_2O_2) content

Hydrogen peroxide content was determined according to previously reported method of Junglee et al. (2014). Powdered samples (150 mg) obtained by grounding wheat shoots in liquid nitrogen were homogenized in the mixture containing 0.25 ml of 0.1% TCA, 0.5 ml of 1 M KJ and 0.25 ml of 10 mM phosphate buffer (pH 7.4), using rotary shaker for 10 min at 4 °C and protected from light. Blanks were prepared with distilled water instead of KJ, to subtract absorbance originating from tissue coloration. Homogenate was then centrifuged at 10,000×*g* and 4 °C for 15 min. Supernatant was incubated at room temperature for 20 min and absorbance was recorded at 350 nm. Concentration of H_2O_2 was calculated using standard curve obtained with H_2O_2 standard solutions (0–15 nM) prepared in 0.1% TCA and expressed as nM per g of fresh weight.

Total phenolic content (TPC)

Total phenolic content (TPC) was measured according to the previously reported method of Julkunen-Tiitto (1985) using Folin–Ciocalteu (FC) reagent with some modifications. Ground samples (100 mg) were extracted with 2 ml of 80% methanol and centrifuged at 10,000×*g* for 10 min. Aliquots of supernatants (20 μl) were mixed with 1580 μl of distilled water and 100 μl of FC reagent, and shaken vigorously. Then, 300 μl of 20% sodium carbonate solution was added to the mixture, which was left 2 h at the room temperature prior to absorbance measurement at 765 nm. Total phenolic content was calculated using gallic acid (GAE) standard curve and expressed as milligrams of gallic acid equivalents per gram of sample fresh weight (mg GAE/g FW).

Radical scavenging capacity (RDSC)

Relative 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity (RDSC) estimation was conducted according to the method of Cheng et al. (2006). Reaction mixture contained 100 μl of grounded plant material diluted with methanol. Five different volumes (10, 20, 40, 60, and 80 μl) of extract and standard (Trolox) have been used. Then, 100 μl of 0.208 mM DPPH solution was added to each microplate well and gently stirred. Blank consisted of 200 μl of methanol and control sample contained 100 μl of methanol and 100 μl of DPPH solution. Absorbance was continuously recorded

each minute for 1.5 h at 515 nm. The percent of DPPH quenched for each time point was calculated as follows:

$$\%\text{DPPH quenched} = \left[1-\left(A_{\text{sample}}-A_{\text{blank}}\right)/\left(A_{\text{control}}-A_{\text{blank}}\right)\right] \times 100$$

The values of DPPH in percent, quenched in different time points for each extract and standard, were plotted against reaction time. Area under obtained curve (AUC) was determined according to the following equation:

$$\text{AUC} = 0.5f_0 + (f_1 + f_2 + f_3 + \ldots. + f_{i-1}) + 0,5f_i$$

where f_0 represents DPPH quenched at the start of the measurement and f_i is DPPH quenched at reaction time i.

Relative DPPH radical scavenging capacity (RDSC) was calculated according to the equation:

$$\text{RDSC (mM TE/g FW)} = \text{AUC}_{\text{sample}}/\text{AUC}_{\text{trolox}} \times \text{molarity}_{\text{trolox}}/\text{weight}_{\text{sample}}$$

where TE represents Trolox equivalents.

Statistical analysis

Treatments were fully randomized in the experiments. Data were subjected to square root transformation and percentage data to angular transformation prior to statistical analysis and inversely transformed for presentation. The standard errors of the means were calculated and denoted in the figures with error bars. The significance of difference between the treatment and the respective control was determined using Student's t test, calculated at the confidence level of $p \leq 0.05$. Statistical analysis was performed using STATGRAPHICS software, version 4.2 (STSC Inc. and Statistical Graphics Corporation, 1985–1989, USA).

Results

R5 phytotoxicity on seed germination and seedling growth of wheat cv. Nataša

The exposure of seeds of wheat cv. Nataša to phytotoxic medium of *C. murale* hairy root clone R5 (R5) affected both seed germination and seedling growth (Table 1). Relative to the control, seed germination was reduced nearly 24% in response to R5. The shoot and root length of wheat seedlings were almost equally and significantly reduced by R5 (approximately 50% comparing to control). The inhibitory effect of R5 was the most pronounced in root fresh weight and in the number of roots per plants (60 and 52%, respectively), while the shoot fresh weight was less affected (43%) (Table 1).

Phytotoxic R5 medium negatively influenced chlorophyll stability too. Significant reduction (26.2%) of total chlorophyll content was detected (Table 1), which was accompanied with growth retardation of both roots and shoots, indicating severe "allelochemical stress."

However, apart from the poor seedlings growth, the impact of R5 on the root gravitropic response was emphasized (Fig. 1). While shoots of control wheat seedlings grew upright with roots permeating filter paper substrate, seedlings exposed to R5 were lying down, with roots mostly orientated upward (Fig. 1a). The curvature angle of treated seedlings ranged from 90° to almost 180°, that indicates the complete absence of gravity response (Fig. 1b). These seedlings were subjected to further analysis in order to identify potentially R5-induced physiological and ultrastructural changes.

R5 changed wheat cell ultrastructure

Transmission electron microscopy observation of control root cells showed typical ultrastructure, with no structural changes (Fig. 2a, b). The cell wall had a uniform shape, the organelles were distributed in cytoplasm, and nucleus with defined nucleolus was located along the cell wall (Fig. 2a). Ultrastructure of xylem complex showed tracheas without living protoplasts, with thick cellulose walls (Fig. 2b).

The R5 treatment caused cellular structural abnormalities, reflecting on the root cell shape and organization. The most significant structural changes were observed in the cell wall. Unlike with untreated control, R5 treatment caused slight cell wall thickening, amoeboid protrusions, and folds leading to its irregular shape (Fig. 2c). The prominent nucleus and enlarged vacuoles that occupied the largest part of the cell were noticed (Fig. 2d). There was an increased number of cell organelles, such as mitochondria and endoplasmic reticulum, distributed near cell wall.

Furthermore, no structural distortion was shown by TEM in the leaf cells of the control wheat plants, characterized by thin cell wall, small vacuoles, numerous plastids with a small starch grains, normal mitochondria, prominent nucleus mixing of fibrillar and granular chromatin and distinct nucleolus (Fig. 3a, b).

Similarly with root cells, the significant visible structural changes were also detected in R5 treated wheat leaf cells comparing to control (Fig. 3c, d). Firstly, the increased number of mitochondria and reorganization of the vacuolar compartment were observed (Fig. 3c, d). R5 also caused undulation of cell walls of closely packed cells, with enlarged intercellular spaces, while the nucleus changed its previous orbicular shape and position, becoming more elliptical, with location close to the cell membrane (Fig. 3d). In addition, some changes on chloroplast organization and distribution were also observed. The chloroplasts were moved and grouped with the plasmalemma into the cell interior, including the change into oval shape (Fig. 3d).

Table 1 Allelopathic effect of phytotoxic medium of *C. murale* hairy root clone R5 on the seed germination, seedling growth parameters, and total chlorophyll content of wheat cv. Nataša after 7 days. Control treatment contained MS, instead of R5 medium

Treatment	Germination (%)	Shoot length (cm)	Fresh weight of shoot (mg)	No. of roots per plant	Root length (cm)	Fresh weight of roots (mg)	Total chlorophyll content (mg g^{-1} fw)
Control	90.6 ± 6.0	8.6 ± 0.6	83.1 ± 7.0	4.8 ± 0.2	10.3 ± 1.1	83.1 ± 11.1	0.69 ± 0.03
R5	66.8 ± 7.7*	4.0 ± 0.7*	46.9 ± 7.8*	2.3 ± 0.3*	4.8 ± 0.8*	34.0 ± 7.1*	0.51 ± 0.04*

Data presented as mean of four or three (total chlorophyll content) replicates ± SE. Asterisks represent a significant difference ($p \leq 0.05$) in relation to MS control (Student's *t* test)

fw fresh weight

R5 induced oxidative stress in wheat

Exposure of wheat seedlings to *C. murale* hairy root exudate has led to a significant increase of total phenolic content (TPC), expressed as a gallic acid equivalents, in roots, as well as in shoots, in comparison to control (Fig. 4a). Comparative analyses of TPC in shoots and roots showed the increment was more pronounced in shoots (41.9%) than in roots (39.8%).

Data of the present study also revealed significant increase in TBARS content, expressed in nanomole per gram of fresh weight, upon R5 treatment in both roots and shoots of 30.5 and 51.2%, respectively (Fig. 4b). Results indicated higher accumulation of lipid peroxides in shoots.

Analysis of antioxidant activity in low molecular fraction of antioxidants, measured by kinetic DPPH radical scavenging assay (Fig. 4c), showed treatment inducing increase of antioxidant activity in the roots (68.2%) and the shoots (78.4%), indicating altered production of low molecular weight antioxidants. Results of total phenolic assay and DPPH assay stood in very high correlation ($r = 0.97$).

Measurement of hydrogen peroxide content using potassium-iodide assay showed intensive accumulation of H_2O_2 in treated plants. Accumulation of H_2O_2, evident in both shoots and roots, was more pronounced in the roots than in the shoots, 64 and 36% increase, respectively (Fig. 4d).

Additional studies on the levels of ROS, which generally accumulates in plant tissue under stress, were performed in roots and leaves of wheat plants under allelochemical stress by imaging the ROS-sensitive fluorescent dye 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA). H_2DCF-DA fluorescence increases as the dye is oxidized by ROS to dichlorofluorescein (DCF). Figure 4e shows a marked increase in fluorescence of treated roots (B), compared to untreated control (A). Observed fluorescence was more intense at the root cap. Elevated fluorescence was also observed in leaves of plants treated with phytotoxic medium (D), in comparison with leaves of control plants (C).

Discussion

The response of wheat cv. Nataša to *C. murale* hairy root clone R5 exudate confirmed its strong allelopathic influence. It is indicative that the main *C. murale* allelopathic target in wheat is post-germination seedling growth, whose shoots and roots were significantly inhibited, similar to the previously tested wheat cv. Vesna (50% or higher) (Mitić et al. 2012). Germination response to allelopathic impact was more genotype dependent, e.g., in cv. Nataša was less affected than in cv. Vesna (24 vs. 55% reduction) (Mitić et al. 2012). Unlike

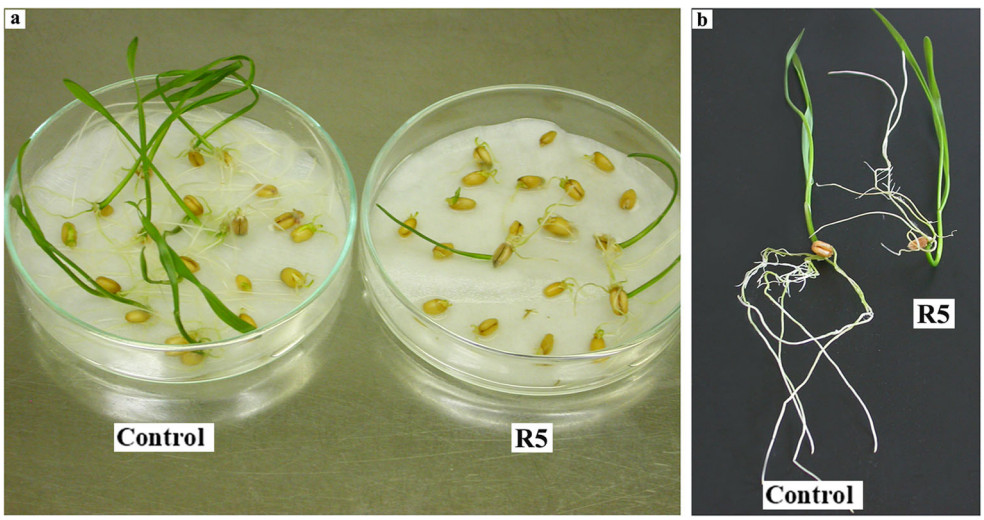

Fig. 1 Wheat seedlings 7 days after exposure to phytotoxic R5 medium lying down compared to control seedlings cultivated on MS which grew upright (**a**). Roots of the seedlings grown on R5 medium showed pronounced agravitropic response compared to MS control (**b**)

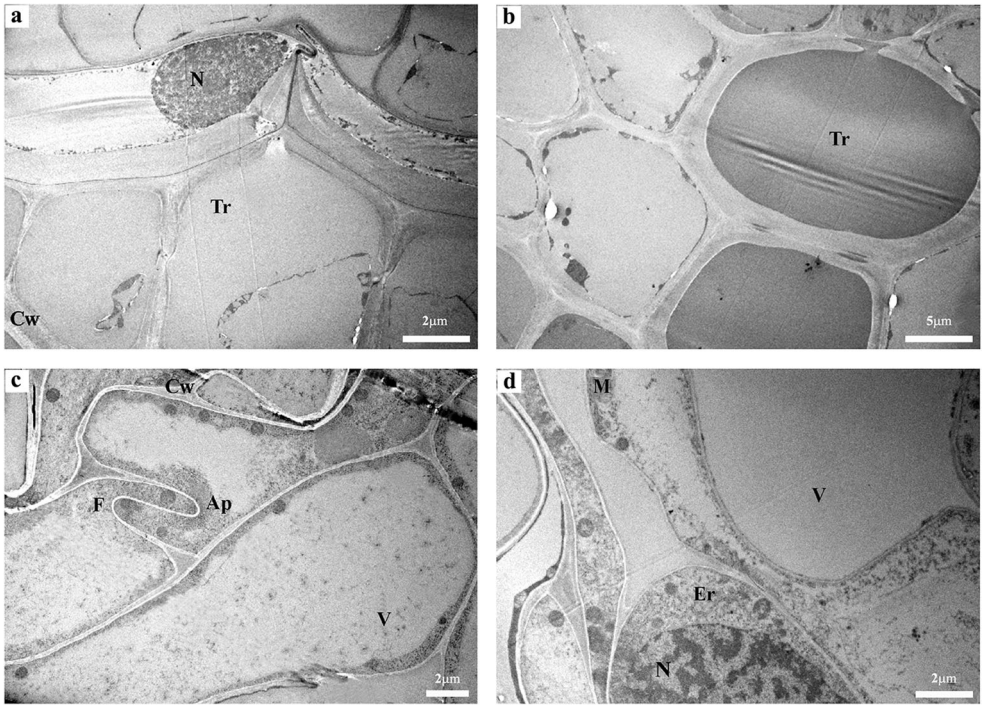

Fig. 2 Transmission electron microscopy (TEM) images of the root cells of the wheat seedlings cv. Nataša, grown for 7 days on control MS (**a**, **b**) and R5 phytotoxic medium causing (**c**) cell wall (Cw) irregular shape with amoeboid protrusions (Ap) and folds (F) and (**d**) prominent nucleus (N), enlarged vacuole (V), increased number of mitochondria (M) and endoplasmic reticulum (Er). Tr, tracheas. *Bars*: a, c, d = 2 μm; b = 5 μm

allelopathic effect on many other plant species, where roots were generally more affected by allelochemicals than shoots, these studies revealed almost equal sensitivity of shoots and roots of wheat seedlings.

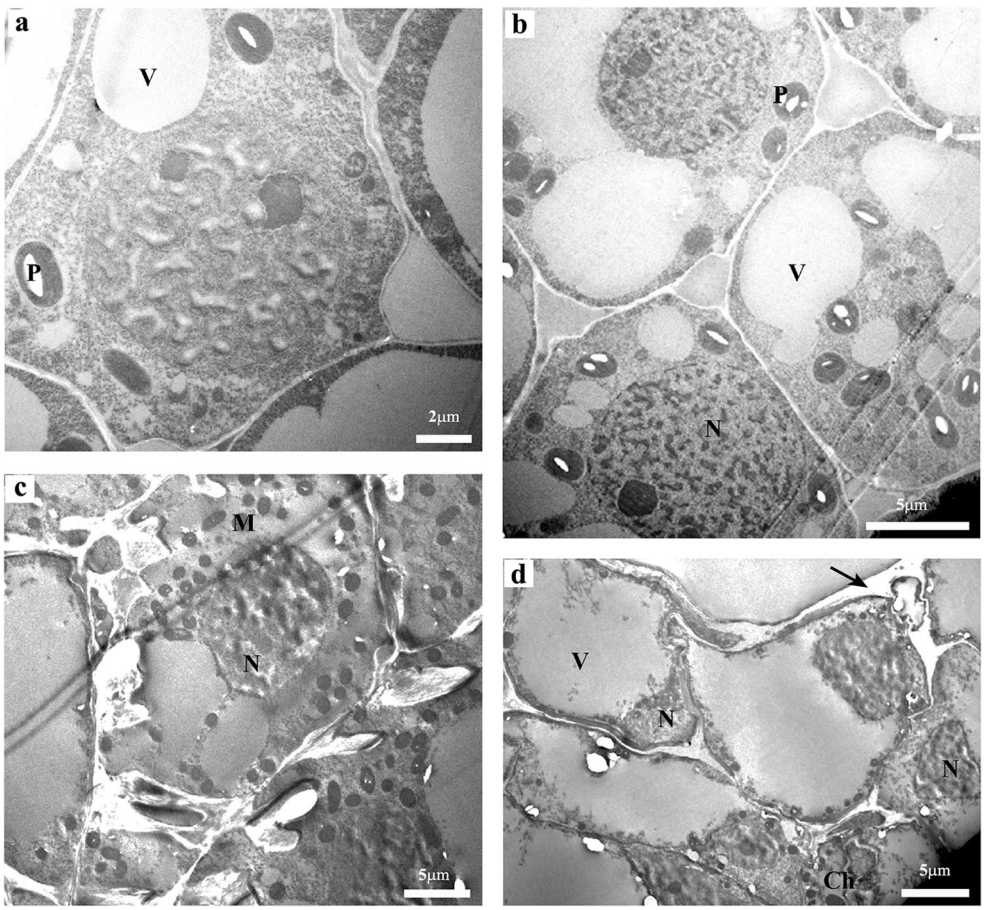

Fig. 3 Transmission electron microscopy (TEM) images of the leaf cells of the wheat seedlings cv. Nataša, grown for 7 days on MS control (**a**, **b**) and R5 phytotoxic medium causing (**c**) increased number of mitochondria (M), (**d**) reorganization of the vacuolar compartment (V), undulation of cell walls of closely packed cells with enlarged intercellular spaces (arrow), elliptical nucleus (N) located directly to the cell membrane and chloroplasts (Ch) grouped with the plasmalemma. (*Bars:* a = 2 μm; b, c, d = 5 μm)

One of the more conspicuous effects of R5 on cv. Nataša seedlings was abnormal gravitropism, similar to that observed in influence of water extracts of fiber hemp on lupine seedlings growth (Pudełko et al. 2014), or in farnesene-treated Arabidopsis where loss of gravitopism was explained by the increment of endogenous auxin and ethylene levels (Araniti et al. 2016).

These visible growth-limiting effects of allelochemicals may be due to their effects on different levels of plant organization, such as reduction/inhibition of mitotic activity, ion uptake, photosynthesis, and enzyme action (Rice 1974). The previous study, related to possible inhibitory effects of *Chenopodium murale* root exudates on Arabidopsis and wheat plants grown in vitro (Dmitrović et al. 2015a), revealed that the plant growth inhibition was, at least partially, based on downregulation of the cell cycle regulators and on generation of oxidative stress in the affected plants.

Despite vast amount of data reporting the changes in oxidative metabolism of plants and altered ROS production due to allelopathy (Macias et al. 1999; Foreman et al. 2003; Lara-Nuñez et al. 2006; Gniazdowska et al. 2015), it is still unclear which are the primary mechanisms of oxidative stress induction in plants and which are the precise molecular targets of ROS (Gniazdowska et al. 2015). In this particular study, the effort was made to establish the link between plant morphophysiological changes and parameters of oxidative stress altered by allelopathic extract.

Ultrastructural analysis of wheat seedlings under the influence of *C. murale* allelochemicals showed significant changes of cell structures, including vacuoles, mitochondria, and cell wall that might be result of increased ROS levels. Vacuolation of cytoplasm is known phenomenon observed in stressed plant cells, including allelopathy (Kaur et al. 2005; Pawlowski et al. 2012). Gzyl et al. (2009) supposed that the volume of vacuoles increase may be in the service of toxic ions capture or phenolic compounds capture, enabling cells to overcome stress conditions. Reorganization of the vacuolar compartment of leaf cells, observed in present study, could also be associated with increased production of soluble phenolic compounds. Support for this claim could be found in recent research of Ferreres et al. (2011) who provided evidence that phenolic compounds found in vacuole could participate in regenerative cycle with ascorbate allowing peroxidase (POX) to scavenge high amounts of H_2O_2. Therefore, removal of organelles with the plasmalemma and reorganization of the vacuolar compartment in R5-treated cells was probably occurring in order to protect the cells when the tonoplast was locally damaged by free radicals.

The next significant structural change, observed in R5 treated wheat root and leaf cells, was increased number of mitochondria, as response mostly observed in plant cells exposed to toxic ions (Gzyl et al. 2009; Kaur et al. 2013). It is contrary to some allelopathic studies where the common reaction to allelochemicals was reduced number of mitochondria (Burgos et al. 2004). Increased number of mitochondria induced by phytotoxicity of R5 medium could be related to energetic metabolism imbalance.

Additionally, distinct changes in cell wall, such as its thickening and formation of protuberances and folds, were observed in both leaf and root cells of wheat seedlings upon R5 treatment. Similarly, the thickness of the cell wall was found in *Arabidopsis* root cell exposed to citral (Grana et al. 2013), while different forms of cell wall undulations were frequently observed in cells stressed with toxic ions (Kaur et al. 2013).

It is possible that stress caused degradation of some components in the cell wall, modifying the cellulose and hemi-cellulose network. Therefore, when pectins or other components are degraded, in the load-bearing cellulose-hemicellulose skeleton space may collapse, resulting in stiffness of the cell wall decrease, and probably caused cell wall irregular shape. Previously published report (Chanliaud et al. 2002), from different stress states in cell walls under in vivo and in vitro conditions, have shown the similar results.

Since the disturbance of photosynthetic apparatus upon action of allelochemicals is well documented (Yu et al. 2003; Weir et al. 2004), decrease in total chlorophyll content observed after the R5 treatment was expected. Present research also revealed the largest structural changes occurred in the chloroplasts, indicating that process of photosynthesis could be disrupted. The thylakoid lumen was not clearly visible, because it is filled with an electron-dense substance (possibly phenolics), as a mode of protecting the thylakoid membranes from oxidative stress.

Despite induction of antioxidant enzymatic system, documented previously by Dmitrović et al. (2015a), increased TBARS content, in both roots and shoots, suggested that generated ROS molecules could not be eliminated completely. This claim is supported by the results of H_2O_2 content measurement and ROS imaging, using H_2DCF-DA probe which also showed increased level of H_2O_2 and other ROS molecules in tissues of R5 treated plants. H_2DCF is applied as H_2DCF-DA, which is taken up by the cell, where unspecific esterases cleave the lipophilic groups, resulting in a charged compound. Oxidation of H_2DCF by ROS converts the molecule to DCF, which is fluorescent. Deacetylated H_2DCF is sometimes retained intracellulary (Rothe and Valet 1990; Shulaev and Oliver 2006), allowing measurement of intracellular ROS production specifically. However, some leakage of the dye is possible (Tarpey et al. 2004). H_2DCF, like many other fluorescent substrates, what is not highly specific and can be an advantage in assays where different types of ROS are produced, as well as in assays where a certain nano-compound induces the release of unknown ROS.

Rapid increase in H_2O_2 level after R5 treatment (about 50% higher compared to control) represents the typical reaction to allelochemicals since the similar effect was annotated after application of numerous other allelochemicals (Soltys et al. 2011, 2014; Guan et al. 2014; Krasuska et al. 2016; Andrzejczak et al. 2018). Different increments of H_2O_2 roots

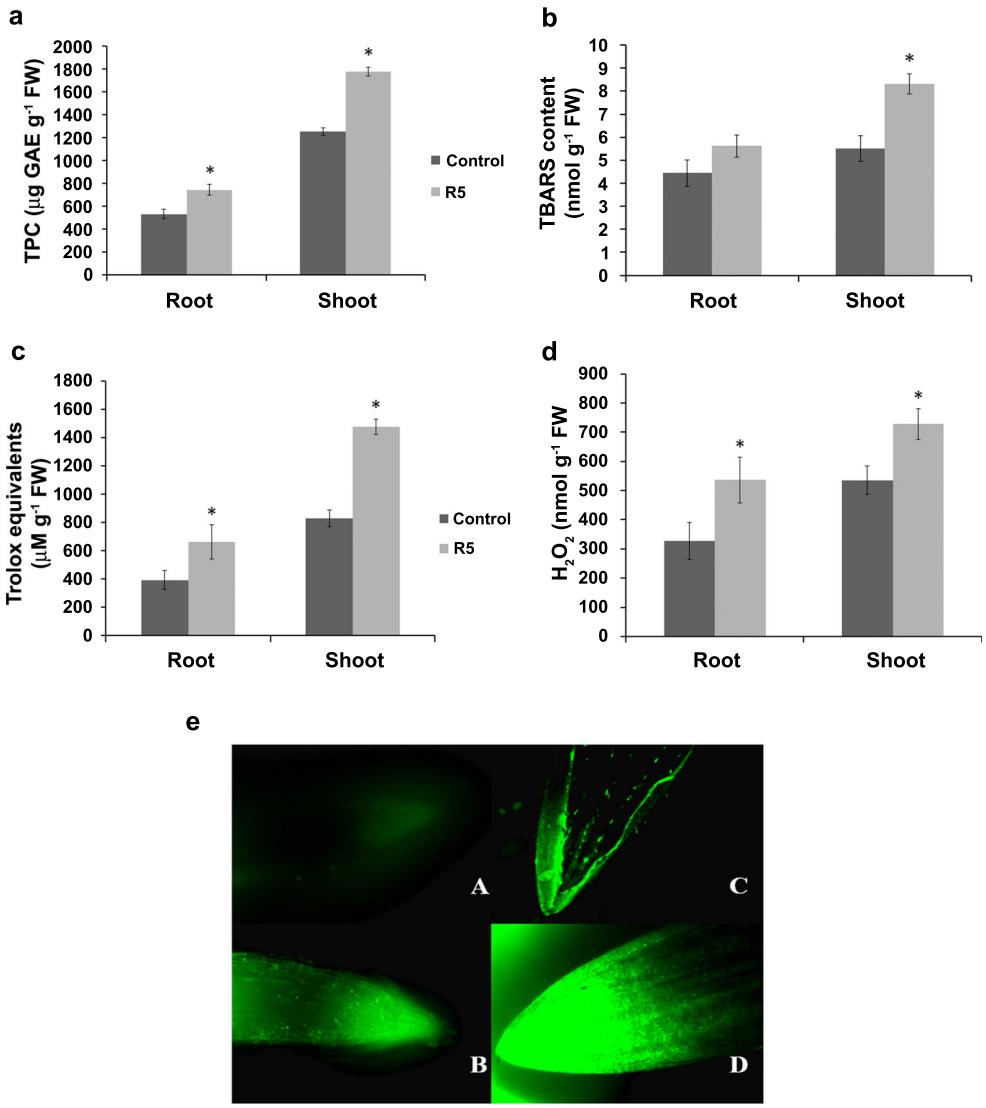

Fig. 4 Biochemical parameters of the wheat seedlings cv. Nataša, grown for 7 days on control MS and R5 phytotoxic medium. **a** Total phenolic content (TPC). **b** Lipid peroxidation—thiobarbituric acid reactive substances (TBARS). **c** Antioxidant activity—DPPH radical scavenging assay. **d** Hydrogen peroxide content. **e** Histochemical detection of ROS using redox-sensitive fluorescent dye H_2DCF-DA. Intensity of green fluorescence indicates ROS accumulation in plant tissue: A-root control, B-root treated by R5, C-leaf control, D-leaf treated by R5. Values are shown as means ± SE from three independent experiments ($n = 3$). Mean values were compared with respective control using Student's t test at $p \leq 0.05$

and shoots content could be explained by differential activation of enzymatic antioxidant defense system, observed in wheat shoot and root in the previous study (Dmitrović et al. 2015a).

It was shown that overaccumulation of H_2O_2 in the roots (particularly in the apical region) induced cellular differentiation and inhibited cell proliferation simultaneously (Tsukagoshi et al. 2010; Soltys et al. 2014). The increased content of H_2O_2 may be just one of many other factors that affect root and shoot growth after R5 treatment. The observed imbalance may contribute to morphological and cytological modifications, causing dysfunction of wheat seedlings.

Total phenolic content (TPC), determined by widely used Folin-Ciocalteu assay, was found to be significantly increased in R5 treated roots and shoots. Increased TPC could be related to the cell wall stiffening process, in which excess phenolic compounds originating from allelopathic extract might be channeled into the phenylpropanoid pathway, contributing to lignification process. On the contrary, in the present study, decreased stiffness of the cell wall was observed. Since the wheat is classified as allelopathic crop species, it is likely that plant itself produces excess phenolic compounds, as a reaction to allelochemical treatment, which had been documented previously. For example, in the field study of Fernandez et al. (2016), allelopathic interaction of *Pinus* extract with *Quercus pubescens* plants provoked an increase of 38.5% in TPC, followed by significant reduction of plant growth and chemical redirection to plant defense. The increase of phenolic compounds under array of environmental stresses has been well documented (Bartwal et al. 2013). Fernandez et al. (2016) hypothesized that production of phenolic compounds and variety of other secondary metabolites, such as terpenoids or aliphatic acids, could serve as an ecological advantage, through supporting the induction of precise class of metabolites, which is the most effective in response to present external threat, that could be allelopathic interaction, competition, or some other type of biotic or abiotic stressor.

It has been established previously that certain compounds react quickly with DPPH radical. However, many other have longer reaction times and more complex mechanisms of interaction with DPPH radical; thus, the assays which use kinetic parameters can provide more reliable data about antioxidant behavior, especially of complex plant extracts, which contain both slow and fast acting antioxidants (Terpinc et al. 2009). In the present study, relative DPPH radical scavenging capacity (RDSC) assay proposed by Cheng et al. (2006) has been employed. This assay measures area under the DPPH radical reaction curve, taking into account kinetic and thermodynamic properties of the reactions between DPPH radical and various antioxidant compounds present in the extract. In R5-treated wheat plants, results of antioxidant capacity assay unambiguously indicated significant increment in relative DPPH radical scavenging capacity (RDSC), which followed the same trend as the total phenolic content with the high correlation ($r = 0.97$). Strong correlation between these two assays could lead to the assumption that phenolic compounds are the major contributors to the increased antioxidant capacity of treated plants. On the other hand, having in mind shortcomings of the Folin–Ciocalteu assay, which shows reactivity towards other reducing agents such as ascorbic acid and aromatic amines organic acids (Prior et al. 2005), the possibility that some other non-phenolic anitoxidants are also responsible for antioxidant capacity increase could not be excluded. Thus, further efforts should be directed towards dissection of antioxidant response of wheat plants to allelopathic stimulus.

Results presented here demonstrated existence of clear defensive response of wheat in the absence of physical stimulus from competitor weed, reflecting intensified production of phenolic compounds in both roots and shoots, followed by increased antioxidant capacity of plant tissues. At the same time, it seems that boosted antioxidant defense neither succeeds to protect the plant from oxidative damage, nor to scavenge excess reactive oxygen species.

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