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New bioassay method to study the allelopathic activity of sugar lungwort (*Pulmonaria saccharata* Mill.)

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ABSTRACT

We develop new method for the bioassay of allelopathic activity of soil and plant materials. The allelochemicals were detected in the sugar lungwort (*Pulmonaria saccharata* Mill.) plant by the gradient bioassay and studied the allelopathic activity of soil under and around the individual plants. In a pot experiment, we studied the effects of 0.2 % and 0.5 % (w/v) of dry leaves and rhizomes of *Pulmonaria saccharata* Mill. as mulch on 3-acceptor plants [red clover (*Trifolium pratense* L.), tall fescue (*Festuca arundinacea* Schreb.) and *Prunella vulgaris* L.] on germination, growth, dry weight and water content. A dual effect was observed in the experiments. Depending on the concentration, the allelochemicals stimulated or inhibited the germination and initial growth of seedlings. Low mulch rates 0.2 % (w/v) stimulated the growth (35-46 %) in 1-5 days after sowing. However, at the subsequent stages of development of acceptor plants, growth was inhibited, water balance and assimilation were affected. The new bioassay made it possible to investigate the mechanism underlying the allelopathic activity of sugar lungwort and the results suggested that allelochemicals from this plant could be used as an herbicide.

Key words: Allelopathy, allelochemicals, clover, dilution, gradient bioassay, fescue *Pulmonaria saccharata*, *Prunella vulgaris*, soil activity, seed germination, seedling growth

INTRODUCTION

Biological activity is usually observed in range of M^{-3} to M^{-6} concentrations. However, one of the fundamental problems in the study of allelopathy is to calculate the concentrations of allelochemicals that approximate to those of natural products that cause specific responses in the acceptor plants. A new gradient bioassay that simulates the natural process of dilution of water-soluble active substances in the soil solution permits the effects of a wide range of concentrations to be determined. One of the major application of allelopathy is the development of natural herbicides to replace synthetic ones (28). For this we should use the weed-suppressing plants (30) rather than herbicide spray. Ground covering plant species are of our interest because they are commonly used in urban landscapes due to their aesthetic appeal (34). Weed suppressing species are required on roadside strips and under the trees canopy in cities (14). The natural habitat of sugar lungwort (*Pulmonaria saccharata* family *Boraginaceae*), [ground-covering plant] (Fig. 1,2) is dry forests of northwestern Italy and southern France (7). In trial plots, in the urban environment of Moscow, it formed a dense carpet, where few weeds were found (9). In our previous experiments we used the theory of Uranov (33) and methods of Grodzinskyi (17) to determine the allelopathic activity of the soil around a single plant, which helped us to use allelopathic plants in urban landscapes with great efficiency (8). The most active allelochemicals are water-soluble substances (5,23) and they move in the

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soil through soil solution. The complexity of soil systems determines, if the allelochemicals concentration is

sufficient or not to affect the receptor plant (5,28). Their transformation, distribution and loss in the soil depends on the rate of sorption, leaching, polymerization, degradation, oxidation, use by soil microflora and plant roots. Thus, the processes of dissolution, movement and accumulation of water-soluble allelochemicals in the soil cannot be ignored. We have modified the bioassay on agar (8,16,30,35) to simulate the process of dilution of allelochemicals in the soil solution. Uranov coined the term 'phytogenic field' in 1965 (10,33) and defined it as the volume of soil and air occupied and modified by a certain plant. The phytogenic field is also an impact zone of the plant in the environment and it extends beyond the space under plant the crown. It has an internal structure due to the distribution of its roots and above-ground shoots in space. Allelopathic activity of the soil in a phytogenic field may have a species-specific arrangement of bands or spots, which demonstrates the mechanism of the phenomenon. The plant species occupies the territory, using underground or aerial shoots. The impact zone of a single plant overlaps that of its neighbour. Therefore, in a population of interconnected plants a complex field is formed with interference zones, in which the allelopathic effects are increased. The functional structure provides the better protection of the colony from invaders. Under abiotic stress, the allelopathic activity of interference zones in the soil can be reduced, which can be used in urban areas as an indicator of the decline of plants' vitality and weed suppression (36). Weed suppression is also affected by plant characteristics [leaf canopy density, growth rate and early growth (14)]. This study aimed to determine the allelopathic activity of *Pulmonaria saccharata* Mill**.** and effects of environmental stress on its allelopathic activity.

MATERIALS AND METHODS

I. Plant material

The experiments were done in Department of Botany: (i) in Moscow (M) (55.815476°N 37.649468°E) and (ii) in rural area of Moscow district (MR) (56.0600°N 37.838459°E). Mean annual temperature: 7 ℃ (Max 38.2, Minimum -42.2 (2019), Mean annual sunshine hours: 1731. Mean annual precipitation [Rainfall + Snow]: 600 to 890 mm, Mean relative humidity: 77 %. Mean annual snow cover (Dec-Feb): 24.6 cm height. The leaves and rhizomes of *Pulmonaria saccharata* and soil samples were collected from the 5yr old identical clonal populations "M" and "MR" of about 500 plants each grown in loamy soil. Both populations were grown with natural leaf litter in partial-shade, away from large trees and shrubs, had identical care (without irrigation), as the *P. saccharata* plants are drought-resistant and the annual precipitation meets its water requirements. These wild plant species are self-sustaining on the natural soil fertility, hence, fertilizer was not added.

For bioassays, 5- soil samples (0-5 cm depth) were collected under the *P. saccharata* plants canopy from each location, these were mixed to prepare 2- homogenized samples: M and MR. The control (Cs) soil samples and soil for the pot experiment were collected from these locations without vegetation (10 m away from the

plants of M and MR populations). The rhizomes and leaves were taken from 50 well-developed plants from each location and were brought to laboratory, washed in running tap water followed by rinsing with distilled water. Soil samples, leaves and rhizomes for bioassay were air-dried at room temperature, ground and sieved through 2 mm mesh before storing in paper bags at 4 °C. The soil samples pH was measured using the soil pH analyzer, Checker® pH Tester HI 98103 'Hanna Instruments'.

Figure 1. *P. saccharata* plants during flowering

Figure 2. *P. saccharata* single plant.

II. Bioassays

The phytotoxicity of soil, leaves and rhizomes were investigated by the gradient bioassay. A 4 mm layer of 0.7 % agar, pH 6-7.5, volume 70 mL, was poured into a sterile 150 mm Petri dish. After its solidification, the test sample comprising soil (2 g), dry leaves or rhizomes (300, 150, 100 mg) was placed in the center of dish using a glass tube. Surface sterilized seeds of cress, *Lepidium sativum* L. (NK Seeds Co) cultivar 'Danskii' were placed on the agar, 50 in total, 10 seeds in each 5 concentric rows, in 10 radially oriented chains, according to the scheme (Fig.3,4). The Petri dishes were placed in the Incubator at 24°C.

After 90 h, the seedlings were removed from the agar with tweezers, sequentially in the rows from 1st to 5th, and the length of hypocotyl and root were measured. The bioassay was done with leaves, rhizomes and soil from two populations: M and MR. Treatments were replicated 5-times. Control soil samples (Cs), M and MR, were used only in the soil bioassay. Control for rhizomes and leaves did not contain these biomasses. The watersoluble compounds of samples moved into the semi-liquid agar aqueous phase with decrease in concentration.

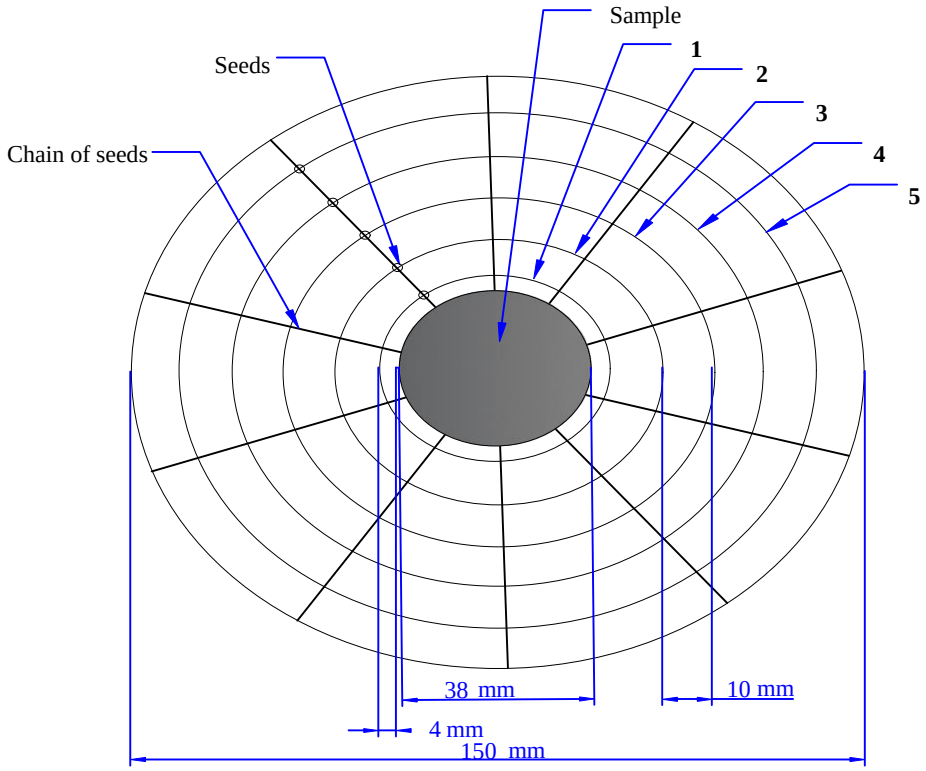


Figure 3. The scheme of Gradient Bioassay in Petri dish (150 mm dia). 1- 5: Rows of seeds. 150 mm: Petri dish dia, 38 mm: Sample position area dia, 10 mm: Distance between seeds rows, 4 mm: Distance between the sample border and the first row of seeds.

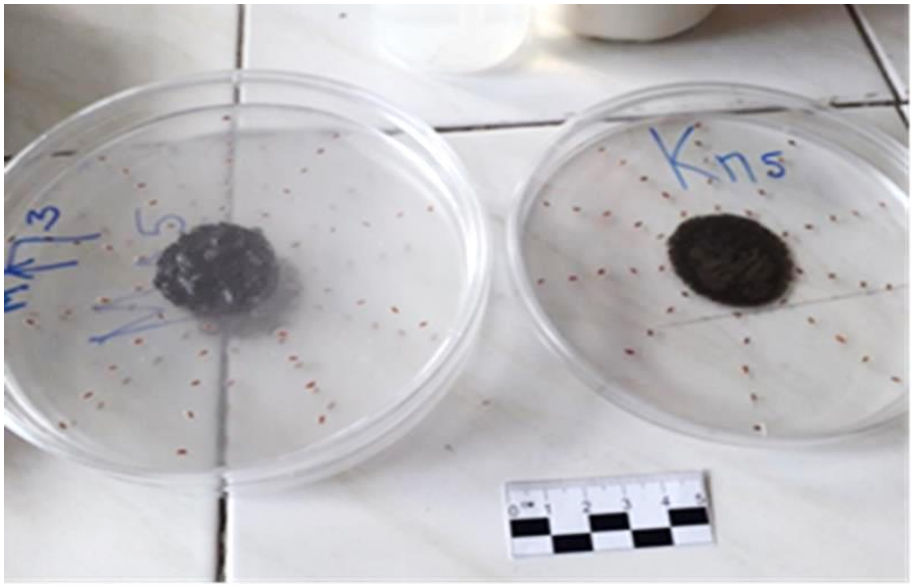


Figure 4. The gradient bioassay.

III. Under-canopy soil activity bioassay

Under and around the 10 well-developed growing single plants soil samples were taken according to the scheme (Fig. 5). Allelopathic activity of this soil upto 5 cm depth was determined as per the method of Furubayashi (16) in 5- replications. Cress was used as test plant. Petri dishes were kept in Incubator at (24 °C, with supplemental light 10 h per day). The control was the soil samples from a location without vegetation at a distance 3-10 m far from the plants.

SEEDS GERMINATION: Before the pot experiment, the seeds germination of Recipient crops was determined as under. In 90-mm Petri dishes, 20- seeds of each test crop were sown on double layers of Whatman filter paper, moistened with 5 mL distilled water, in separate Petri dishes, in 5- Replications. These Petri dishes were kept in growth chamber (25 °C). After 5-days, germination capacity was calculated as the percentage of seeds germinated.

POT EXPERIMENTS: The pot experiments were done to study the effects of allelochemicals on recipient plants. Grodzinsky (17) had calculated the amount of dry material from the donor plant as 0.4 % and 1.6 % weight to soil volume as relatively "low" and "high" concentrations of allelochemicals used as mulch in pot experiments. In bioassays we had found that 100-300 mg per 70 mL agar volume was effective application. The Experimental treatments consisted of 4 Factors: (i). Soil Sources: 2 (Moscow city, outside Moscow city), (ii). Mulch Doses: 3 (0, 200, 500 mg per pot), (iii). Activated carbon: 1 (500 mg per pot added and mixed to the soil) and (iv). Recipient Plants: 3 (T - *Trifolium pratense*, F - *Festuca arundinacea*, P - *Prunella vulgaris)* and

replicated 5-times in Completely Randomized Design. We calculated the dose of dry leaves or rhizomes added as mulch per pot and it was 0.2 % and 0.5 % (w/v). Thus we added 200 and 500 mg plant material of *P. saccharata* as mulch per pot. The pots soil was sieved through 3 mm mesh sieve. The pot experiment was done in 120 cm^3 plastic pots 5 x 4 cm, depth of pot 6 cm, depth of soil 5 cm, air-dried soil per pot was 134 g. Five seeds per pot were sown at 2-3 mm depth and the pots were placed in growth chamber at room temperature and in natural light with additional light for 4-6 h per day. Pots were irrigated daily with 7 mL Distilled water for 60 % of total water capacity of soil. Seedlings emergence and the shoot length of clover and fescue were recorded daily, however, the root and shoot length were measured and the fresh and dry weights were determined at end of experiment i.e. at 15th day after sowing asper (26).

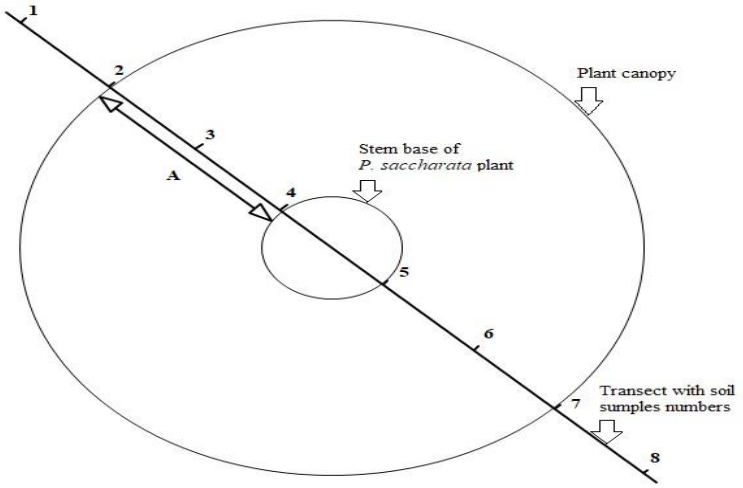


Figure 5. The scheme of soil sampling points 1-8 under and around *P. saccharata* plants canopy. Points 2,4,5,7 under the plant canopy are the key points. Distance between two sampling points are A/2.

Table 1. The Treatment details of pot experiment

M			MR		
Trifolium pratense (T)	*Festuca arundinacea* (F)	*Prunella vulgaris* (T)	*Trifolium pratense* (T)	*Festuca arundinacea* (F)	*Prunella vulgaris* (T)
Control	Control	Control	Control	Control	Control
M T 200	M F 200	M P 200	MR T 200	MR F 200	MR P 200
M T 500	M F 500	M P 500	MR T 500	MR F 500	MR P 500
M T 500+	M F 500+	M P 500+	MR T 500+	MR F 500+	MR P 500+

Sampling sites: M: Soil and *P. saccharata* biomass samples from Moscow City, MR: Soil and *P. saccharata* biomass samples from Moscow Rural area. **Acceptor plants**: T: *Trifoloium pratense*, F: *Festuca arundinacea*, P: *Prunella vulgaris*. ***P. saccharate*** **biomass Mulch rates (mg per pot**): 200: (leaves 150 mg + rhizomes 50 mg), 500: (leaves 350 mg + rhizomes 150 mg), 500+: (leaves 350 mg + rhizomes 150 mg) + activated carbon 500 mg (mixed with soil) per pot.

IV Biomass and water content

The fresh weight (FW) of plants was determined at the end of pot experiment after removing the plants from the soil, washing, and drying with filter paper. The dry weight (DW) of plants was determined after drying in an oven at 70°C for 48 h, cooling in desiccator with glass lid and weighing. The water content (W) expressed as amount of water (mg) per mg of dry weight was calculated as under:

$$W = \frac{FW - DW}{DW}.$$

V. Germination, growth and allelopathy indices

Interpretation of pot experiment data and the precise description of allelopathic effects depends on the choice of germination and growth indices (1). Two were calculated for germination (G_T and S_5).

(i). Total Germination or final germination percentage (G_T) was calculated as under: Germination Percentage G_T (%) = Germinated seeds×100/Total seeds

(ii). Speed of Germination (S_5) at 1-5 days after sowing, showed the mean number of seeds germinated in one day and was calculated as under:
Speed of Germination (S) = $N_1 + (N_2 – N_1)/2 + (N_4 – N_3)/3 + (Nn – Nn\text{-}1)/n$
Where, Nn: Number of germinated seeds observed on the nth day after sowing.

(iii). Relative Growth Rate (RGR) was calculated for the initial stage (1-5 days after sowing) and for the remaining 10 days of the pot experiment (6-15) and was calculated as under:
Relative Growth Rate (RGR)1-5= $(H_5 - H_1)/[H_1 \times 4]$ and (RGR)6-15= $(H_{15} - H_6)/ [H_6 \times 9]$
Where, H_1, H_5, H_6, H_{15}**:** Mean shoot length of acceptor plants on day 1, 5, 6, 15 in pot experiment. 4, 9: Number of days (Duration) of pot experiments.

(iv). Allelopathic Effect expressed as allelopathic index (AI) indicates the intensity of allelopathic effect and was calculated as under: Allelopathic Index (AI) = 1 – C/T
When $T \geq C$, and AI = T/C – 1,
When $T < C$, where C: Control data and T: Treatment data.

(v). Allelopathic index (AI): AI > 0 indicates stimulation, AI < 0 indicates inhibition. AI was calculated for G_T, S_5, RGR1-5 and RGR6-15.

(vi). Root/Aerial Part Ratio (RA) was calculated as under:
RA = Root length (mm) /aerial part length (mm).

VI. Statistical Analysis of Data

Statistical analysis of data was done by calculating significant differences using oneway ANOVA with the *t*-test, using $p < 0.05$ as the level of significance. The tables and graphs show arithmetic mean values with standard errors (M ± SE).

RESULTS AND DISCUSSION

Gradient bioassay

Preliminary testing of the gradient bioassay was necessary to select the optimum sample weight, identifying the physiological concentration range of allelopathic agent and

preventing the 'edge effect' (overabundance of hydrophilic organic compounds for a given volume of medium). The dose of 100 mg leaves (M) stimulated, while dose of 300 mg leaves inhibited the receptor seedling growth (Fig. 6). Similarly, samples of rhizomes caused inhibition. The first row of seedlings was exposed to high concentrations of substances dissolved (300 mg of leaves and 100 mg of rhizomes) from mulch, hence, the seedlings died. Applied doses of leaves, 150 mg (M), 100 mg (MR), soil (M, MR) and occasionally rhizomes (M, MR) caused variable effects on growth. When 150 mg (M) of leaves were applied the seedlings growth was less inhibited (61.3±3.6 %) in first row (Fig. 6,7), while, in the third row growth was stimulated (84.7±5.6 %), there was little inhibition (34.9±4.1 %) in the fourth row (Fig. 6,7) over the control. Thus the water soluble *P. saccharata* allelochemicals changes the biological activity during the dissolution and dilution, the inhibitory effect was changed to stimulatory effect, and then again to inhibitory effect.

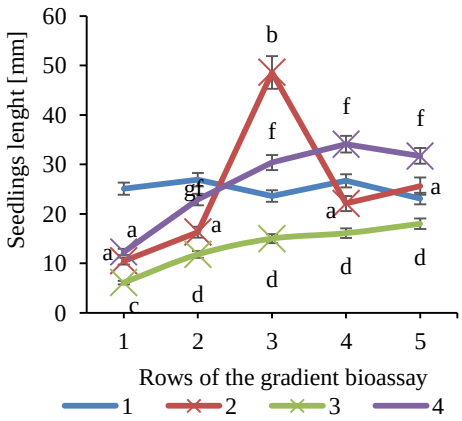


Figure 6. Effects of *P. saccharata* leaves weight (1: Control; 2: 150 mg; 3: 300 mg; 4: 100 mg) from M population, on the seedlings (Root + Shoot) length in rows of gradient bioassay.

Noted line markers – p < 0.05 (*t*-test) compared with control. Bars denote ± standard error. Data marked with different letters were significantly different under *p* level as 0.05 (*t*-test).

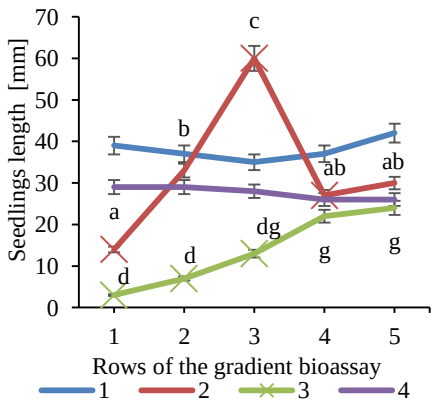


Figure 7. Effects of water-soluble allelopathic substances of *P. saccharata* rhizome and under canopy soil of population MR on seedlings (Root + Shoot) length in rows of gradient bioassay in 4- treatments (1: Control soil (Cs); 2: *P. saccharata* under canopy soil; 3: rhizome 100 mg; 4: Control). Noted line markers – *p* < 0.05 (*t*-test) compared with control. Bars denote ± standard error. Data marked with different letters were significantly different under *p* level as 0.05 (*t*-test).

Allelochemicals are often found as complex of substances with different biological activities (17,19). The most common allelochemicals are phenolic substances (4,16,23,27). Some phenolics can form unstable light-dependent isomers when illuminated with an ultra violet light. Isomers of hydroxycinnamic acids are of two types: (i). Light-dependent unstable, with high solubility and biological activity, (ii). Stable, low-soluble, inactive or inhibitor (12,15,18,20,28).

Schubert *et al.* (32) reported the formation of unstable isomers in an aqueous solution of hydroxycinnamic acids at M^{-3}–M^{-7} concentrations under the action of ultraviolet radiation by fixing the transformations of absorption spectra (32). However, the isomers appeared in solution even in the dark during dissolution and dilution. The unstable ferulic acid isomer, in the dark or with further dilution, transformed back to the stable isomer within 30-60 minutes. Li *et al.* (22) found an inhibiting effect of a stable isomer of cinnamic acid regardless of its concentration (22). Phenolic acids and their conjugates were found present in the *Pulmonaria* species (21). We assume that isomerization in the Biotest during dilution, produced an unstable isomer as growth promoter in third row, and the stable isomer was inhibitor in fourth row. Inhibition in the first row may be attributed to the non-specific effects of relatively high concentrations of water-soluble substances. This phenomenon can be studied when the Biotest is done in light and dark, and in pot experiment. Hence, the bioassay was done in light, and then the Petri dishes were kept in dark incubator.

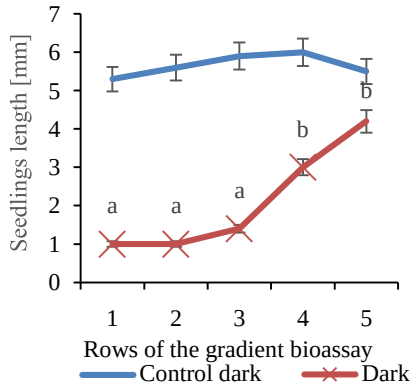


Figure 8. Effects of *P. saccharata* leaves (M) allelochemicals on the seedlings (Root + Shoot) length in the gradient bioassay in darkness. Control dark: Control bioassay, kept in the dark.
Noted line markers – $p < 0.05$ (*t*-test) compared with control. Bars denote ± standard error. Data marked with different letters were significantly different under *p* level as 0.05 (*t*-test). *Note*: Samples of dry leaves were kept in the dark for 72 h, all preparations for the bioassay were held in the dark.

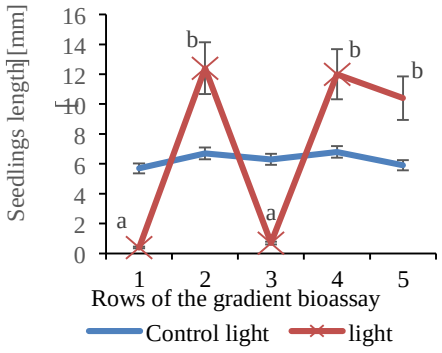


Figure 9. Effects of *P. saccharata* leaves (M) allelochemicals on the seedlings (Root + Shoots) length in the gradient bioassay done in light. Control light: Control bioassay, kept in the light.
Noted line markers – $p < 0.05$ (*t*-test) compared with control. Bars denote ± standard error. Data marked with different letters were significantly different under *p* level as 0.05 (*t*-test).

In the following bioassays experiments, replications with 150 mg dry leaves (M) were prepared and exposed to the light, while others were kept in dark (Fig. 8,9). In the bioassays done in light, we found alternate stimulation and inhibition of growth, with predominance of stimulation. The seedlings died completely in the first and third rows. While in bioassay done in dark, only inhibition of growth was observed. The bioassay

results showed dependence of allelopathic activity on the concentration of *P. saccharata* active substances. While it revealed the phenomenon of a non-linear dependence on concentration and sharp change in biological activity during the dilution of substances in an aqueous solution or light exposure. The above studies of isomers and their physiological activity were done using the chemically pure phenolic substances (2,4,12,19,23,32). Blum (4) found that when using samples of plants biomass or extracts for biotests, we must consider the action of other substances with biological activity and also the additive, antagonistic or synergistic effects on acceptor plants. The observed processes are associated with the influence of a group of biologically active substances, their various concentrations and chemical interference, assumed isomerization and different effects of isomers. We have added brief description of phenolic isomers, as this is one of the possible ways to interpret the Biotest and pot experiment data. In this study, we wanted to make possible assumptions, without discussing the chemical nature of isomers, as this requires a separate research. These results showed the presence of specific substance in the leaves, rhizomes and soil of M and MR populations. The bioassay indicated the different contents of allelochemicals in the leaves and rhizomes (more in rhizomes), in the plant tissues of M and MR populations (more in MR). The influences on the length of the roots and hypocotyls were non-significant.

Under-canopy soil activity bioassay

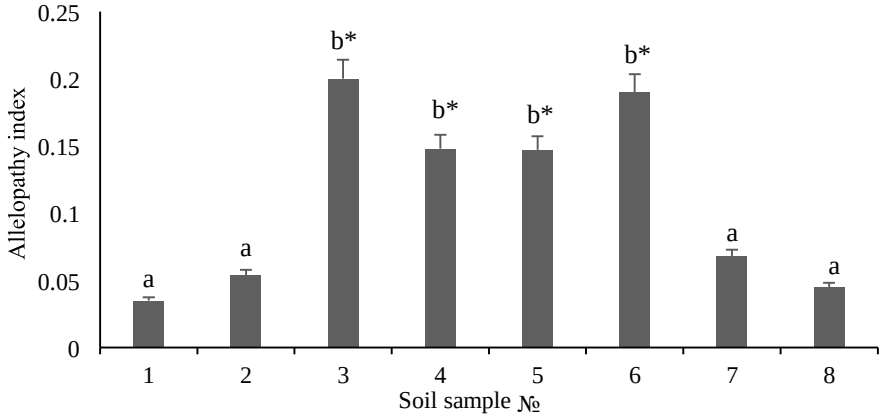


Figure 10. The allelopathic activity (Allelopathy Index) of *P. saccharata* under canopy soil. Soil samples 1-8 were taken along the transect through the center of *P. saccharata* plants. Bars denote ± standard error. Different letters indicate statistically significant differences under *p* level as 0.05 (*t*test). * - statistically significant differences compared to control ($p < 0.05$, *t*-test).

Allelopathic activity of the soil under *P. saccharata* plants canopy was stimulatory, with insignificant activity beyond the plants canopy in soil samples 1 and 8 (Fig.10). Soil samples taken from the points 3,4,5,6 at different times of year showed statistically significant activity. Drying and storing samples for two years did not affect the allelopathic activity. Stimulatory activity was associated with low concentrations of allelochemicals in the field soil, or with isomerization, or both of these factors and are in

agreement with our Biotest data. We can assume the transfer of allelochemicals (i). from leaves with rainwater (water soluble components), (ii) with leaf litter and (iii) excretion from rhizomes in soil.

POT EXPERIMENT

Germination: The application of allelochemicals to the seeds of susceptible species inhibited their germination and later affected their seedlings growth and caused metabolic disorders (3,13,21,24,27). The allelochemicals stimulated the seeds germination (G_T) of fescue and clover in M treatments but inhibited in MR treatment (Fig. 11).

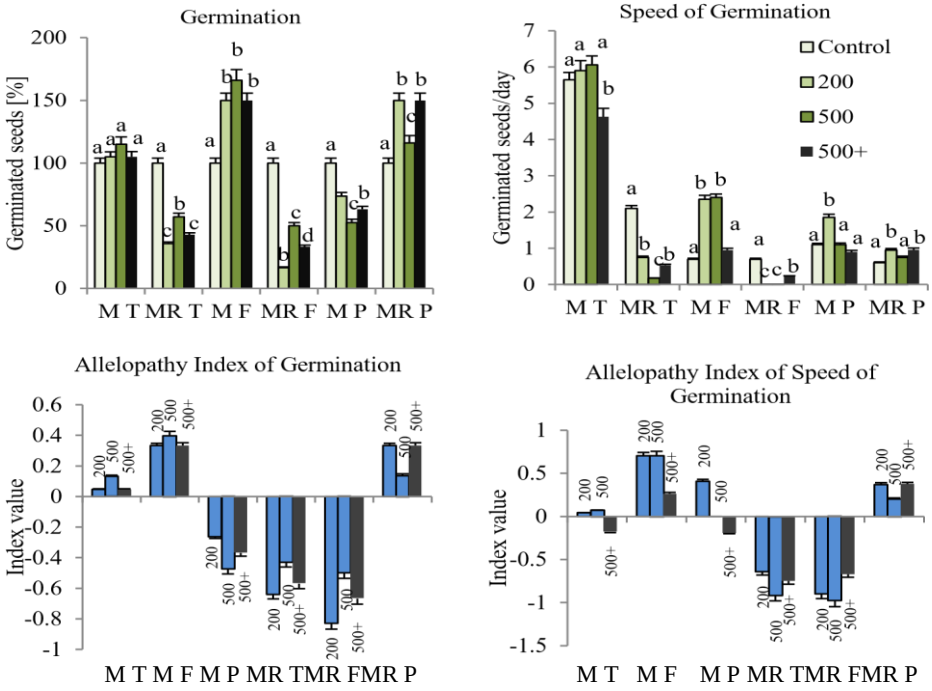


Figure 11. Effects of *P. saccharata* allelochemicals on the total germination percentage (G_T) and speed (S_5) of acceptor plants seeds. **Sampling sites;** M: Soil and *P. saccharate* biomass samples from Moscow City, MR: Soil and *P. saccharata* biomass samples from Moscow Rural area. **Acceptor plants**: T: *Trifoloium pratense*, F: *Festuca arundinacea*, P: *Prunella vulgaris*. ***P. saccharata* biomass Mulch rates (mg per pot)**: 200: (leaves 150 mg + rhizomes 50 mg), 500: (leaves 350 mg + rhizomes 150 mg), 500+: (leaves 350 mg + rhizomes 150 mg) + activated carbon 500 mg (mixed wit soil) per pot. Data marked with different letters were significantly different under *p* level as 0.05 (*t*-test) in the same column. Bars denote ± standard error.

The bioassay results confirmed that the germination depended on the different content of allelochemicals in *P. saccharata* tissues in M and MR populations. The allelochemicals contents decreased in urban environment due to the reaction of plants to abiotic stress (25). The soil characteristics and microbial communities influences the effectiveness of allelochemicals (5,17), although the pH was similar (6.3±0.01 and

6.7±0.14) in M and MR soil samples. The effects of allelochemicals on the seed germination depended on their concentration, low concentration caused stimulation, while, high one caused inhibition.

The response of clover in treatment M was weak due to its high seed germination (92±2.0 %), while, in fescue and *P. vulgaris* seeds it was lower, 84±6.0 % and 70±4.0 %, respectively. Thus final germination is not sensitive enough to validate the allelochemical effects. Speed of germination is considered as the key indicator among the germination indices in allelopathic studies (13). In the treatment MR at relatively high concentration of allelochemicals, germination of clover was decreased (43-64 %) than control. Germination in MR 500 was 60 % higher than in MR 200. In MR, the germination speed decreased (60-90%), but at MR 200 it was 77% higher than in MR 500.

Fescue germination in M treatment was stimulated (50-65%). However, in the MR treatment the germination was drastically inhibited (79-82%) than control. In M treatment, the germination speed increased (23-25%) but decreased (86-92%) in the MR treatment. Germination in MR 500 was significantly higher (50%) than in MR 200 (16.6%).

Clover and fescue response to the mulch application was similar. The non-linear dependence of the effects on mulch rate in MR 200 and MR 500 suggested a simultaneous action of two substances in the MR 200. The inhibitor reduced the seeds germination, while, the activator increased the seeds germination speed.

P. vulgaris reaction was quite different. In M treatment, germination was inhibited (47-51%), while in M 200 germination speed was increased (68%) than control. In the MR treatment, germination was stimulated (16-50%) over the control. The high activity of the antioxidant complex enzymes and the action of endogenous isomerases involved in the rosmarinic acid synthesis may cause such significant changes (29).

Activated carbon absorbs and neutralizes the water-soluble low-molecular substances and has great affinity to phenolics (23). By comparing the M 500 and M 500+ (with Activated carbon) data, we estimated the changes in the reactions of test plants to the absorbent Activated carbon. The M treatment with Activated carbon reduced all 3-test plants seeds germination (8-18%) and the seeds germination speed (18-50 %) compared to the M 500. The Activated carbon is not inhibitory but it absorbs the stimulatory compounds. Probably, the Activated carbon absorbed some part of seed germination promotor compound(s), while the inhibitor was not absorbed. In MR treatment with Activated carbon, clover and fescue germination was decreased (23-50%) than MR 500 but speed of seeds germination was increased (23-28 %). A dual effect in MR 500+ can be obtained, when two substances (an inhibitor and a stimulator) simultaneously affected the test plants. The *P. saccharata* allelochemicals at low concentrations accelerated the germination speed of all seeds and also stimulated the germination of weak and dormant seeds of fescue.

Growth rate

In clover seedlings, in the initial stage (1-5th days), the relative growth rate in M 200, M 500 and MR 200, MR 500+ treatments were increased by 35-70 % (Fig. 12). While in next stage (6-15th day), the growth rate was decreased (67-73 %) to control

values. A similar reaction was observed in fescue growth rate in M 200 and M 500 treatments increased in the initial stage by 79 % and 30 %, respectively. Contrarily in the next stage (6-15 days) growth was inhibited (67 % and 10 %, respectively). In clover and fescue, the major decrease in the growth rate occurred after the activation of germination speed and initial growth rate in M 200, MR 200, MR 500+. Externally induced accelerated growth of seedlings in the initial stages of seedlings development may deplete their nutrients reserves and hence, disrupts the important metabolic processes at their beginning stage.

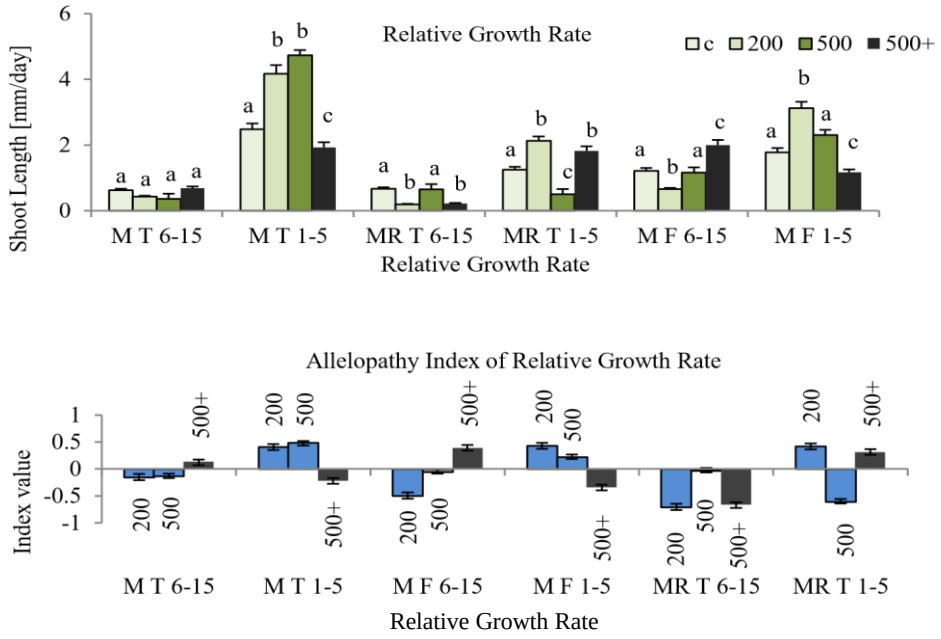


Figure 12. Effects of *P. saccharata* allelochemicals on the relative growth rate (RGR) of the acceptor plants at 6-15 and 1-5 days after sowing. **Sampling sites;** M: Soil and *P. saccharata* biomass samples from Moscow City, MR: Soil and *P. saccharata* biomass samples from Moscow Rural area. **Acceptor plants**: T: *Trifoloium pratense*, F: *Festuca arundinacea*, P: *Prunella vulgaris*. ***P. saccharata* biomass Mulch rates (mg per pot)**: 200: (leaves 150 mg + rhizomes 50 mg), 500: (leaves 350 mg + rhizomes 150 mg), 500+: (leaves 350 mg + rhizomes 150 mg) + activated carbon 500 mg (mixed with soil) per pot. **RGR Growth Stages:** 1-5 and 6-15: days after sowing. Data marked with different letters were significantly different under *p* level as 0.05 (*t*-test) in the same column. Bars denote ± standard error.

Comparing the 500 and 500+ data, we found that in the first 5 days after sowing regardless of the mulch rates and different content of allelochemicals in the M treatments, the growth rate was decreased (49-59 %) in the presence of activated carbon. The stimulator was absorbed, and the inhibitor decreased the growth rate below the control. In the subsequent growth stage (6-15 days) in the presence of Activated carbon, the growth rate was increased (72-91 %) in treatment M, or decreased in MR treatment as compared to M 500 and MR 500 respectively (Fig.12). In treatments with high mulch rate, the high

concentrations of allelochemicals could block the further dissolution of substances or isomerization, or both. Probably absorption of some allelochemicals, changed the concentration of active substances in soil solution, or changed the isomers in soil solution. It takes time, to saturate the entire soil volume with dissolved allelochemicals, hence, this effect is developed in the later stage of the experiment. This phenomenon may be due to the inhibitory concentrations, the allelochemicals act more like contact agents, than systemic agents (4,5). The very high concentration of allelochemicals allows them to easily contact the seeds. The seeds and young seedlings had low ability to avoid the contact with allelochemicals in close proximity.

The ANOVA results of data dispersion of shoot length of acceptor plants showed the inhibition and stimulation in M 200 in clover and fescue M 200 and not in M 500 and M 500+ (Fig. 13). In MR treatments, less number of plants made the analysis incorrect.

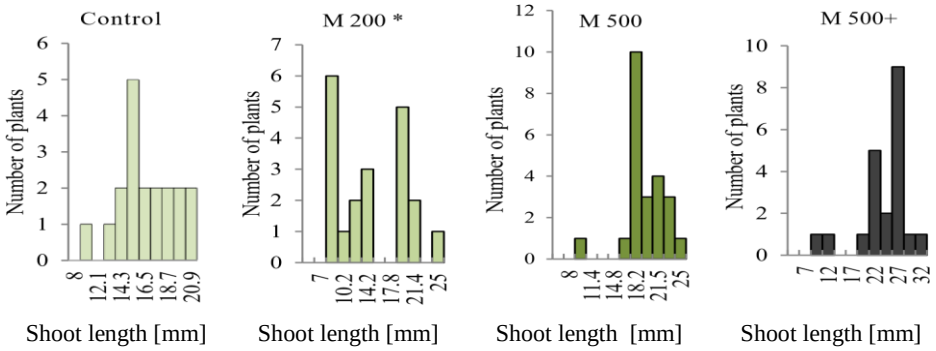


Figure 13. ANOVA results of data dispersion of shoot length (mm) of acceptor plants. *T. pratense* in M treatments on 8th day in pot experiment. *P. saccharata* biomass Mulch rates (mg per pot): 200: (leaves 150 mg + rhizomes 50 mg), 500: (leaves 350 mg + rhizomes 150 mg), 500+: (leaves 350 mg + rhizomes 150 mg) + activated carbon 500 mg (mixed with soil) per pot. * - $p < 0.05$ (*t*-test) for data groups.

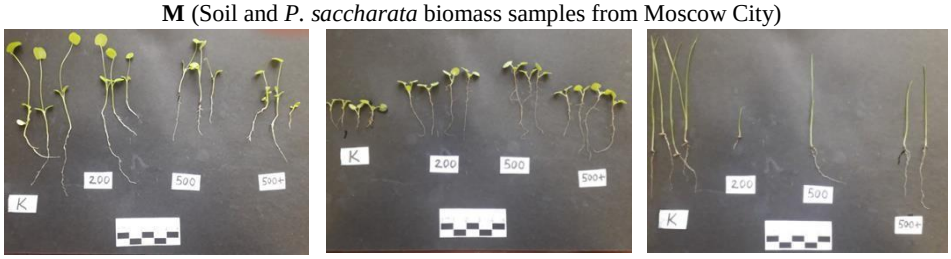
Li *et al.* (22) reported that chemically pure phenolic acids in concentrations less than M^{-3} ("low" concentration) were stimulatory, while, higher concentration were inhibitory (22). While in natural conditions there are mixtures of conjugated phenolic compounds with complex characteristics. We arranged the treatments with different mulch rates according to the test plants reactions into the assumed gradient concentrations of allelochemicals as M 200 < M 500+ < M 500 < MR 200 < MR 500+ <MR 500 from lowest to highest. Probably, as in the Biotest, at relatively low concentrations of allelochemicals in M 200 and MR 200 formed the most suitable conditions for isomerization. Activated carbon partially absorbed the allelochemicals and promoted the isomerization in M 500+ and MR 500+ treatments. Despite the growth inhibition followed by stimulation, or *vice versa*, at the end of the experiment the plants were identical (in M 200) or with a slight difference (M 500+ and MR 200) in height and the fresh mass compared to the control. However, assumed isomerization reduced the germination and vitality of acceptor plants. The reaction of the test crops to treatments MR 500+ and MR 500 was different, at the end of experiment, the plants differed significantly in height and biomass from the control plants (Fig.14,15).

Roots growth

Roots are more susceptible to the impact of allelochemicals than shoots (4). Various effects of phenolic compounds on the root growth of tested plants are known, they are often species-specific. The ferulic acid increases the number of secondary roots, the root-shoots ratios for cucumber, or inhibited rhizogenesis, root growth and secondary roots formation in Vigna (20,31). Allelopathically induced changes in the shoot and underground parts of the acceptor plants indicate a serious decline in their vitality. The ratio of roots and shoots of acceptor plants was changed according to the species specificity (Table 2). In clover, the relative root length decreased in treatments M 200 and MR 500. The fescue response was variable. The *P. vulgaris* roots in the treatments M and MR were very long but thin (Fig. 14). The biomass of plants grown in the soil of M and MR differed than control plants, but their root length were similar. The presence of activated carbon in M 500+ caused the formation of root 'brushes' in clover seedlings. In MR treatments there were more lateral roots of *P. vulgaris* than in M. In MR treatments 500 and 500+AC (Activated Carbon) all plants showed etiolation and necrosis of cotyledons and treated leaves showed very high concentration of allelochemicals (Fig. 14).

Trifolium pratense *Prunella vulgaris* *Festuca arundinacea*

M (Soil and *P. saccharata* biomass samples from Moscow City)



Trifolium pratense *Prunella vulgaris* *Festuca arundinacea*

MR (Soil and *P. saccharata* biomass samples from Moscow Rural area)

Figure 14. The Photographs showing the effects of *P. saccharata* biomass allelochemicals on seedlings growth of acceptor plants, *Trifoloium pratense* (T), *Festuca arundinacea* (F), *Prunella vulgaris* (P) at the 15th day in pot experiment. **Sampling sites:** M: Soil and *P. saccharata* biomass samples from Moscow City, MR: Soil and *P. saccharata* biomass samples from Moscow Rural area. ***P. saccharata* biomass Mulch rates (mg per pot**): 200: (leaves 150 mg + rhizomes 50 mg), 500: (leaves 350 mg + rhizomes 150 mg), 500+: (leaves 350 mg + rhizomes 150 mg) + activated carbon 500 mg (mixed with soil) per pot.

Table 2. Effects of allelochemicals in *P. saccharata* biomass on the root /shoot ratio (RS) of the acceptor plants.

Treatment	M		R	
	Trifolium pratense	*Festuca arundinacea*	*Trifolium pratense*	*Prunella vulgaris*
Control	0.80±0.02 a	0.43±0.03 a	0,81±0.02 a	2,59±0.22 a
200	0.57±0.02 b	0.48±0.05 a	0,69±0.01 b	6,7±0.08 b
500	0.67±0.01 a	0.38±0.03 a	0,70±0.01 b	7,2±0.16 b
500+	0.80±0.02 a	0.51±0.04 a	0,76±0.01a	5,0±0.10 b

M: Soil and *P. saccharata* biomass samples from Moscow City, MR: Soil and *P. saccharata* biomass samples from Moscow Rural area. Values are the mean ± SE. Data marked with different letters were significantly different under *p* level as 0.05 (*t*-test) in the same column.

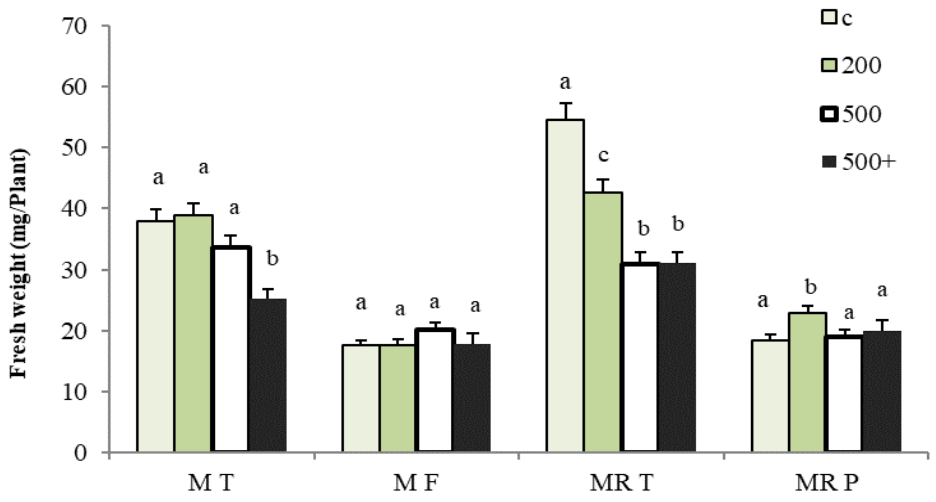


Figure 15. Effects of *P. saccharata* biomass allelochemicals on fresh weight (FW) of acceptor plants *Trifoloium pratense* (T), *Festuca arundinacea* (F), *Prunella vulgaris* (P) at the 15th day in pot experiment. **Sampling sites:** M: Soil and *P. saccharata* biomass samples from Moscow City, MR: Soil and *P. saccharata* biomass samples from Moscow Rural area. ***P. saccharata* biomass Mulch rates (mg per pot**): 200: (leaves 150 mg + rhizomes 50 mg), 500: (leaves 350 mg + rhizomes 150 mg), 500+: (leaves 350 mg + rhizomes 150 mg) + activated carbon 500 mg (mixed with soil) per pot. Data marked with different letters were significantly different under *p* level as 0.05 (*t*-test) in the same column. Bars denote ± standard error.

Dry weight and water content

The rate of dry weight accumulation by clover and fescue seedlings decreased only in the treatments M 200 and M 500+ (Fig. 16). The water content in fescue and clover tissues increased (up to 122 %) in M 200 and M 500+ treatments. (Fig. 16). At the same time, clover and *P. vulgaris* in MR treatments showed water deficit/witling in their tissues

on 15th day. This indicates that the analyzed compounds affected the plants metabolism. Allelochemicals affects the different metabolic sites of plants (23). Application of high concentrations of phenolic acid disrupts the water balance and caused wilting of seedlings (4,31).

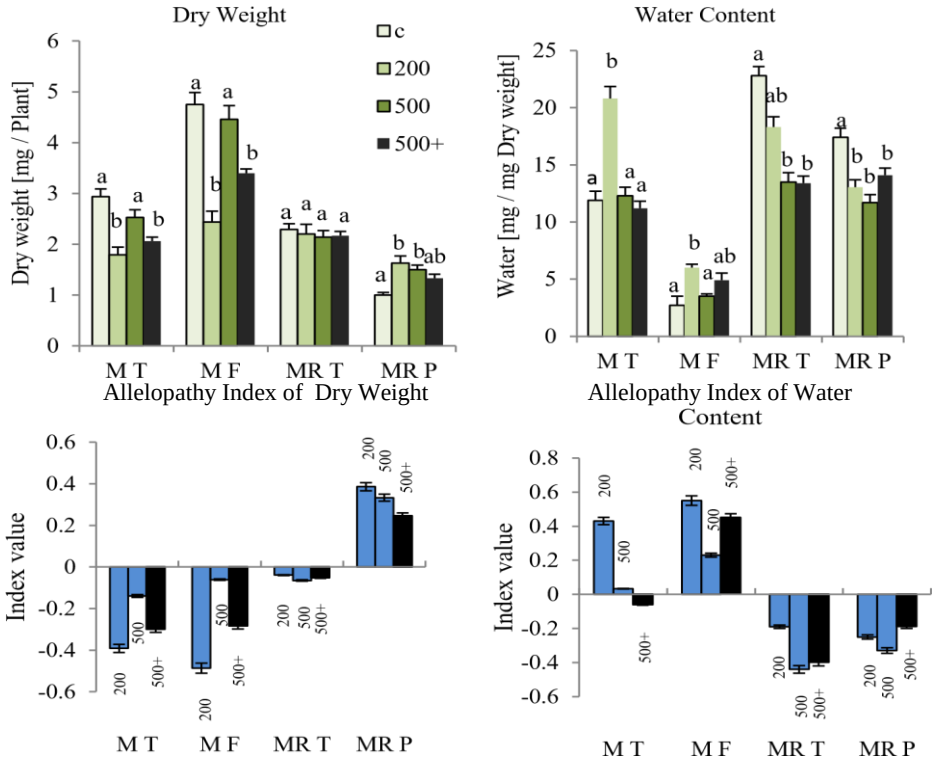


Figure 16. Effects of *P. saccharata* soil allelochemicals on dry weight (DW) and water content (W) of the acceptor plants on the 15th day of pot experiment. **Sampling sites:** M: Soil and *P. saccharata* biomass samples from Moscow City, MR: Soil and *P. saccharata* biomass samples from Moscow Rural area. **Acceptor plants**: T: *Trifoloium pratense*, F: *Festuca arundinacea*, P: *Prunella vulgaris*. ***P. saccharata* biomass Mulch rates (mg per pot)**: 200: (leaves 150 mg + rhizomes 50 mg), 500: (leaves 350 mg + rhizomes 150 mg), 500+: (leaves 350 mg + rhizomes 150 mg) + activated carbon 500 mg (mixed with soil) per pot. Data marked with different letters were significantly different under *p* level as 0.05 (*t*-test) in the same column. Bars denote ± standard error.

Presumably, the activation effect of allelochemicals on germination speed and growth at the initial stage, disrupts the assimilation and caused the water balance malfunction. The effects of *P. saccharata* allelochemicals should be associated with low concentrations, stimulation of germination, accelerated growth of seedlings at early stages of growth and their subsequent metabolic disorders (metabolic failure). The evidence showed two physiological strategies (hormone-like and inhibitory) of *P. saccharata* allelochemicals in decreasing the growth of acceptor plants. The results obtained so far showed the synergistic interactions of allelopathic agents (11,19,22). The question is,

whether it is one substance, two or more different substances, or isomers of the same substance. All species of acceptor plants were susceptible to allelochemicals. The active substances were found not only in the tissues of donor plant, but also in the under-canopy soil. Environmental stress affected the allelopathic activity of *P. saccharata*, however, it was at low concentrations and adversely affected the acceptor plants. Gradient bioassay allows us to detect the physiologically active concentrations of allelochemicals and peculiarities of their transformation during dissolution and dilution The future research needs to identify the allelochemicals and mechanism of their transfer into the soil, to determine the seasonal dynamics of quantitative content of allelochemicals in the soil, these could be very useful, as allelochemicals of *Pulmonaria saccharata* may be used as herbicides.

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