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ALLELOPATHIC AND CYTOTOXIC ACTIVITY OF *ORIGANUM VULGARE* SSP. *VULGARE* GROWING WILD IN BULGARIA

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Abstract. *Origanum* L. (Lamiaceae) is a valuable aromatic plant. Recently aromatic plants have gained interest as potent allelopathic plants. Secondary metabolites in allelopathic plants may lead to discovery of new classes of herbicides. On the other hand, these toxic secondary metabolites could affect adversely the consumers. Influence on mitotic cells has shown to be of importance for humans. The present study aimed to evaluate allelopathic and cytotoxic effects of water infusions made from the aerial parts of *Origanum vulgare* ssp. *vulgare* growing wild in Bulgaria in laboratory conditions. For monitoring the allelopathic effect germination and root elongation test were used. The effect on mitotic cells was determined using *Allium cepa* L. test. Infusions had minor effect on germination, but significantly decreased root length of *Cucumis sativus* L. and *Triticum aestivum* L. Oregano exerted mitodepressive and genotoxic effect in *Allium cepa*. These results demonstrated the potential of infusions as a source of bioactive substances that could be use in agriculture. The observed effects provide also information on safety evaluation of plant tested.

Keywords: *Origanum vulgare* ssp. *vulgare*, root growth inhibition, mitodepressive and genotoxic effect

Introduction

Nowadays, there is a renewed interest in medicinal plants as sources of variety of bioactive compounds. The beneficial health effects of plants result from constituents which have specific physiological action. Plants produce numerous secondary metabolites involved in biochemical defence mechanisms. The phenomenon of allelopathy, where plants influence the growth and survival of other organisms has been known from centuries (Kruse et al., 2000). The growing interest in allelopathy in recent years is connected with environmental problems associated with pollution due to agrochemicals (De Almeida et al., 2010). Studies on secondary metabolites in allelopathic plants may lead to discovery of new classes of herbicides (Kruse et al., 2000). Allelopathic activity

of medicinal plants is of great importance also as a caution for humans: secondary metabolites in medicinal plants could affect adversely the consumers (Teixeira et al., 2003; Liman et al., 2012; Neelamkavil & Thoppil, 2014).

Recently, aromatic plants have gained the interest of many research groups as potent allelopathic plants. Several studies have demonstrated the allelopathic activity of essential oils (Angelini et al., 2003; Arminante et al., 2006; De Almeida et al., 2010). There are also some data about phytotoxic effects of water extracts of plants (Economou et al., 2007; Dhima et al., 2009; Kakati & Baruah, 2013). Bioassays to detect allelopathy can be carried out in the laboratory conditions: germination and root length are typically evaluated parameters (Belinelo et al. 2008). These visible effect.f allelochemicals depend on effects at the cellular level as photosynthesis, respiration, cell division, production of plant hormones, specific enzyme activities *etc.* (Reigosa et al., 1999). Mechanism of action of allelochemicals has been widely studied. Among the damages caused by these toxic compounds effects on dividing cells have shown to be of importance for humans (Sousa & Viccini, 2011; Neelamkavil & Thoppil, 2014). *Allium cepa*-test has been proved to be a useful tool for the assessment of cytotoxicity (Leme & Marin-Morales, 2009).

Origanum L. (Lamiaceae) is a valuable aromatic plant. Crude extracts and essential oils of oregano species were used from ancient times. The growing popularity of oregano at present time is a result of scientific research (Raduðienë et al., 2005). Numerous studies report the antimicrobial, antifungal, insecticidal and antioxidative effects of this herb (Raduðienë et al., 2005; Hussain et al., 2011; De Falco et al., 2013). *Origanum vulgare* L. is an important culinary herb and medicinal plant commonly known as „oregano" (Hussain et al. 2011). One of the most usually used and consumed subspecies is *Origanum vulgare* ssp. *vulgare* L. (Viturro et al., 2010). This plant is widely distributed in Bulgaria (Anchev, 1989).

The objective of this study was to evaluate allelopathic and cytotoxic effects of water infusions made from the aerial parts of *Origanum vulgare* ssp. *vulgare* L. growing wild in Bulgaria in laboratory conditions.

Materials and methods

Plant material

Origanum vulgare ssp. *vulgare* growing wild in the vicinity of Shumen (Velino, Bulgaria) (latitude 43°18´ N; longitude 27°01´ E, altitude 227 m) was used in this study. The aerial parts of oregano plants were collected at the flowering stage. The plant specimens were identified and authenticated by Zh. Nanova (Taxonomist), Faculty of Natural Sciences, Shumen University, Bulgaria. Collected plant materials were dried at a room temperature.

Plant water infusions

Aerial parts of oregano plants, collected in June-July, cut about 20 cm from the top, were used in laboratory tests. The dried stems, leaves and flowers were covered with boiling distilled water, left for 60 min and then allowed to cool to room temperature. Oregano Water Infusions (OWI) were prepared at concentrations 3.5 g/l normally used by population (Nikolova & Manolov, 2002), 17.5 g/l (5× more concentrated), 35.0 g/l (10× more concentrated) and 52.5 g/l (15× more concentrated).

Germination rate and root elongation tests

Twenty seeds of *Cucumis sativus* L. cv. Gergana and *Triticum aestivum* L. cv. GTW, respectively, were placed on filter paper in each of ten Petri dishes (11 cm in diameter). Five ml of each infusion or distilled water, as a control, was applied to the seeds. The dishes were sealed and incubated at 25 ± 1°C for 72 h. The percent of germinated seeds was recorded, and the length of the roots of germinated seeds was measured. The percentage root growth inhibition in relation to the control for each extract was determined. Seeds that did not germinate were not included in the root elongation test. Three replications of each treatment were done.

Allium cepa-test

Thirty seeds of *A. cepa* were placed on filter paper in each of three Petri dishes (11 cm in diameter), containing 5 ml of distilled water. The Petri dishes were sealed and incubated at 25 ± 1°C for 48 h. Twenty germinated seeds with equal length of roots (~1 cm) were removed and placed on filter paper in each of another three Petri dishes. Five ml of water infusion (3.5 g/l) were added to two dishes, and incubated at 25 ± 1°C for 3 h. Distilled water was used as a negative control and methyl methanesulfonate (11 mg/l, for 24 h) was used as a positive control. After treatment, the roots were fixed in Clarke's fixative (95% ethanol: acetic acid glacial, 3:1) for 90 min, hydrolysed in 3N HCl for 8 min and in 45% acetic acid (CH_3COOH) for 30 min at room temperature and stained for 40 min in 2% acetoorseine. After staining, the terminal root tips (1-2 mm) were cut off and squashed in 45% CH_3COOH. Each sample consisted of six root meristems. At least 1000 cells of each root meristem were analysed. The microscopic analysis included estimation of the mitotic indices and aberrant cells. The mitotic index was determined as a ratio between the number of cells in mitosis and the total number of analysed cells. The index of each phase of mitotic division was calculated as a ratio between the cell number in the respective period and the number of dividing cells. Abnormalities in mitotic cells were evaluated. Interphase cells were analysed for the presence of micronuclei.

Statistics

Experimental data were processed by Student's t-test. In germination and root inhibition test we choose as an experimental unit the root. The calculations were carried out on the assumption that roots used in each treatment made one sample, and each sample was tested against the control sample. In *Allium cepa* test we choose as an experimental unit the cell, instead of the root. The calculations were carried out on the assumption that all the cells of the six root meristems made one sample, and each sample was tested against the negative control.

Results and discussion

Germination rate and root elongation

In present study germination and root growth inhibition of *C. sativus* and *T. aestivum* were used as index of general toxicity (Sousa et al., 2009; Oyeyemi & Bakare, 2013). Both test objects were reported as sensitive biosensors in other allelopathic studies (Belinelo et al., 2008; Chandra et al., 2012).

Fig. 1 shows the results of the influence of OWI on the germination of *C. sativus* and *T. aestivum* seeds. Seeds of both species germinated to above 90% in distilled water after 3 d. Seed germination of *C. sativus* was not influenced by treatment with concentrations 3.5 g/l and 17.5 g/l. Treatment with infusions at concentrations 35.0 g/l and 52.5 g/l exerted negative effect (inhibition by 6.67 and 8.33 percent units respectively in comparison to control).

Treatment with the concentration 3.5 g/l and 17.5 g/l had negligible effect on germination of *T. aestivum.* OWI at concentration 35.0 g/l inhibited germination of *T. aestivum* at the same rate as those observed of *C. sativus* L. (inhibition by 5.00 percent units). This inhibitory effect increased at the highest concentration tested (inhibition by 16.66 percent units).

Root length of both test objects was reduced significantly in comparison with the control (Table 1). Root growth was inhibited by 21.91% and by 16.80% respectively upon the treatment with the lowest concentration (3.5 g/l). OWI at concentration 17.5 g/l decreased root growth by about 50% compared to the control. This negative effect notably increased at concentration 35.0 g/l and 52.5 g/l.

Allelochemicals could cause inhibition of seed germination and/or seedling growth (Kruse et al., 2000). The results showed that OWI exerted notable effect on germination of both test objects only at highest concentration tested. In contrast, root growth was inhibited significantly at all concentrations tested. These observations lead to the conclusion that root growth is more sensitive than germination. Similar results was also obtained in other studies (Kakati & Baruah, 2013). The established root growth reduction by oregano could be attributed to inhibitory effects of allelopathic substances present in the water

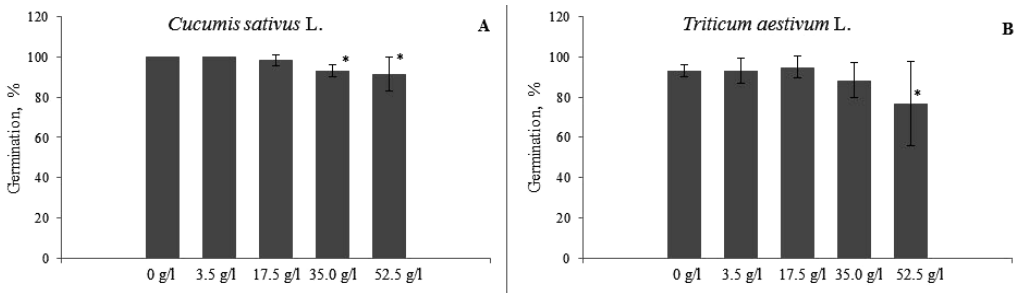

Fig. 1. Germination of *C. sativus* L. and *T. aestivum* L. seeds after 72 h of exposure to water infusions *of Origanum vulgare* ssp. *vulgare*. Data are expressed as means ± SD (standard deviation), *P ≤ 0.05, Control (0 g/l) – distilled water

Table 1. Root lengths of *Cucumis sativus* L. and *Triticum aestivum* L. after 72 h of exposure to *Origanum vulgare* ssp. *vulgare* water infusions

	Cucumis sativus L.		*Triticum aestivum* L.	
Sample	Root length, mm ± SD	% compared to the control	Root length, mm ± SD	% compared to the control
0 g/l	60.61 ± 9.45	100	63.27 ± 15.57	100
3.5 g/l	47.33 ± 11.55***	78.09	52.64 ± 9.94***	83.20
17.5g/l	33.08 ± 7.34***	54.58	32.12 ± 9.37***	50.77
35.0g/l	21.84 ± 6.48***	36.03	18.28 ± 6.35***	28.89
52.5g/l	13.44 ± 4.61***	22.17	14.25 ± 5.66***	22.52

Data are expressed as means ± SD (standard deviation), ***P ≤ 0.001; Control (0 g/l) – distilled water

infusions. The results of present study confirmed observations that many plants which are used in traditional medicine shows allelopathic effect (Kakati & Baruah, 2013). Plants with proven allelopathic activity are an important potential source for alternative pest management strategies (Macias et al., 2003). From this point of view, the possibility of utilization of oregano water infusions in agriculture could be further studied.

Allium cepa-test

Growth inhibitory macroscopic effects are consequence of primary effects at the cellular or molecular levels. Cytogenetic analysis could provide information about

mechanism of action of allelochemicals. Moreover, a study of genotoxic effects gives important information about potentially deleterious effects in humans of secondary metabolites in medicinal plants (Sousa & Viccini, 2011).

Table 2 shows the results of the influence of OWI on the mitotic index and on the frequency of mitotic phases. Upon the treatment the mitotic index was significantly decreased in comparison with negative control. The observed inhibition of mitotic activity indicates the occurrence of a cytotoxic effect of OWI (Leme & Marin-Morales, 2009). These data can explain the observed reduction of the root development (Table 1). The treatment changed the mitotic phase distribution in comparison to the control. The notable effect caused by OWI was an increase of prophase index. The interference in the cell cycle kinetics is accepted as a sign of cytotoxic influence (Liman et al., 2012).

Treatment with oregano significantly increased the frequency of abnormal mitotic cells (Table 3). OWI induced a variety of different chromosome aberrations (Fig. 2). Spindle abnormalities in metaphase and ana-telophase were the most frequent kinds of aberrations. Anaphases and telophases with bridges and fragments were the second. Anaphases with laggard chromosomes were also scored.

The evaluation of different kinds of aberrations allows assessing the action mechanisms of the tested compounds. Structural aberrations may be consequence of DNA breaks, inhibition of DNA synthesis and replication of altered DNA (Leme & Marin-Morales, 2009). The result of breaks is occurrence of fragments and bridges. Normally the ends of chromosomes (telomeres) prevent chromosome fusion. The loss of telomere may lead to fusion with other broken chromosome ends and formation of bridges (Maluszynska & Juchimiuk, 2005). According to Leme & Marin-Morales (2009) chromosome bridges and breaks in mitotic cells are indicators of a clastogenic action. Abnormal metaphases and anaphases indicated that OWI caused inhibition of spindle formation (Liman et al., 2012). Laggard chromosomes also indicate spindle disturbances (Rank, 2003). Janicke et al. (2007) defined a laggard as "a chromosome that did not overlap along the long axis of the spindle with any of the properly segregating chromosomes". Lagging chromosomes have been a regular feature of other studies with medicinal plant extracts (Sousa & Viccini, 2011). The observed increase of prophase index (Table 2) also may be due to disturbance of spindle apparatus.

The percent of interphase cells with micronuclei also increased significantly upon the treatment with oregano (threefold in comparison to control) (Table 3). The result of laggard chromosomes, fragments and bridges observed in mitotic cells of the first cell cycle after treatment are micronuclei in the interphase cell in the next cell cycle (Maluszynska & Juchimiuk, 2005; Sousa &Viccini, 2011). Both fragments and entire chromosomes cannot be incorporated into the main nucleus during the cell cycle. Micro-

Table 2. Effect of treatment with OWI (3.5 g/l, for 3 h) on mitotic index and phase indices in root tip meristems of *Allium cepa* L.

Sample	Cells analysed (number)	Dividing cells (number)	MI% ± SD	Prophase PhI% ± SD	Metaphase PhI% ± SD	Anaphase PhI% ± SD	Telophase PhI% ± SD
NC	8987	507	5.64 ± 0.23	25.84 ± 0.44	26.04 ± 0.44	18.74 ± 0.39	29.39 ± 0.46
OWI	11472	426	3.71 ± 0.19***	35.68 ± 0.48***	20.89 ± 0.41*	19.72 (± 0.40)	23.71 ± 0.43*
PC	7226	270	3.74 ± 0.19***	20.37 ± 0.46	31.85 ± 0.46	26.30 ± 0.44**	21.48 ± 0.41**

Sample: NC: negative control (distilled water, 0 g/l); PC: positive control (methyl methanesulfonate, 11 mg/L); OWI: water infusion made from the aerial parts of *Origanum vulgare* ssp. *vulgare*; MI: Mitotic index; PhI: Phase index. Data are expressed as means ± SD (standard deviation), *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

nuclei are extranuclear bodies of chromatin material (Fig. 2). The frequency of cells with micronuclei has been considered by many authors a good indicator of the cytogenetic effects of chemicals (Leme & Marin-Morales, 2009).

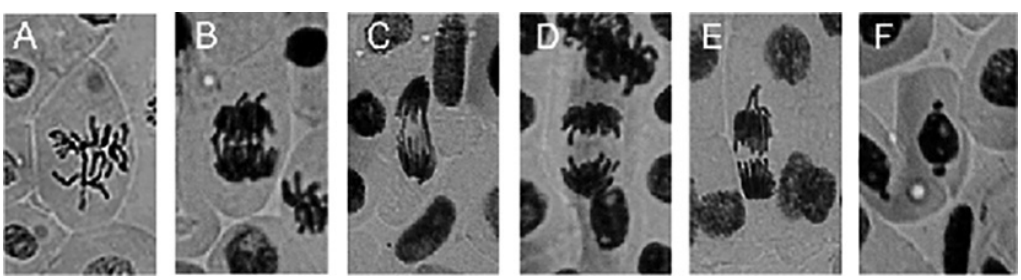

Fig. 2. Aberrations induced by OWI in *Allium cepa* root tips: A – abnormal metaphase; B – spindle abnormalities in anaphase and bridge; C, D – anaphase-telophase with fragment; E – laggard chromosome in anaphase; F – micronuclei in interphase cell

Table 3. Mitotic abnormalities and interphase cells with micronuclei in root tip meristems of *Allium cepa* L. after treatment with OWI (3.5 g/l, for 3 h)

Sample	Abnormalities, % of mitotic cells				Total abnormalities in mitotic cells, % ± SD	Micronuclei, % of interphase cells ± SD
	Spindle abnormalities in metaphase	Spindle abnormalities in ana-telophase	Bridges and fragments	Laggard chromosomes		
NC	–	1.58	0.39	0.20	2.17 ± 0.15	0.13 ± 0.04
OWI	0.70	1.88	1.88	1.64	6.10 ± 0.24 **	0.36 ± 0.06 ***
PC	0.36	–	11.11	4.82	16.29 ± 0.37 ***	0.46 ± 0.07 ***

Sample: NC: negative control (distilled water, 0 g/l); PC: positive control (methyl methanesulfonate, 11 mg/l); OWI: water infusion made from the aerial parts of *Origanum vulgare* ssp. *vulgare*. Data are expressed as means ± SD (standard deviation) ,**P ≤ 0.01, ***P ≤ 0.001

Herbal medicines are widely considered to be safe, but recently there has been increased discussion on the safety assessment of herbs (Tedesco & Laughinghouse IV, 2012). Numerous studies have been provided in order to evaluate genotoxic potential of medicinal plants in recent years. We used the classical *Allium* test for studying the effects of *Origanum vulgare* ssp. *vulgare*. Important features of this test system are: 1) sensitivity; 2) the presence of an oxidase enzyme system, which enables evaluation of promutagens and 3) good correlation with other test systems (Fiskesjo, 1985). Keeping the abovementioned in mind, the observed effects in this study could serve as an indicator of safety for humans. The results revealed that OWI induced chromosome aberrations in *Allium cepa* root tips. Compared with the positive control, oregano had less genotoxic effect. However, the different types of aberrations produced by oregano suggest possible genotoxic potential. In addition, these results could contribute to better understanding of the mechanism of the macroscopic effects of OWI.

Conclusion

Oregano water infusion decreased root length of *C. sativus* L. and *T. aestivum* L., in addition inhibiting cell division and inducing chromosomal alterations in *Allium cepa*. These results demonstrated the potential of these infusions as a source of active biological substances that could be use in agriculture. The observed effects in this study provide information regarding the safety evaluation on this plant.

Acknowledgments. This work has been supported by the National Science Fund, Grant No. RD-08-266/14.03.2013.

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