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The phenols content and phytotoxic capacity of various invasive plants

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Abstract

The phytotoxicity testing was intended to demonstrate specific traits of invasive plants, namely their ability to remove native species. For this purpose, we determined the effect of extracts obtained from Amaranthus retroflexus L., Helianthus tuberosus L. and Ailanthus altissima Mill. Swingle on root elongation and the mitotic film cell by Triticum assay. The method consists in determining the maximum dilution of the extracts studied which, depending on the duration of action, influence root elongation and the caryokinetic film. Microscopic examination allows determining the changes induced in the mitotic film by the extracts studied on radicular tips of Triticum aestivum L. The staining method is based on the affinity of orcein to chromatin in acid environment. The occurrence of significant mitotic abnormalities may be due to the interaction of phenolic compounds with proteins involved in mitosis, to clastogenic effects or due to cell wall degradation leading to the release of genetic material from the cell. The extracts obtained from Ailanthus altissima showed the highest level of phytotoxicity.

Keywords: flavonoids, invasive plants, phenolic compounds, phytotoxicity, *Triticum* assay.

1. Introduction

One of the main hypotheses for the success of the alien species invasion consists in the release of chemicals that have harmful effects on native plant communities (allelopathic substances) [1, 2]. The most important aspect of allelopathic substances consists in the production and release into the environment of different phytotoxic compounds.

Recently, allelopathic interactions were considered useful tools for organic weed management based on natural compounds, but for invasive plants such possibility doesn't exist, because these species also showed the ability to inhibit the growth of agricultural plants of interest [3].

Non-native species show the ability to establish, spread and successfully invade an environment where they have not previously been exposed. Because the invaded environments are new, the alien species should not be adapted to these specific areas, due to a lack of evolutionary history, but a large number of these species dominate new habitats eventually both in terms of numerical abundance and of biomass. Therefore, these allelophatic "invaders" produce chemicals that native species cannot adapt to. These phyto-chemicals may inhibit germination, growth or reproduction of native species and can persist in the soil for many years [4].

The allelophatic compounds resulted from invasive species can have negative impacts on the ecosystem, directly and indirectly, in several ways, such as: changing the natural cycles of nutrients in the invaded area, impairing mycorrhizal fungi, changing the chemical composition of the soil, changing the structure of plant communities, damage to local habitats, reduction of nutrients for native flora, changes in the sequence phytocoenoses, increased incidence of pathogens and the emergence of exotic diseases [5], eventually leading to disappearance of native plants and altering the ecosystem functions.

The main compounds known for allelopathic properties, especially for their phytotoxic activity are phenolic acids, coumarins and flavonoids [6, 7]; these compounds could lead to an increase in the invasiveness character and maybe could be considered responsible for this effect [2]. According Gallet and Pellisier [8], the role of allelopathic activity of phenolic compounds requires an ecosystemic approach, and it was demonstrated that these compounds possess biologic toxicity to other organisms such as microflora and soil animals [9].

In most of the agricultural research experiments, the allelochemical effects were tested on different species of weeds or crops that could be affected by these substances.

Throughout the time, several hypotheses have been advanced on the possible mechanism of action for phenolic allelopathic substances in plant cells [10]: changes in membrane permeability and inhibition of nutrient uptake; inhibition of cell division, root elongation and submicroscopic structure; effects on photosynthesis and respiration; effects on different functions and enzymatic activities; effects on endogenous hormone synthesis and effects on protein synthesis.

Since in Romania a black list of invasive species is not yet available, we intend to study the phytotoxicity character of a few alien species against an agricultural interest, namely *Triticum aestivum* L. to include those plants in the invasive category. These species were included in the black list for neighboring countries [11]. The plants were chosen such that one species to be a type of C-4 species native to the tropical America (*Amaranthus retroflexus*), a C-3 warm-season species of sunflower native to North America (*Helianthus tuberosus*) and a tree native to the northeast and central China and Taiwan (*Ailanthus altissima*, tree-of-heaven) [12, 13, 14]. The propagation strategy of *H. tuberosus* is known as phalanx [15]. While the invasiveness of the tree-of-heaven consists in high production of seeds and rapid seedling development, pollution and drought are favouring factors [13], at *A. retroflexus* it has been noticed that it shows a high plasticity through rapid development and production of many viable seeds [14].

In this knowledge context we decided to test phytotoxicity of invasive plants for prove this specific trait, namely their ability to remove native species. The aim of the paper consists in studying the extract effects on root elongation and the influence on plant cell mitotic film using the *Triticum* bioassay. The proposed objectives consist in obtaining the plant extracts, determining the total phenols and flavonoids content, following up the influence of the extracts obtained on root elongation and examining the changes induced by these in the mitotic film on young root tips of *Triticum aestivum.*

2. Materials and Methods

Plant material. The plant material was harvested from the Romanian spontaneous flora (*A. retroflexus* and *H. tuberosus* were collected from Jiblea Veche, Valcea and *A. altissima* from Botanical Garden, Bucharest) and identified by the Department of Botany staff from the Faculty of Biology, University of Bucharest. The vouchers were deposited in the herbarium of the Botanical Garden "Dimitrie Brandza": *Amaranthus retroflexus* (400637), *Helianthus tuberosus* (400639), and *Ailanthus altissima* (400634). The plant material was manually sorted and dried at room temperature.

Alcoholic extract. The extractions were performed without heating with ethanol 70% (v/v) using an ultrasonic bath (Elma Sonic 80H), with frequencies ranging from 20 kHz to 2000 kHz, making possible the extraction of active compounds by increasing the permeability of cell walls and causing cell lysis [16]. The extract thus obtained was filtered. The residual plant material was extracted for three times and brought to 100 mL with 70% ethanol. The extracts were stored in tightly closed brown bottles at 4°C.

Determination of total phenols content (TP). TP was done by the Folin-Ciocalteu method [17]. The total phenolic content was assayed by homogenizing 0.5 mL sample or standard (gallic acid) in 5 mL Folin-Ciocalteu reagent and adding 4 mL of 1 M sodium carbonate. Absorbance was measured after 15 minutes compared with a blank sample (containing ethanol 70% v / v, instead of the sample) in 1 cm glass cuvettes. The absorbance of samples (standard / test) was determined at 746 nm with a Shimadzu UV-1240 Spectrophotometer (Japan). A calibration curve was plotted with standard solutions of gallic acid with concentrations varying between 5 and 150 mg / L. TP content was expressed as mg of gallic acid equivalents (GAE) per gram of plant.

Determination of total flavonoids. The total flavonoids content of the alcoholic extracts was determined by the aluminium chloride method [18] with some modifications. Briefly, to 10 mL solution of alcoholic extract (or standard of different concentrations) were added 10 mL of 10% sodium acetate, stirred and filtered. At the filtered solution (5 mL) were added 3 mL 2.5% $AlCl_3$ solution and filled with ethanol in a 25 mL volumetric flask. After stirring and incubation at room temperature for 45 min, the absorbance of the reaction mixture was measured at 430 nm with a Shimadzu UV-1240 Spectrophotometer (Japan), in a 1 cm cuvette. The amount of aluminum chloride from samples was substituted by the same amount of ethanol in blank. Similarly, different volumes of standard solution (quercetin) were reacted with 10% sodium acetate and 2.5% aluminum chloride for determination of flavonoid content as described above. The flavonoids content was expressed in mg quercetin (QE) / g of plant and it was calculated using the calibration curve obtained for concentrations of quercetin that ranged between 0 and 120 mg / mL.

RP-HPLC analysis of phenolic compounds. Phenolic compounds were evaluated by reversed phase - high performance liquid chromatography (RP-HPLC) with direct injection. Chromatographic analysis was carried out with a Thermo Finnigan Surveyor Plus equipped with a Surveyor Photodiode Array Detector (PDA), Surveyor autosampler, Surveyor LC Pump (Quaternary gradient) and Chrome Quest Chromatography Workstation. Separation was performed at 30°C with an Accuacore PFP (2.6 µm, 100 × 2.1 mm) column. The flow rate was 0.3 mL/min and an injection volume 1 µL. Gradient elution of two solvents was used: solvent A consisted of water with 0.1% formic acid and solvent B: acetonitrile with 0.1% formic acid. The gradient programme used is given in Table 1. The detection was made at 280 nm.

Table 1. Solvent gradient conditions with linear gradient

Time (minutes)	A%	B%
Initial	98	2
30	70	30
35	25	75
40	98	2
55	98	2

The plant extracts were injected into the HPLC system after filtering through a 0.45 µm pore size membrane filter. The amounts of phenolic compounds in the extracts were

calculated as mg/L using external calibration curves, which were obtained for each phenolic compound. Each determination was carried out in triplicate and the mean was reported. Blank solution and control samples were analyzed in order to monitor performance related to variable factors or random error.

All standards (gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, caffeic acid, chlorogenic acid, (+)-catechin, (−)-epicatechin, syringic acid, p-coumaric acid, ferulic acid, ellagic acid, resveratrol, rutin and quercetin) were purchased from Sigma-Aldrich (Steinheim, Germany). Stock solutions of all the standards were prepared in methanol. Working standards were made by diluting the stock solutions in a mixture of methanol: water (50: 50, v/v). The formic acid, acetonitrile and methanol were LC grade and were obtained from Merck. Twice distilled and demineralised water produced by a Milli-Q Millipore system (Bedford, MA, USA) was used for preparation of the aqueous solutions.

Macroscopic determination of the phytotoxic character. The plant extracts were evaporated to dryness and the residue was resuspended with the same volume of distilled water. Wheat kernels selected to be homogeneous were soaked in distilled water for 24 hours and then placed in Petri dishes with a diameter of 15-20 cm for germination, on a substrate of filter paper and cotton moistened with water and left at 25°C for 24 hours. When the main root length reaches 10 mm, 10 of the caryopses germinated are introduced in a Petri dish with a diameter of 10 cm, containing 15 mL aqueous plant extract and stored at room temperature. The influence of aqueous extracts on root elongation involves measuring linearly the main root length of *Triticum aestivum* from baseline, every 24 h over 5 days; this period is actually the most active phase of development. The results were compared to a control containing distilled water.

Statistical analyses. The statistical analyses were carried out with the computing language and environment, R and several packages developed for R (version 3.0.3). Descriptive statistics parameters were computed using packagedoBy [19]. As the distribution was not normal for several groups, bootstrapping with 2000 bootstrap replicates was applied to generate bootstrap bias-corrected accelerated (BCa) 95% confidence intervals for the mean. Multiple comparisons were performed through *Kruskal-Wallis test*, as a parametric ANOVA was not possible due to the violation of its prerequisites. Comparison of the root length for each concentration level of an extract and the control group was performed for each extract using a *Wilcoxon test* with Holm adjustment for multiple comparisons. Structural validity of ANOVA models and normality were verified through the visual examination of the residuals (qq-plots and histograms); for more confidence in the visual interpretation of these data we also applied the K2 omnibus *d'Agostino test* of normality¸ using the fBasics package (version 3010.86) [20]. The assumption of homoscedasticity was investigated through *Levene's test*, using the package car [21]. For each of the two experiments, the effect of extract (differences between different extracts) was tested through *Kruskal-Wallis* and for each extract the effect of the concentration (differences between concentrations) was tested by means of the same test (*Kruskal-Wallis*). The effect size for *Kruskal-Wallis* was estimated through the epsilon squared parameter, computed by us in R. Post-hoc multiple comparisons were done at an alpha level of 0.5, adjusting the p value through the Holm method [22], as implemented in the R package, *agricolae* [23]. The inhibition index (in percentages) was calculated on the median values according to the following formula: II=100-(T-1)/(C-1) x100, where: II – inhibition index; T – median value of the test group; C – median value of the control group (1 cm is the root length at the starting of experiment and 100 is used to express the result as a percentage). All analyses were based on values measured at 72 hours after treatment.

Microscopic Examination of Mitotic Film Changes. Microscopic examination allowed determining the mitotic film changes induced by the extracts studied on root tips of *Triticum*

aestivum. The determinations were compared to a blank containing distilled water, maintained under similar conditions with the samples. Embryonic roots of *T. aestivum* were sectioned at approximately 5 mm from the top and stained with dilute acetic orcein according to the *La Cour* procedure. The method is based on the affinity of the chromatin for orcein in acid pH, necessary for chromatic material hydrolysis of meristems which turn in red-purple. The samples were examined using a Nikon microscope Labophot 2, using 40x and 100x objectives (the latter with cedar oil immersion).

3. Results and discussion

Determination of total phenols content

The extract obtained from the leaves of *H. tuberosus* showed the highest concentration of phenols (53.29 mg GAE / g dry plant) followed by extracts obtained from *A. altissima* leaves (24.66 mg GAE / g dry plant) and *A. altissima* flowers (17.94 mg GAE / g dry plant) (Table 2). The leaves extract of *A. retroflexus* (7.19 mg GAE / g dry plant) showed a similar content of total phenols compared with literature data [24, 25]. Yuan et al. (26) observed a significant difference ($P < 0.05$) in the total phenolic content of the *H. tuberosus* tuber and leaf extracts (22.40 ± 0.63 and 101.07 ± 1.61 mg GAE/g of dry extract, respectively). There were also considerable differences between these concentrations for the extracts obtained by Yuan et al. (26). These differences could be attributed to the otherness between extraction methods and the different climatic conditions of plant development.

To our knowledge, this is the first report on the total phenols content of the extract obtained from the *A. retroflexus* inflorescences. Comparing with other papers, the total phenolic content of *A. retroflexus* leaves extract has showed some differences, probably given by the phenol used for expression the total phenolic content [27, 28]. A few data about phenolic compounds of *H. tuberosus* leaves were found [29].

Table 2. The concentrations of total phenols and flavonoids content

	The total phenolic content (mg GAE/g dry product)	The total flavonoids content (mg QE/g dry product)	The flavonoids content from total phenolic content (%)	The phenolic compounds identify by HPLC (%)
H. tuberosus leaves	53.29	0.71	1.33	56.66
H. tuberosus tubers	0.34	0.02	6.58	1.61
A. retroflexus leaves	7.19	0.20	2.75	46.93
A. retroflexus flowers	7.56	0.16	2.08	20.69
A. altissima leaves	24.66	1.82	7.39	38.48
A. altissima flowers	17.94	1.11	6.20	27.07

Determination of total flavonoids

The concentrations of flavonoids are presented in Table 2. The species richest in flavonoids was *A. altissima* which contained 1.82 mg QE / g dried herb (leaves) and 1.11 mg QE / g dry plant (flower). The *A. altissima* proved to be the richest in flavonoids representing approximately 7.39% of the total phenols for leaves and 6.2% for the flowers.

Among the species studied the percentage of flavonoids from total phenols is approximately 2.75% for *A. retroflexus* leaves and 2.08% for flowers; *H. tuberosus*: 1.33% leaves, 6.58% tubers (Table 2). The literature mentions a higher concentration of flavonoids than that obtained in our study for leaves [30].

RP-HPLC analysis of phenolic compounds

In Figure 1 are shown the chromatograms of standard substances (a) and of the hydro-alcoholic extract of *A. altissima* flower (b). The concentrations of phenolic compounds of the hydro-alcoholic extracts of the vegetative and reproduction organs of invasive species (mg phenolic compound/ g of plant) are listed in Table 3. Among the identified compounds mostly are phenolic acids known for their allelopathic effect.

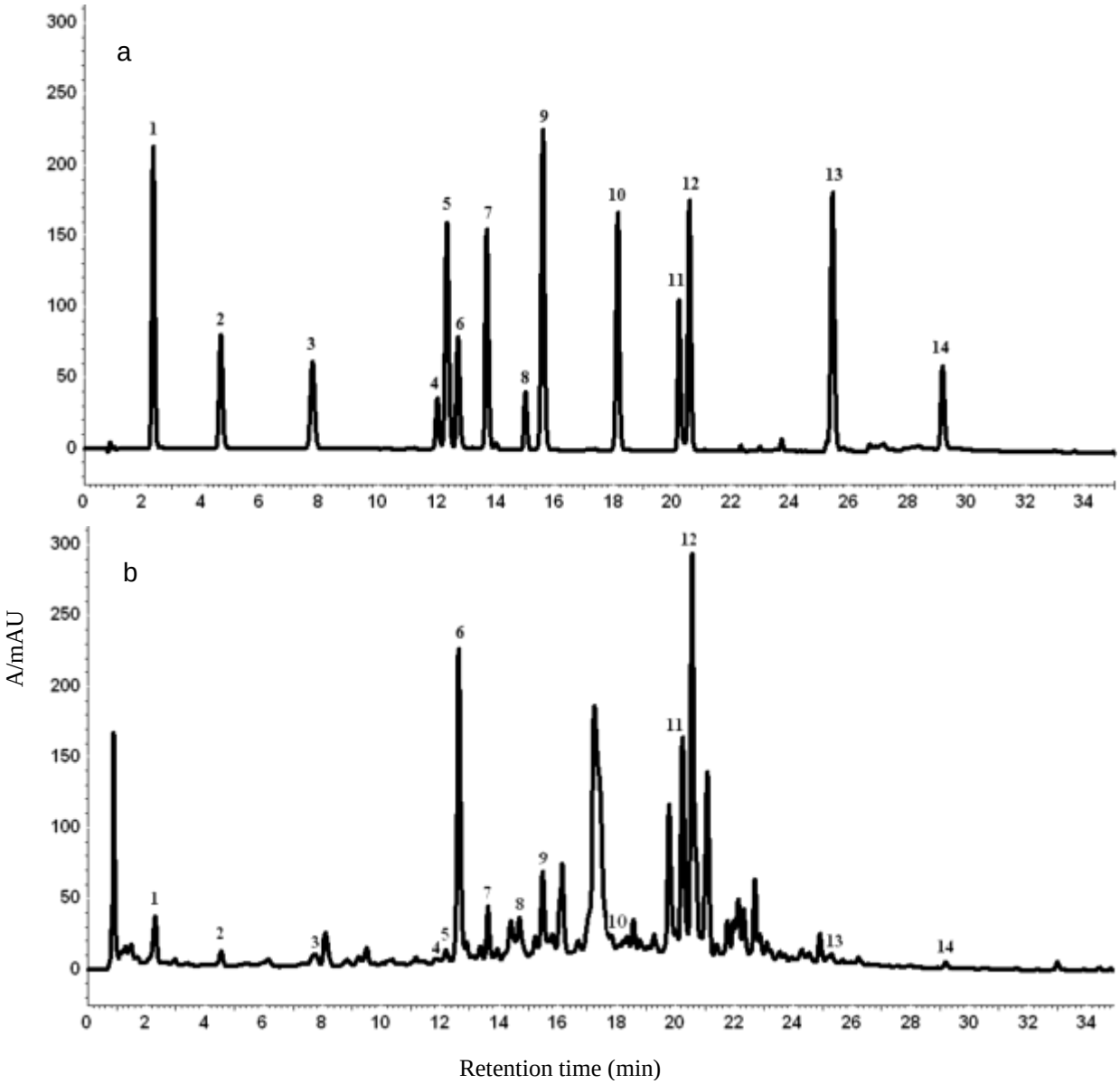

Figure 1. HPLC chromatograms at 280nm: a) standard substances and b) the hydro-alcoholic extract of A. altissima flower. Peak ID numbers correspond to the compounds listed in Table 3.

The main components found in the extract of *H. tuberosus* leaves were: chlorogenic acid (28.07 mg/g leaves) and 4-hydroxybenzoic acid (1.35 mg/g leaves), only first compound was reported previously [12]. From this extract 56.66% of phenols were quantify by HPLC from the total phenols determined by Folin-Ciocalteu method. It is obvious that the concentrations of phenolic compounds of the hydro-alcoholic extracts of tubers were very low. The extracts of *A. retroflexus* are rich in (-) – epicatechin (2.56 mg/g leaves and 1.09

mg/g flower). The following substances having high quantities in extracts of *A. altissima* were evidenced: ellagic acid (2.75 mg/g leaves and 1.08 mg/g flower), 4-hydroxybenzoic acid (2.27 mg/g leaves and 0.11 mg/g flower), chlorogenic acid (1.39 mg/g leaves and 1.96 mg/g flower), and p-coumaric acid (1.20 mg/g leaves and 0.13 mg/g flower).

In the case of alcoholic extract of *A. altissima* leaves, vanillic, caffeic, p-coumaric, syringic, chlorogenic and ellagic acids were reported, but the gallic acid and quercetin have not been previously identified [30].

Table 3. The phenolic compounds concentrations of the hydro-alcoholic extracts of the vegetative and reproduction organs of invasive species (mg phenolic compound/ g of plant)

Standards	*H. tuberosus* leaves	*H. tuberosus* tubers	*A. retroflexus* leaves	*A. retroflexus* flowers	*A. altissima* leaves	*A. altissima* flowers
Gallic acid	nd	nd	nd	nd	0.69	0.15
3,4-Dihydroxybenzoic acid	0.06	nd	0.01	0.02	0.12	0.11
4-Hydroxybenzoic acid	1.35	nd	0.03	0.01	2.27	0.11
(+) - Catechin	0.18	nd	0.15	0.03	0.03	0.06
Caffeic acid	nd	nd	0.02	0.02	0.05	0.03
Chlorogenic acid	28.07	0.01	0.01	0.03	1.39	1.96
Syringic acid	0.04	nd	0.02	0.02	0.04	0.16
(-) – Epicatechin	0.08	nd	2.56	1.09	0.25	0.14
p-Coumaric acid	0.06	nd	0.01	0.01	1.20	0.13
Ferulic acid	0.03	nd	0.03	0.01	0.02	0.01
Ellagic acid	0.02	nd	0.02	nd	2.75	1.08
Rutin	0.01	nd	0.50	0.29	0.59	0.86
Resveratrol	0.09	nd	0.01	0.03	0.01	0.01
Quercetin	0.20	nd	nd	nd	0.08	0.04

Macroscopic determination of the phytotoxic character

In order to assess the phytotoxic potential of phenolic compounds present in the invasive species studied, the effect of extracts obtained from various reproductive and vegetative organs on *T. aestivum* seed germination were examinated. Macroscopic changes in the degree of development of the embryonic root after 72 h depending on the type and concentration of the solution tested are synthetically shown in Table 4.

Among the extracts tested it can be seen that those obtained from the vegetative organs are highly phytotoxic demonstrating one of the specific features of invasive plants, namely their ability to remove native species or agricultural interest.

For the investigation of the substance effect, two-way or one-way analysis of variance (ANOVA) for both extract and concentration could not be used, because the *Levene test* showed that the homogeneity of variance was not satisfied ($p<0.01$ for various groups tested), although normality was confirmed for several comparisons. Descriptive statistics for the data collected are shown in Table 4. 95% confidence intervals were obtained for the mean by bootstrapping with 2000 replications by the bias-corrected and accelerated method, a statistical procedure that allows estimation of confidence intervals without specific assumptions about the sample distribution [31].

There is a statistically significant difference in the inhibitory effect on the root growth for the extracts assessed in the first experiment (*Amaranthus* leaves, *Helianthus tuberosus* tubera) and the control group ($H=24.50$, $df=2$, $p<0.0001$, $n=52$, KW). The effect size for the extract, as expressed by epsilon squared was 0.4803, which means that about half of the variability seen in the root length in this experiment is related to the extract tested. The post-

hoc analysis with Holm adjustment for multiple comparisons has indicated that all extracts have an inhibitory effect on the root growth in comparison with the control group (p=0.0023 for *Amaranthus* leaves and p<0.0001 for *Helianthus* tubera). Post-hoc comparison also showed that the two extracts differed significantly among themselves (p=0.0003).

Table 4. Descriptive statistics for the main root measurements

Sample	Concentration (mg dry plant/100 mL distilled water)	Root length mean ± SD* (mm)	95% confidence interval for the mean (BCA)	Median	p (*Wilcoxon-test* versus control, adjustment by Holm method)	Inhibition index (%)
***Amaranthus retroflexus* leaves**	1.66	34.86±12.86	24.00-47.00	28.0	<0.0001	70.00
	0.84	41.71±2.43	39.00-43.20	43.0	0.0001	45.00
	0.17	72.71±15.23	59.40-85.44	74.0	1.0000	-6.67
***Helianthus tuberosus* tubera**	5.17	12.71±2.63	10.20-14.60	13.0	<0.0001	95.00
	2.59	24.86±3.93	22.00-28.80	24.0	<0.0001	74.67
	0.52	49.00±13.65	35.40-59.00	55.0	<0.0001	25.00
Control	N/A	72.4±10.70	66.00-79.00	70.0	N/A	N/A
***Amaranthus retroflexus* flowers**	1.67	13.8±2.30	12.17-14.83	13.0	<0.0001	94.54
	0.83	40.4±19.44	25.57-54.67	43.0	0.0272	40.00
	0.06	75.9±19.54	62.83-90.48	79.0	0.1500	-25.45
***Ailanthus altissima* leaves**	3.47	10.00±0.00	N/A	10.0	<0.0001	100.00
	1.74	10.70±1.89	N/A	10.0	<0.0001	100.00
	0.23	59.40±13.61	50.33-67.83	61.0	0.5696	7.27
Control	N/A	61.29±18.09	43.60-76.20	65.0	N/A	N/A
***Helianthus tuberosus* leaves**	1.86	30.29	24.14 - 39.92	30	<0.0001	76.19
	0.93	71.86	61.58 - 76.86	72	<0.0001	26.19
	0.13	88.57	73.14 - 96.67	92	0,1409	2.38
***Ailanthus altissima* flowers**	3.26	10.14	10.00 - 10.57	10	<0.0001	100
	1.63	11.57	10.86 - 12.57	11	<0.0001	98.81
	0.23	36.57	33.00 - 39.57	36	<0.0001	69.05
Control (distilled water)	N/A	94.5	87.33 - 101.17	94	N/A	N/A

N/A - All values were equal to 10.00, therefore a confidence interval was impossible to compute.
SD- standard deviation

The effect is also statistically different globally between the extracts assessed in the second experiment (*Amaranthus* flowers, *Ailanthus altissima* leaves) and the control group (H=15.5786, df=3, n=67, p=0.0004, *Kruskal-Wallis*). For this group, the effect size for the extract variable, as expressed by epsilon squared was 0.2360, which means that only about a quarter of the variability seen in this experiment was attributable to the extract. The extract obtained from *Amaranthus* flowers hadn't statistically significant inhibitory effect on root growth. The absence of a statistically significant difference for the extract prepared from *Amaranthus* flowers is related to the difference of effects seen with the three different concentrations: at the highest concentration an almost complete inhibition was seen, at the lower concentration a moderate inhibitory effect was observed, while at the lowest concentration a stimulatory effect on root growth was recorded. Therefore, globally there is no difference from the control group, but to understand correctly the effects of this extract it is necessary to look into the specific effects of each concentration level.

A statistically significant difference was seen in the inhibitory effect on the root growth of the extracts assessed in the third experiment (*Helianthus tuberosus* leaves, *Ailanthus altissima* flower) and of the control group (H=41.43, df=2, p<0.0001, n=64, KW). The effect size for the extract, as expressed by epsilon squared was 0.6576; in other words,

about two thirds of the variability seen in the root length in this experiment is related to the extracts tested. The post-hoc analysis using *Wilcoxon test* with Holm adjustment for multiple comparisons has indicated that abstraction making of the concentration level, with the exception of the *Helianthus tuberosus* leaves extract, all other extracts have an inhibitory effect on the root growth when compared with the control group. In the case of *Helianthus* leaves extract, the comparisons looking at concentration as an independent factor, show that the non-significant difference between this extract and the control group is driven mainly by the lowest concentration level (0.13%), as at the higher levels (0.93%, 1.86%) the root length is still different between the test and control groups (Table 4).

We also tested the influence of concentration on the root length through *Kruskal-Wallis* and found a significant effect ($H=43.383$, $df=6$, $p<0.0001$, $n=52$ – for the first experiment; $H=53.3918$, $df=6$, $p<0.0001$, $n=67$ – for the second experiment; $H=58.0642$, $df=6$, $p<0.0001$, $n=64$ – for the third experiment). Epsilon squared for the models with concentration as the independent variable was higher than for the models with extract as the independent variable: 0.8506 for the first experiment, 0.8090 for the second and 0.9217 for the third one. This indicates that the effect on root growth is considerably dependent on concentration.

The effect of the concentration for each of the extracts tested, by comparing the root length measured at three different concentration levels was investigated. The *Kruskal-Wallis* test was applied to examine the global influence of the concentration variable, and because in all cases this indicated a significant effect of the concentration, the post-hoc test was used to identify the sub-groups for which the differences were significant.

For *Amaranthus* leaves, there are significant differences between the effects seen with the lowest concentration and the higher ones (0.84%, 1.66%), but not between the latter ones. In the case of *Helianthus tuberosus* tubers extract, the differences in the effects on root growth were statistically significant for all three concentrations investigated.

For the *Ailanthus altissima* leaves extracts, the differences were significant between the lowest concentration and the higher ones, but not between the latter. In the case of the *Ailanthus altissima* flower extract was observed that $p<0.0001$ for the lowest against higher levels and 0.0006 for the comparison of higher levels among themselves.

In the case of *Helianthus tuberosus* leaves extract, the largest difference was recorded between the lowest and the highest concentration ($p<0.0001$), while the lower and higher values have smaller differences among themselves, but still statistically highly significant ($p=0.0025$ for 0.13% versus 0.93% and $p=0.0014$ for 0.93% versus 1.86%). The phenolic compounds showed synergistic effect of phytotoxicity on seed germination capacity of *T. aestivum*. Thus, it was observed that the aqueous extracts obtained from reproductive organs are highly phytotoxic, inhibiting totally the root elongation.

The following compounds were previous tested individually for their allelophatic effect: chlorogenic, gallic, syringic, vanillic, p-hydroxybenzoic, protocatechuic, caffeic, p-coumaric acids and catechin, quercetin, rutin and 3,4 -dihydroxybenzaldehyde and they inhibited the growth of root elongation, which was a concentration-dependent effect [32, 33, 7]. The allelophatic properties were confirmed for most of the phenolic compounds [34, 10]. Some of the compounds listed above were also identified in our extracts (Table 3).

The extracts of *A. altissima* leaves and flowers had the highest concentrations in hydroxybenzoic acids. The same samples have also had the highest values of the inhibition index (100%) on root growth which is in line with the results of Reigosa and Pazos-Malvido [7]. They have demonstrated that the hydroxybenzoic acids (gallic and syringic acids) have a

stronger effect on root growth inhibition than the hydroxycinnamic acids (ferulic and p-coumaric acids). There have tried to give some explanation about the mechanism action and one hypothesis was that oxidative damages are lower in plants treated with hydroxycinnamic acids because they have higher antioxidative effect. A possible explanation could also be that hydroxybenzoic acids have better interactions with cellular enzymes [35].

Among the phenolic compounds identified in the studied extracts, catechin and epicatechin (identified in almost all extracts studied) could be also important allelochemicals. These compounds may affect: soil biota, soil chemistry, the impact on nitrification effect on specific fungal cell wall for soil strains, biogeographical differences in the sensitivity of native and alien species, abiotic factors (light) [36].

Microscopic Examination of Mitotic Film Changes

For a better understanding of the extract effects on plant cells, a microscopic examination was carried out, which allowed the determination of the changes induced in the mitotic film in the cells of the embryonic root tips of *Triticum aestivum*. All determinations were made in comparison with blank containing distilled water maintained under similar conditions with the samples.

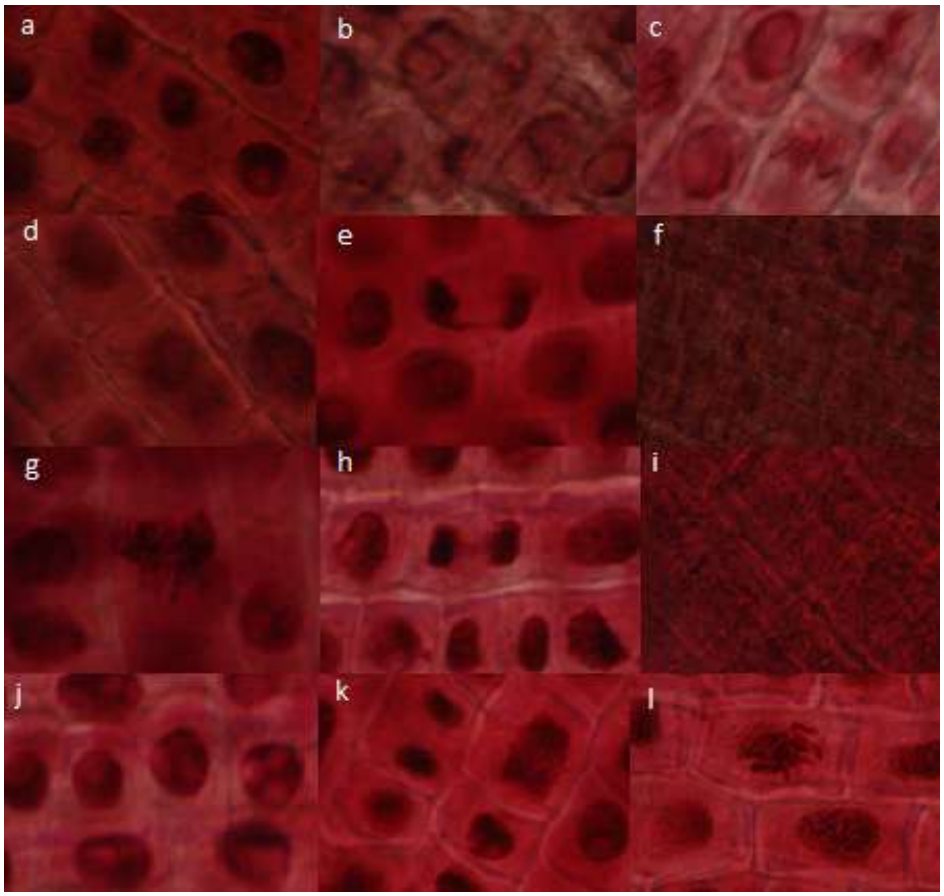

Figure 2. Microscopic images of changes induced by different concentrations of the studied extracts a. Kinesis inhibition. Nucleus with hypertrophy nucleolus. Early prophase; b. Nucleus with hypertrophy nucleolus, prophases, cytotoxicity; c. Prophases, normal metaphases; d. Prophase, corrugated wall; e. Telophase in deck, normal prophase; f. Cytotoxic aspect, nuclear material dispersed; g. Prophase, normal metaphase; h. Telophase with retarded chromosomes; i. Cytotoxicity aspect; j. Kinesis inhibition. Nucleus with 2-3 hypertrophy nucleolus. Early prophase; k. Anaphase with bridges, normal telophase; l. Disorganized metaphase, normal prophase.

Primarily for the interpretations of changes induced by tested solutions on mitotic (Figure 2) were observed the normal mitosis phases of the control: interphase, prophase, metaphase, anaphase and telophase.

In addition to affecting the root elongation, the allelochemicals can induce the occurrence of abnormal seedlings with stunted roots [37, 38], as observed in this study. The most phytotoxic extract was *A. altissima* flowers extract which proved to contain approximately 6.2% flavonoids from total phenols, followed by *A. altissima* leaves (7.4%). The main caryokinetic changes produced were kinesis inhibition, dehydration aspect and nucleus with 2, 3 hypertrophied nucleoli for *A. altissima* flowers extract; all stages of cell division were observed, the fragmoplast was obvious, presences of normal and tropokinesis telophase were recorded for *A. retroflexus* flowers extract; for *H. tuberosus tubers* extract no division phases were observed and the interphase nuclei had strongly hypertrophyed nucleoli (Figure 2). For vegetative organs extracts all the stages of cell division were seen, with smaller number of abnormalities, corresponding to the lower rate of *T. aestivum* root elongation in comparison with the control group.

Changes in the germination and development patterns can lead to alterations in the membrane permeability, the transcription and translation of DNA in the formation of secondary messengers, breathing in the conformation of enzymes and receptors, and of course, cell division [39].

4. Conclusion

This study demonstrated one of the main features of invasive plants and it is notice that the extracts studied alter powerfully the cell division, inducing significant mitotic abnormalities such as telophases with chromosome bridges, hypertrophied nucleoli, deformed cell walls and nuclear contents outside the cell and totally dispersed. The occurrence of these abnormalities may be related to phenol compounds interacting with proteins involved in mitosis [40, 41], clastogenic effects (by formation of bridges or the nuclear material totally dispersed) [42] or due to cell wall degradation leading to the release of the genetic material from the cell. To our knowledge, this is the first paper describing the changes induced by the aqueous extracts obtained from *A. retroflexus, H. tuberosus and A. altissima* on the mitotic film, in the cells of the embryonic root tips of *Triticum aestivum*. These results demonstrated one of the main features of invasive plants: their phytotoxic potential against agricultural interest species.

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References

1. A. CSISZAR, Allelopathic effects of invasive woody plant species in Hungary. *Acta. Silv. Lign. Hung*., 5: 9-17 (2009).
2. R.M. CALLAWAY, W.M. RIDENOUR, Novel weapons: invasive success and the evolution of increased competitive ability. *Front. Ecol. Environ*. 2(8): 436- 443 (2004).
3. D. SOLTYS, R. BOGATEK, A. GNIAZDOWSKA, Phytotoxic effects of cyanamide on seed germination and seedling growth of weed and crop species. *Acta. Biol. Cracov. Ser. Bot*. 54(2): 87- 92 (2012).

4. A.L. SMITH, A.R. DYER Exploring the mechanisms of allelopathic interaction in the invasive annual plant, *Phyllanthus urinaria*. *JSC Acad Sci*. 11(2): 13- 16 (2013).
5. C. SÎRBU, A. OPREA, P. ELIAS, P. FERUS, New contribution to the study of alien flora in Romania. *J. Plant. Develop.* 18: 121- 134 (2011).
6. G.F. XU, F.D. ZHANG, T.L. LI, D. WU, Y.H. ZHANG, Induced effects of exogenous phenolic acids on allelopathy of a wild rice accession (*Oryza longistaminata*, S37). *Rice Sci*. 17(2): 135- 140 (2010).
7. M.J. REIGOSA, E. PAZOS-MALVIDO, Phytotoxic effects of 21 plant secondary metabolites on *Arabidopsis thaliana* germination and root growth, *J. Chem. Ecol*., 33(7): 1456-1466 (2007).
8. C. GALLET, F. PELLISSIER, Phenolic compounds in natural solutions of a coniferous forest. *J. Chem. Ecol.* 23(10): 2401-2412 (1997).
9. M. KRUSE, M. STRANDBERG, B. STRANDBERG (2000) Ecological effects of allelopathic plants – a review, *National Environmental Research Institute, Silkeborg, Denmark. – NERI Technical Report*, pp 35-40.
10. Z.H. LI, Q. WANG, X. RUAN, C.D. PAN, D.A. JIANG, Phenolics and plant allelopathy. *Molecules*. 15: 8933-8952 (2010).
11. P. PYSEK, P.W. LAMBDON, M. ARIANOUTSOU, I. KÜHN, J. PINO, M. WINTER (2009).Alien vascular plants of Europe. In: J.A. Drake (ed) Handbook of Alien Species in Europe. Invading Nature - Springer Series in Invasion Ecology, *Springer-Verlag GmbH, Heidelberg*. pp. 43-61.
12. P.C. SUN, Y. LIU, Y.T. YI, H.J. LI, P. FAN, C.H. XIA, Preliminary enrichment and separation of chlorogenic acid from *Helianthus tuberosus* L. leaves extract by macroporous resins, *Food Chem*. 168:55-62 (2015).
13. L.J. CALL, E.T. NILSEN, Analysis of interactions between the invasive tree-of-heaven (*Ailanthus altissima*) and the native black locust (*Robinia pseudoacacia*). *Plant. Ecol*. 176(2): 275-285 (2005).
14. D. IAMONICO, Biology, life-strategy and invasiveness of *Amaranthus retroflexus* L. (*Amaranthaceae*) in central Italy: preliminary remarks. *Bot. Serb*. 34(2): 137-145 (2010).
15. C.J. SWANTON, D.R. CLEMENTS, M.J. MOORE, P.B. CAVERS The biology of Canadian weeds. 101. *Helianthus tuberosus* L. *Can. J. Plant. Sci*. 72(4): 1367- 1382 (1992).
16. I.C. MARINAS, E. OPREA, E.I. GEANA, C. CHIFIRIUC, V. LAZAR, Antimicrobial and antioxidant activity of the vegetative and reproductive organs of *Robinia pseudoacacia*. *J. Serb. Chem. Soc*. 79(11): 1363 - 1378 (2014).
17. V.L. SINGLETON, J.A. ROSSI, Colorimetry of total phenolics with phosphomolybdic- phosphotungstic acid reagents. *Am. J. Enol. Vitic*. 16(3): 144- 158 (1965).
18. R. WOISKY, A. SALATINO Analysis of propolis: some parameters and procedures for chemical quality control. *J. Apic. Res*. 37: 99-105 (1998).
19. S. HŘJSGAARD, U. HALEKOH, J. ROBISON-COX, K. WRIGHT, A.A. LEIDI doBy: doBy - Groupwise summary statistics, general linear contrasts, population means (least-squares-means), and other utilities. R package version 4.5-9. Website: http://CRAN.R-project.org/package=doBy (2013).
20. D. WUERTZ, P. CHAUSS, R. KING, C. GU, J. GROSS, D. SCOTT, T. LUMLEY, A. ZEILEIS, K. AAS, A. GEBHARDT, fBasics: Rmetrics - Markets and Basic Statistics. R package version 3010.86. Website: http://CRAN.R-project.org/package=fBasic (2013).
21. J. FOX, S. WEISBERG, An R Companion to Applied Regression, Second Edition. Sage Publications. Website: http://socserv.socsci.mcmaster.ca/jfox/Books/Companion (2011).
22. M. AICKIN, H. GENSLER, Adjusting for multiple testing when reporting research results: the Bonferroni vs Holm methods. *Am. J. Public. Health*. 86(5): 726-728 (1996).
23. F. MENDIBURU, Agricolae: Statistical Procedures for Agricultural Research. R package version 1.1-8. Website: http://CRAN.R-project.org/package=agricolae (2014).
24. S. PACIFICO, B. D'ABROSCA, A. GOLINO, C. MASTELLONE, S. PICCOLELLA, A. FIORENTINO, P. MONACO, Antioxidant evaluation of polyhydroxylated nerolidols from redroot pigweed (*Amaranthus retroflexus*) leaves. LWT – *J. Food Sci. Tech*. 41(9): 1665- 1671 (2008).
25. R.Y. YANG, S. LIN, G. KUO, Content and distribution of flavonoids among 91 edible plant species. *Asia Pac. J. Clin. Nutr*. 17(S1): 275- 279 (2008).
26. X. YUAN, M. GAO, H. XIAO, C. TAN, Y. DUA, Free radical scavenging activities and bioactive substances of Jerusalem artichoke (*Helianthus tuberosus* L.) leaves. *Food Chem*. 133(1): 10 – 14 (2012).
27. F. CONFORTI, M. MARRELLI, C. CARMELA, F. MENICHINI, P. VALENTINA, D. UZUNOV, G.A. STATTI, P. DUEZ, F. MENICHINI, Bioactive phytonutrients (omega fatty acids, tocopherols, polyphenols), *in vitro* inhibition of nitric oxide production and free radical scavenging activity of non-cultivated Mediterranean vegetables. *Food Chem*. 129(4): 1413- 1419 (2011).
28. T. OZEN, Antioxidant activity of wild edible plants in the Black Sea region of Turkey. *Grasas Y Aceites*. 61(1): 86- 94 (2010).

29. F. CHEN, X. LONG, Z. LIU, H. SHAO, L. LIU, Analysis of Phenolic Acids of Jerusalem Artichoke (*Helianthus tuberosus* L.) Responding to Salt-Stress by Liquid Chromatography/Tandem Mass Spectrometry. *Sci. World J.* doi:10.1155/2014/568043 (2014).
30. A. LUIS, N. GIL, M.E. AMARA, F. DOMINGUES, A.P. DUARTE, *Ailanthus altissima* (MILLER) SWINGLE: A source of bioactive compounds with antioxidant activity. *BioResources*. 7(2): 2105- 2120 (2012).
31. D.S. FAY, K. GEROW, A biologist's guide to statistical thinking and analysis. WormBook. 9: 1-54 (2013).
32. C.H. CHOU, L.L. LEU, Allelopathic substances and interactions of *Delonix regia* (BOJ) RAF. *J. Chem. Ecol*. 18(12): 2285- 2303 (1992).
33. D.R. BATISH, S. KAUR, H.P. SINGH, R.K. KOHLI, Role of root-mediated interactions in phytotoxic interference of *Ageratum conyzoides* with rice (*Oryza sativa*). *Flora*. 204(5): 388-395 (2009).
34. C.H. CHOU, Y.F. LEE, Allelopathic dominance of *Miscanthus transmorrisonensis* in an alpine grassland community in Taiwan. *J. Chem. Ecol*. 17(11): 2267- 2281(1991).
35. F.M. CAMPOS, J.A. COUTO, T.A. HOGG, Influence of phenolic acids on growth and inactivation of *Oenococcus oeni* and *Lactobacillus hilgardii*. *J. Appl. Microbiol*. 94(2): 167- 174 (2003).
36. E.H. INDERJIT, C. CROCOLL, D. BAJPAI, R. KAUR, Y. FENG, C. SILVA, J.T. CARREÓN, A. VALIENTE-BANUET, J. GERSHENZON, R.M. CALLAWAY, Volatile chemicals from leaf litter are associated with invasiveness of a neotropical weed in Asia. *Ecol*. 92(2): 316 - 324 (2011).
37. G.S. GUSMAN, A.H.C. BITTENCOURT, S. VESTENA, Alelopatia de *Baccharis dracunculifolia* DC. sobre a germinação e desenvolvimento de espécies cultivadas. *Acta. Sci. Biol. Sci*. 30(2): 119-125 (2008).
38. J. BORELLA, A.C.D. WANDSCHEER, L.C. BONATTI, L.H. PASTORINI, Efeito alelopático de extratos aquosos de *Persea americana* Mill. sobre *Lactuca sativa* L. *Rev. Bras. de Biocienc.* 7(3): 260 - 265 (2009).
39. D.M. SILVESTRE, R.M. KOLB, F. FREI, C. dos SANTOS, Phytotoxicity of organic extracts of *Turnera ulmifolia* L. and *Turnera diffusa* Willd. ex Schult. in cucumber seeds. *Acta Bot. Bras*. 27(3): 476 - 482 (2013).
40. O. AKSOY, T. ERBULUCU, S. ONER, I.B. TEKELI, Phytotoxic and genotoxic effects of water samples taken from the eastern channel of kocaeli on *Vicia faba* and *Zea mays*. *Fresen. Environ. Bull*. 21(7): 1819-1826 (2012).
41. S.K. TRIPATHY, S. BIJAYINEE, I. SAMAD, R.K. DAS, Endosulfan: A potential genotoxicant on *Allium cepa* root tip cells, *J. Agric. Biotech. Sustain. Dev.* 5(2): 29 - 35 (2013).
42. A. JITĂREANU, S. PĂDUREANU, G. TĂTĂRÎNGĂ, C. TUCHILUȘ, U. STĂNESCU, Evaluation of phytotoxic and mutagenic effects of some cinnamic acid derivatives using the *Triticum* test, *Turk. J. Biol*. 37(6): 748 - 756 (2013).