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Phytotoxic activity of *Calia secundiflora* (Ortega) Yakovlev

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ABSTRACT

It was evaluated the phytotoxic activity of *Calia secundiflora* seeds extracts on germination and seedling growth of *Amaranthus hybridus* and lettuce (*Lactuca sativa*). MS). Biotest was done at different concentrations of organic extract. The evaluated characters [germination speed index, germination (%), root length, shoot length, total plant length and abnormal plants] showed that the extract was more phytotoxic than isolated alkaloids. Cytisine was isolated and identified in these extracts and its concentration was 24.48 %. The phytotoxic activity of extract was due to the synergic effects of other alkaloids. Lettuce and *A. hybridus* germination and seedling growth showed different responses to seed extracts. The phytotoxic effects of extracts were probably due to synergic effects of all alkaloids present in it.

Key words: *Calia secundiflora*, cytisine, extract, Fabaceae, phytotoxicity, quinolizidine alkaloids.

INTRODUCTION

Chemicals that from plants or microorganisms influence many organisms in ecosystem and the term allelopathy refers to the activity of such chemicals on plants or microorganism (7). These plant chemicals suppresses the germination and growth of weed species (16,20,24). Inhibitory substances released from the allelopathic plants can control weeds in a sustainable manner and reduces the labour costs (22). There is need to develop cost-effective, efficacious, selective and environmentally safer agrochemicals (herbicides) in agriculture (2). Many allelopathic and medicinal plants controls the weeds and could thus be developed as an alternative for weed management. Allelopathic chemicals alters the plant growth and development through multiple effects on physiological processes (7). The alkaloids are non-toxic to the organisms producing them but are toxic to other organisms. The biotoxicity of alkaloids is selective and depends on their chemical structures and organisms (3). Unlike plant-insect interactions, only a few alkaloids have been studied in allelopathy (7).

Calia secundiflora (Ortega) Yakovlev [Syn. *Sophora secundiflora* (Ortega) Lag. ex DC] (Fabaceae) is a bush plant in America, Africa and Asia. In México, it is found in eastern Sierra Madre and in states of Sonora, Chihuahua, Coahuila, Nuevo León, San Luis Potosí, Tamaulipas, Zacatecas, Querétaro and Hidalgo (21). It is also known as 'skunk

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weed' due to its characteristic quinolizidine alkaloids content, hence, considered a toxic specie. The ecology and quinolizidine alkaloid chemistry of *Calia secundiflora* (Ortega) Yakovlev plants growing at two sites in México has been studied (8). Seventeen species of plants were found in Hidalgo and 21 in Queretaro; *Flourensia resinosa* and patol (*Calia secundiflora*) dominated the vegetation in Hidalgo. The profile of quinolizidine alkaloids in plant samples of Hidalgo were different than those of Queretaro. The seeds of *C. secundiflora* from each site accumulated similar quinolizidine alkaloids, but the alkaloids in the leaves and roots were different. The leaves and roots of plants at Hidalgo accumulated similar alkaloids to seeds, but cytisine and/or *N*-methylcytisine were most abundant. The leaves and roots of plants from Querétaro accumulated lupinine, while other alkaloids were in lower concentration. These differences suggests a chemical defence against herbivores and competing plants (15,27,28). It is assumed that quinolizidine alkaloids are potential compounds of plant-plant interaction (i.e. allelopathy) besides their role in plant-herbivore interrelations (31). The extracts and their components of different species have shown the phytotoxic and allelopathic effects (5,6). However, the great diversity of plants on earth justifies the need to investigate other species, which have never been studied especially those, such as *Calia secundiflora.* We have studied it in its growing habitat and have showed diverse biological activities. Pérez-Laínez *et al.* (17) evaluated the activity of organic extract and cytisine on several phythopatogenic fungi (A*lternaria solani, Fusarium oxysporium* and *Monilia fructicola*) and bacteria (*Pseudomonas* sp, *Xanthomonas campestris* and *Erwinia carotova*). The crude extract of *Calia secundiflora* was moderately active against bacteria and more potent on phythopatogenic fungi, in contrast cytisine showed the opposite effects. Despite its relatively wide distribution, there are no ecological or phytochemical studies on *Calia secundiflora*.

MATERIALS AND METHODS

Plant material was collected randomly from the Cardonal-Santuario and Cardonal counties (20° 36' N and a 99° 07' W, altitude 2130 m) state of Hidalgo. A crude alkaloid extract was prepared as per Harris and Wilson (9). The mature plants of *C. secundiflora* seeds were ground in Thomas-Wiley mill (Thomas Scientific, Swedesboro, NJ, USA). The powder (1300 g) was defatted with hexane in Soxhlet apparatus for 24-h. After evaporation, it was shaken for 24-h with 5 % trichloroacetic acid solution and filtered. The resulting mixture was basified to pH 10 using a 10 M sodium hydroxide. The final supernatant was extracted with dichloromethane (3 x 100 ml). The organic phase was recovered and dried by evaporating the solvent in a Büchi rotary evaporator at 40-45 °C under reduced pressure. We obtained 6.8 g extract. The crude alkaloid extract (6.8 g) was separated by column chromatography (CC) with silica gel (G 60 Merck 70-230 mesh). A dichloromethane/methanol gradient (100:0 to 89:11 v/v) was used to collect 384 fractions and monitored by thin layer chromatography (25).

The alkaloids were identified by thin layer chromatography (25) and liquid chromatography-mass spectrometry (LC/MS). The analyses were done in reverse phase in a Waters 600 high resolution liquid chromatograph attached to a Finnigan Mat LCQ mass spectrometer. A 250 mm x 4.6 mm C18 Sun Discovery column was used for

chromatography. The flow speed was 1 ml/min, and a 0.1 % (pH = 4.7) ammonium acetate/MeOH/ACN /75:20.5:50:45.5 mixture was used as the mobile phase (corresponding to the programmed times, with the UV detector at 230 nm. Mass spectrometry (electronic impact source at 70 eV; temperature at 180 °C, registered at 0.75 s/scan at the 38-600 m/z interval) was used to confirm and identify the alkaloids, comparing their spectra with those of standard samples and those of the spectra library (29,30).

Bioassays

The phytotoxicity assays were done as per Anaya *et al*. (1). Seven concentrations (0.02, 0.05, 0.1, 0.2, 0.3. 0.4 and 0.5 %) of organic extract and three concentrations (2.63 x 10^{-2}, 3.15 x 10^{-2} and 3.68 x 10^{-2} mol/l) of cytisine were prepared using 1 % methanol as solvent. Water + 1 % methanol were used as control. The germination test was done in 10 cm dia Petri dishes using filter paper Whatman No.1 saturated with 3 ml of each test solutions. Fifty seeds were distributed on the filter paper of each Petri dish; these were covered and placed in a Seed germination chamber for 120-h [25 °C, continuous light at 15.39 µmol s^{-1} m-^{2} and 85 % RH]. Seed viability was determined prior to biotest and selecting those seeds with 95 % viability. Each Petri dish was considered as an experimental unit. The experiment was done in randomized complete block design with four replications. Parameters measured were: Germination speed index (GSI), germination (% G), abnormal plants (% AP, plants showing damage or stunting), shoot length (LAP) and total length (TPL) [root + shoot lengths]. The GSI was calculated as under:

$$GSI = \sum_{i}^{k} \frac{X_i - X_{i-1}}{X_i}$$

Where, *i :* 1, 2, 3,....k; k =5 counts **,** GSI: Germination speed index , X_i*:* Total number of seeds germinated at i-esim count , X_{i-1}*:* Total number of geminated seeds at i-1 count

Statistical Analysis: The data were analyzed employing the ANOVA and LSD means comparison using Statistical Analysis System (SAS) software, version 8.0, 2002. A correlation test was done on the variables evaluated.

RESULTS AND DISCUSSION

Using the method of Harris and Wilson (9), 7.72 g organic extract (dichloromethane) (0.6 % of original sample) was obtained from 1.3 kg ground *C. secundiflora* seeds. The alkaloid extract was fractioned by preparative column chromatography. A dichloromethane/methanol gradient (100:0 to 89:11 v/v) was used to collect 384 fractions; similar ones were identified using TLC and combined. A brown solid obtained in the fractions 101-144 in dichloromethane-methanol 96:4 v/v was purified and identified as cytisine, the most abundant alkaloid (1.67 g, 0.13 % of original sample). Preliminary identification of alkaloids was done in the extract using thin layer chromatography (25).

The liquid chromatography-mass spectrometry showed the profile of alkaloids identified in seeds extracts of *C. secundiflora* with their relative retention times. Cytisine, lupinine, anagyrine, sparteine, N-methylcitysine, 5,6-dehydrolupanine and lupanine were identified by liquid chromatography-mass spectrometry in seeds extract, the mass spectra of alkaloids showed fragmentation patterns characteristic of the quinolizidine alkaloids (8,15). Cytisine was identified by comparing the standard sample spectra of ^{1}HNMR and Mass spectrometry.

Bioassays

I. Germination (Subsections: Lettuce, *A. hybridus.*

The organic extract inhibited the seed germination, shoot and root length in seedlings of *Lactuca sativa* and *Amaranthus hybridus*. The low concentration (0.02 %) slightly stimulated the germination of *L. sativa* while, the higher concentrations (0.4 y 0.5 %) inhibited the germination and seedling growth of *A. hybridus* (Fig. 1). The inhibitory effects of extracts on root growth were concentration dependent. However the lower concentrations (0.2%) stimulated the shoot length of both test species (Fig. 1).

The cytisine had little effect on the germination of *A. hybridus* and *L. sativa*. The seeds of *L. sativa* were highly sensitive to cytisine. The cystine concentration (2.63×10^{-2} mol/l) caused 2.1 times inhibition in germination in *A. hybridus*, but at higher concentration (3.68×10^{-2} mol/l) caused 3.5 times inhibition in germination over the control. The cystine stimulated the shoot growth of *A. hybridus* at lower concentration but caused inhibition at higher concentration. The inhibitory effects of cytisine on radicle length of *A. hybridus* were concentration dependent (Fig. 2).

Except the shoot length, the other variables were more affected in *L. sativa* than in *A. hybridus* (Table 1). The effects of cytisine content in extracts varied between the species. The extracts decreased the germination in both species, but cytisine inhibited the length of the shoot length and total plant length in both test species. The cytisine increased the development of abnormal plants (primary roots with cracked holes or absent, hypocotyls or coleoptiles short, thick or deformed), these plants could not survive in field. Higher rates of cystine (3.68×10^{-2} mol/l)) greatly effected the root length of *L. sativa* than the shoot length. While these doses of cytisine stimulated the radical growth in *A. hybridus*.

Table 1. Effects of seed extracts of *C. secundiflora* and cytisine on germination and seedling growth of *A. hybridus* and lettuce

Test Species	Solution	GSI [y]	G(%)	Inhibition (%)	AP(%)	RL (cm)	SL (cm)	TL (cm)
A. hybridus	Extract	29.1 a[z]	92.6 b	6.9	38.6 b	3.0 b	1.8 c	4.7 b
A. hybridus	Cytisine	30.3 a	97.4 a	1.1	50.2 a	3.5 a	1.5 d	4.2 c
Lettuce	Extract	28.8 a	85.9 c	12.3	39.2 b	2.5 c	2.6 a	5.2 a
Lettuce	Cytisine	17.7 b	90.8 b	2.9	49.5 a	2.2 d	2.3 b	4.4 bc
LSD		2.0	4.0		0.9	0.1	0.2	0.3

[y] GSI: Germination Speed Index; % G: % of Germination; % AP: % Abnormal Plants; RL: Root Length; SL: Shoot Length; TPL: Total Plant Length. LSD: Least Significant Difference. [z]Values having similar letters within columns are not significantly different at P=0.05.

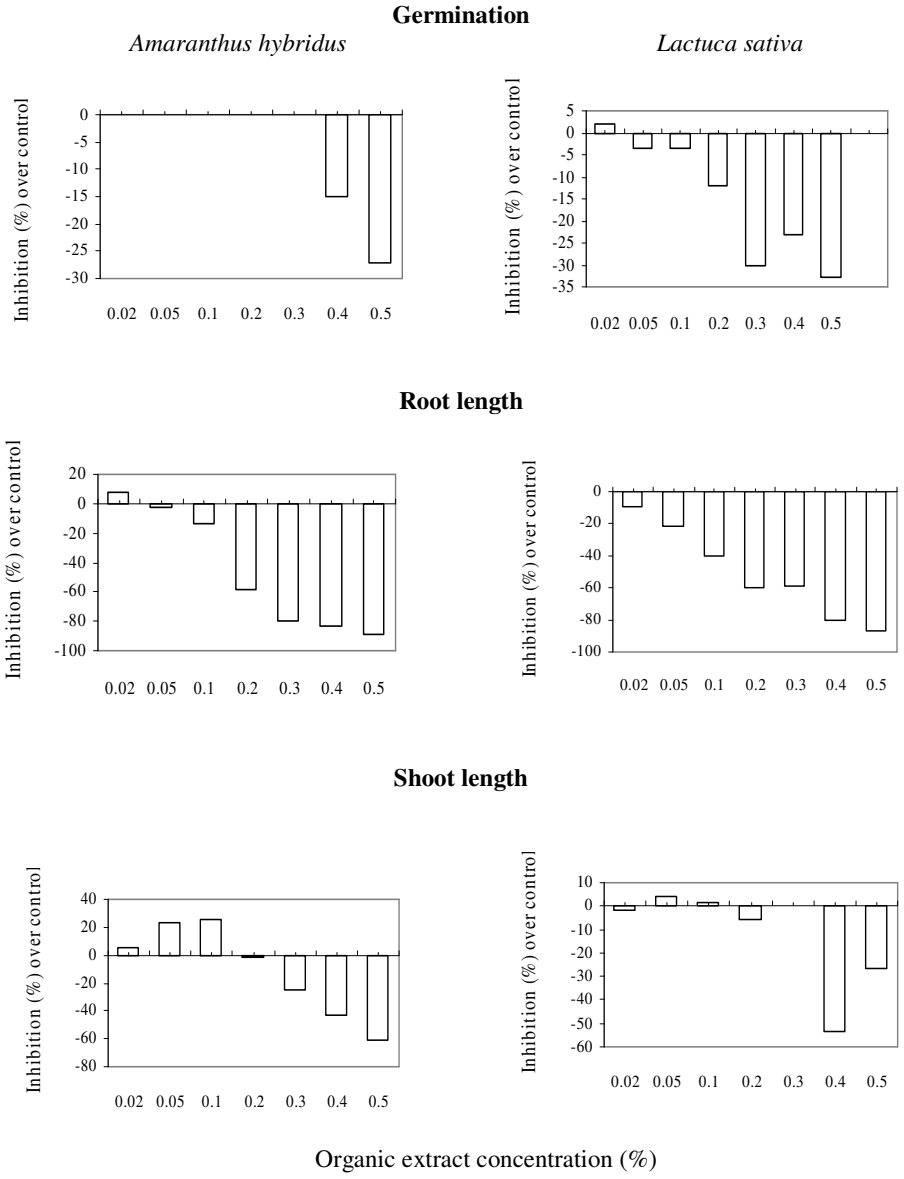

Figure 1. Effects of seed organic extract on germination and seedling growth of test crops. Along Y-Axis the values < 0.0 indicate the inhibition (%) over control and values > 0.0 indicate the stimulation (%) over control.

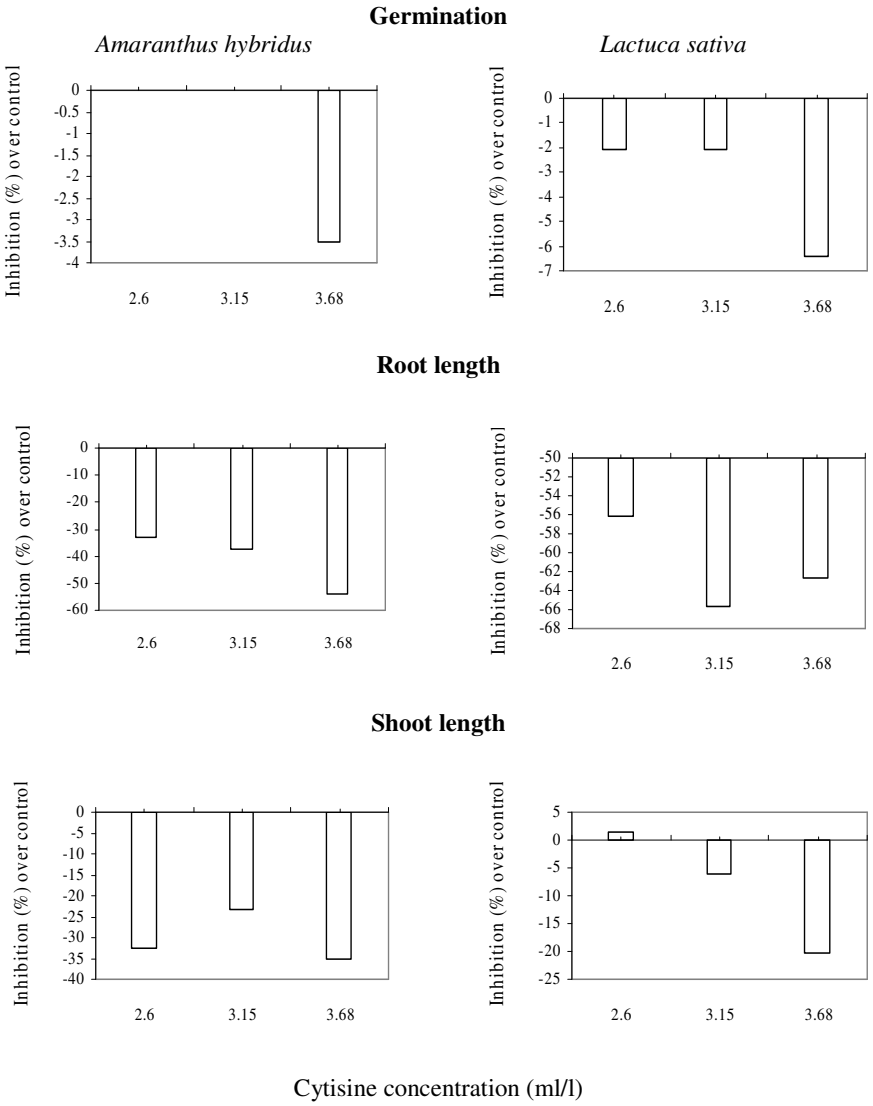

Figure 2. Effects of cytisine application on germination and seedling growth of test spp. Along Y-Axis the values < 0.0 indicate the inhibition (%) over control and values > 0.0 indicate the stimulation (%) over control.

The extract was more toxic to *L. sativa* than to *A. hybridus* (Table 2). The highest concentrations caused more phytotoxicity (0.4 and 0.5 %) in both species. The germination (%) was decreased above 0.3% concentration, while at low concentration of extract (0.1 %), *A. hybridus* germination (%) was higher and abnormal plants population was decreased (Table 2).

Table 2. Effects of organic extract concentrations on germination and seedling growth of *A. hybridus* and *lettuce*

Organic extract Conc (%)	GSI [y]	G(%)	AP(%)	RL (cm)	SL (cm)	TPL (cm)
	----------*A. hybridus*----------					
0	44.5 a[z]	99.5 a	0.0 f	4.93 b	1.93 c	6.86 b
0.02	40.3 b	99.0 a	0.0 f	5.23 a	2.04 c	7.28 a
0.05	39.9 b	98.0 a	1.5 e	4.86 b	2.37 bc	7.23 a
0.1	40.6 b	99.0 a	0.7 e	4.27 c	2.43 b	6.70 b
0.2	18.3 g	97.5 a	6.5 c	2.07 f	1.96 c	4.03 e
0.3	19.9 g	90.5 b	100.0 a	1.04 g	1.44 d	2.49 g
0.4	15.4 h	84.5 c	100.0 a	0.81 g	1.09 e	1.91 gh
0.5	13.4 h	72.5 d	100.0 a	0.57 gh	0.75 f	1.32 h
	---------- *L. sativa* ----------					
0	38.6 b	98.0 a	0.00 f	4.49 c	2.88 ab	7.37 a
0.02	37.3 bc	96.0 a	0.00 f	4.09 cd	2.80 ab	6.90 ab
0.05	26.8 e	94.5 ab	0.50 ef	3.51 d	3.09 a	6.61 b
0.1	29.5 d	94.5 ab	2.75 d	2.68 e	2.96 a	5.64 c
0.2	30.5 d	86.0 c	10.20 b	2.03 f	2.59 b	4.62 d
0.3	24.3 f	76.5 d	100.0 a	1.86 f	2.89 a	4.75 d
0.4	23.4 f	75.5 d	100.0 a	0.90 g	2.24 bc	3.15 f
0.5	19.7 g	66.0 e	100.0 a	0.58 gh	1.59 d	2.18 g
LSD	2.25	4.44	0.95	0.24	0.21	0.34

[y] GSI: Germination Speed Index; G (%): Germination (%), AP(%): Abnormal Plants (%); RL: Root Length; SL: Shoot Length; TPL: Total Plant Length. LSD: Least Significant Difference. [z] Values having similar letters within columns are not significantly different at P=0.05.

The cytisine caused phytotoxic activity at higher concentration of 3.68 x 10^{-2} mol/l in *L. sativa*, but did not inhibit the germination, however, it damaged the seedlings.

A plant is source of alkaloids, if it contains more than 0.05 % on dry weight basis (15). Hegnauer (11) lowered the minimum to 0.01 %. Hatfield *et al.* (10) reported 0.25 % of cytisine isolated of seeds from *Sophora secundiflora.* In this work, the most abundant compound isolated of *C. secundiflora* was cytisine (0.13 %). The presence of alkaloids in *C. secundiflora* explains why the plant is toxic. The alkaloid concentration obtained in the present study was below the minimums listed by us, possibly because half of quinolizidine alkaloids that accumulated in fruit tissues were due to synthesis *in situ* and half was translocated through phloem. These data confirm that the majority of quinolizidine alkaloids are synthesized in shoot tissues (23). García-Mateos *et al.* (8) found that the leaves and roots of plants from Hidalgo accumulated a minor range of alkaloids in the seeds (0.03 %), with cytisine and/or *N*-methylcytisine the most abundant.

Table 3. Influence of cytisine concentrations on germination and seedling growth of *A. hybridus* and *lettuce*

Cytisine Conc. (mol/l)	GSI [y]	G (%)	AP (%)	RL (cm)	SL (cm)	TPL (cm)
	---------- *A. hybridus* ----------					
0	38.675 a[z]	98.5 a	0.0 d	5.048 a	1.875 bc	6.923 a
2.63 x 10^{-2}	30.250 b	98.0 a	4.7 c	3.373 c	1.263 c	4.635 b
3.15 x 10^{-2}	27.550 bc	98.0 a	96.0 b	3.143 d	1.440 c	4.583 b
3.68 x 10^{-2}	24.725 c	95.0 ab	100.0 a	2.315 e	1.215 c	3.530 cd
	---------- *L. sativa* ----------					
0	19.175 d	93.5 ab	0.0 d	4.0 b	2.4 a	6.4 a
2.63 x 10^{-2}	15.6 d	91.0 ab	0.0 d	1.8 f	2.5	4.2 bc
3.15 x 10^{-2}	19.3 d	91.0 ab	98.0 ab	1.4 g	2.3 ab	3.6 cd
3.68 x 10^{-2}	16.6 d	87.5 b	100.0 a	1.5 g	1.9 b	3.4 d
LSD	5.0	9.9	2.1	0.2	0.5	0.8

[y] GSI: Germination Speed Index; G (%) : Germination (%) ; AP (%): Abnormal Plants (%); RL: Root Length; SL: Shoot Length; TPL: Total Plant Length. LSD: Least Significant Difference. [z]Values having similar letters within columns are not significantly different at P=0.05.

The total content of quinolizidine alkaloid varies in *L. exaltatus* depending on the analyzed organ, stage of growth and period of cultivation, but there are no findings on *C. secundiflora* (32). Quinolizidine alkaloid contents in *Lupinus* species ranges from 0.6 to 6 % of dry weight with high contents in flowers and fruits, which are important for reproduction and survival (12). The mature legume fruits have the highest concentrations of alkaloids, as the legumes accumulates alkaloids in fruit (26). In *C. secundiflora,* the alkaloids identified were: cytisine, lupinine, anagyrine, sparteine, N-methylcytisine, 5,6-dehydrolupanine and lupanine. Hatfield *et al.* (10) described a similar profile of seeds from *Sophora secundiflora.* The alkaloids content found in this study was different from other studies (8,14) .

The *Lactuca sativa* and *Amaranthus hybridus* showed different response to phytotoxic activity. *L. sativa* has low germination capacity, lowest index of germination velocity, germination (%) and length of root. In contrast, in *A. hybridus* there was less shoot growth, other variables were less affected to compete and survive (Table 1). Previous studies of phytotoxicity done on seeds of *L. sativa* and *A. hybridus* point out constantly major sensibility of the first species than the amaranth (18,33). The differences in germination observed in lettuce and amaranth, were in agreement with Chou and Yang (4), they determined the germination and root hairs growth. The differences were found not only between the varieties, but also among the species in this research, only one variety of each specie was tested.

The cytisine at higher dose decreased the development of lateral roots in *L. sativa* than the aerial part on the contrary, it stimulated the radicle growth in *A. hybridus* seeds. Robinson (19) reported that the alkaloids can activate or deactivate some enzymes that could explain this effect. Besides allelopathic compounds influence the diverse enzymatic functions and stimulates their enzymatic activity, when the allelopathic compounds are in

low concentrations, but at higher concentration, enzymatic activity is reduced, blocking or lowering many metabolic processes in plant. The alkaloids of quinolizidine type can have inhibitory or stimulatory effects (26) (Figure 1 and Table 1).

II. Seedling growth (Subsections: Lettuce, *A. hybridus*)

The abnormal behavior of *A. hybridus* at low concentrations of extract can be due to higher concentrations of some alkaloids, which inhibited some processes, however the lower concentrations stimulated the development (Figure 1). The difficulty in studying the effects of alkaloids as growth regulators is similar to the problem of variability in alkaloid content in plants. The concentrations of these compounds in plants influence their activity as growth regulators (3). The alkaloids in plants can act as growth regulator and they influence the plant growth, both as stimulators and regulators (3,23). Numerous studies have proved that quinolizidine alkaloids in crude lupine extracts effects both the yields and quality. Foliar application of lupine extract on several crops increase the yield (17-20 % and 15-25 %), because the crude lupine extract (with quinolizidine alkaloids) influenced the balance of nitrogen compounds in plants (3). Protein and amino acid contents were increased. After foliar application of extract, the snap bean (*Phaseolus vulgaris* L.) seed yield increased by 16.4% and the biological value of proteins and essential amino acids increased by 2.87%. because, the alkaloids stimulated the nitrogen metabolism.

There is scarce information about the mechanisms to explain the phytotoxic properties of several kind of alkaloids. Although these metabolites affect many organisms, their interaction in specific sites of plant cells are not known. To understand the phytotoxic effects of alkaloids in plant-plant interactions, they are defensive compounds. All alkaloids occurring in Fabaceae have both biological and ecological significance (3).

According to studied variables, Table 2 showed the interaction of each species with the concentration of applied extract, the extract was more toxic to *L. sativa* than to *A. hybridus.* The lateral roots are an absorption organ, through which enters the applied solution and these are more sensitive to inhibitors which alters the physiological development and this provoked higher amount of abnormal plants.

These results agree with the effect of aqueous extract of *C. secundiflora*, leaves at 0.5 and 1.0 % concentrations on the germination of *L. sativa* and *A. hybridus*, respectively (33). The reduction in growth of lateral roots and shoots in both species indicates that the extract affected the cell division and elongation, as observed with other metabolites (13). These factors (concentration of different alkaloids, synergic action of involved components or polarity of extract) can affect the penetration capacity into the seeds tissue and the dispersion and accumulation in intracellular compartments influences the germination and development (18). It was found that in extract cystine was 24.48 %, hence, the phytotoxic activity of extract is due to the synergic effect of all alkaloids.

The cytisine did not inhibit the germination of more than 50 % seeds of *L. sativa*, but the application of *L. albus* extract containing sparteine, lupanine and cytisine caused 100 % inhibition in seeds germination.

It can be assumed that in phytotoxicity bioassay on *Amaranthus hybridus* and *Lactuca sativa* seeds, the alkaloid extract was more toxic than cytisine, thus inhibited and retarded the seed germination, inhibited the root and shoot growth and resulted in large percentage of abnormal plants. The LC-MS analysis identified 7-quinolizidine alkaloids

(cytisine, lupinine, anagyrine, sparteine, N-methylcytisine, 5,6-dehydrolupanine, and lupanine). The cytisine was major compound in extract.

These results support the assumption that quinolizidine alkaloids have broad spectrum of biotoxic properties and showed the importance of *Calia secundiflora*. The study of organic extracts and natural compounds makes it possible to identify the substances or metabolites, with greater phytotoxic potential, that may serve as a model for the synthesis of new natural herbicides.

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