Allelopathic effect of the *Cladonia verticillaris* lichen extracts and fumarprotocetraric acid on the early growth of germinating seeds and seedlings in *Allium cepa* L.

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Summary: The allelopathic activity of the different extracts of the lichen Cladonia verticillaris and fumarprotocetraric acid on the early growth of A cepa (IPA 6) seedlings depends on their chemical composition and concentration, respectively. It was observed that the length of the radicle was significantly stimulated by fumarprotocetraric acid at high concentrations and by the total extract of C. verticillaris thalli, which contained a high level of fumarprotocetraric, acid confirmed by HPLC-technique. In addition, it was found, that the phosphate buffer extract, which contained high level of methyl β-orcinol carboxilate measured by HPLC, reduced the length of the hypocotyls significantly. Under our experimental conditions there was no influence of the different types of extract and fumarprotocetraric acid on the ratio of seed germination, in relation to the control. From the study of HPLC it was found that fumarprotocetraric acid and methyl β-orcinol carboxilate were present in all extracts at different concentrations, depending on the method of extraction.

Abbreviations: HPLC= high pressure liquid chromatographic, i.d.= inner diamater, UV= ultraviolet , FUM= fumarprotocetraric acid.

Introduction

The lichens, as a group of the symbiotic organisms, composed by the association of algae (generally *Cyanobacteria* and *Chlorophyta*) and fungi (most *Ascomycetes* and few *Basidiomycetes*), produce a range of secondary compounds, most of which specific (*Rundel*, 1978). Among these substances produced by lichens, there are depsides, depsidones, dibenzofurans and usnic acid. Some of these products, possibly, play an important role in the physiology of defense mechanism. The fumarprotocetraric acid is an aldehyde derivative form of depsidones, of the series meta of the type b-orcinol (*Huneck*, 1973), present in the medullae of lichenic thallus (*Huovinen* et al., 1990). Some works refer to this acid as responsible for pedogenesis in rocky sites, where the lichens are present (*Schatz*, 1963 and *Syers*, 1969). Despite of being one the lichenic acids of high solublility (*Iskandar* and *Syers*, 1971), it is leached in the soil in small amounts due to its medullar localization (*Garcia-Junceda* and *Xavier-*

Filho, 1986). Other works emphasize its importance against colds, bronchitis, asthma and gastric disorders (*Huovinen* et al., 1983), as for its ecological significance, *Reutimann* and *Scheidegger* (1987) found that the lichens which produce the fumarprotocetraric acid were repellent to mites on the alimentary diet. Furthemore, *Giez* et al. (1994), showing that fumarprotocetraric acid, when fed to neonate larvae during the first six days of their development, caused a pronounced increase of the larval period and/or a high incidence of developmental malformations, indicating deleterious long term effects. Molisch in 1937 defined allelopathy as the detrimental effects of one plant species (the donor) on the process of germination, growth, or development of another plant species (the recipient) (*Putnam*, 1985). Furthermore, Rice (1974, cit. *Roger* and *Ocaña*, 1992), declares that this effect can be stimulatory or inhibitory, depending on the concentration.

Up to now, few attempts were made to show the allelopathic activity of the lichens (*Pyatt*, 1967; *Brown* and

Mikola, 1974; *Dauriac* and *Rondon*, 1976; *Fisher*, 1979; *Whiton* and *Lawrey*, 1982, 1984 and *Goldner* et al., 1986). However, studies concerning fumarprotocetraric acid and allelopathy are few. The goal of the present work was to study the allelopathic influence of different extracts obtained from *C. verticillaris* and of fumarprotocetraric acid on the germination and early growth of *A. cepa* seedlings.

Materials and methods

Preparations of lichenic extracts

The following extracts were prepared: aqueous, phosphate buffer and total from *C. verticillaris*. Samples of 20g of lichenic thallus dried at room temperature (28 ^{0}C) were pulverised and utilised for the preparation of extracts. The procedures of obtaining the aqueous and buffered extracts were similar, differing only in the solvents utilised, composed, respectively, of distilled water and phosphate buffer 50 mM in pH 7.0. For the total extract, the following solvents were used: ethylic ether → acetone → ethanol: water (8:2 v/v). All solvents were shaked with the lichenic thallus for one hour. The preparation of the total extract was done by cold percolation, utilising the solvents in increasing order of their polarity for a better extraction of the substances present in the thallus.

Germination tests

The following concentrations of fumarprotocetraric acid were used: 72.6 μM, 290.6 μM and 726.7 μM. The concentration of extracts was 127 mg/l, corresponding to 290.6 μM of fumarprotocetraric acid in the total extract, according to the HPLC results. The onion (*Allium cepa* cv. IPA 6) seeds were placed and germinated in Gerbox plates containing filter paper (100 seeds in each Gerbox plate, for each replication), under laboratory conditions (+/- 28^{0}C, diffuse natural light). The evaluations were performed on the sixth and tenth day after the start of the experiment: germination rate, and radicle and hypocotyl length were measured. The experimental design was completely randomised with four replicates.

Isolation and purification of the fumarprotocetraric acid

The fumarprotocetraric acid was isolated from *C. verticillaris* with 20g of the pulverised lichenic thallus by the methodology of *Asahina* and *Shibata* (1954).

HPLC of the lichenic extracts

The chromatographic analyses were performed in a VARIAM 5000 apparatus, under the following conditions: RP-C8 column of 40 x 0.4 cm i.d., mobile phase acetonitril: acetic acid (80:20 v/v): water (98:2 v/v), flux of 0.3 cm/min., 26 ^{0}C of temperature, 84 atm of pressure, and UV detector at 280 nm.

Result

Germination tests

Germination rate – The *C. verticillaris* extracts and fumarprotocetraric acid neither showed inhibitory nor stimulatory effect on the germination rate of *A. cepa*. Despite the percentage values of the phosphate buffer and total extracts, respectively, 95.26% and 94.75%, being above the ones obtained in the control (93.5%), they were not significantly different by the Tukey test (*Table 1*).

Table 1 – Germination rates of *Allium cepa* L. (cv. IPA 6) seeds with fumarprotocetraric acid and *Cladonia verticillaris* extracts.

TREATMENTS	GERMINATION RATE (%)	
1. FUM–290.6 μM	96.0	a
2. FUM–72.6 μM	95.75	a
3. Phosphate buffer	95.25	a b
4. Total extractl	94.75	a b
5. FUM–726.7 μM	93.75	a b
6. Control	93.5	a b
7. Aqueous extract	91.25	b

means followed by the same letter do not differ statistically by the Tukey test (0.05).

Radicle length – The evaluation of radicle length showed growth stimulus in the treatment with fumarprotocetraric acid at 726.7 μM and 290.6 μM concentrations and in the total extract, since they exhibited values significantly higher than the control. The other treatments also showed longer radicles than those found in the control, but not significant, statistically (*Table 2*).

Table 2 – Mean radicle length of *Allium cepa* L. (cv. IPA 6) with fumarprotocetraric acid and *C. verticillaris* extracts.

TREATMENTS	RADICLE LENGTH (cm)	
1. FUM–726.7 μM	3.90	a
2. FUM–290.6 μM	3.41	a b
3. Total extract	2.95	b c
4. Aqueous extract	2.75	b c d
5. FUM–72.6 μM	2.47	c d
6. Phosphate buffer extract	2.13	d
7. Control	2.07	d

means followed by the same letter do not differ statistically by the Tukey test (0.05).

Table 3 – Mean hypocotyl length of *Allium cepa* (cv. IPA 6) with fumarprotocetraric acid and *C. verticillaris* extracts.

TREATMENTS	HYPOCOTYL LENGTH (cm)	
1. FUM–726.7 μM	5.38	a
2. FUM–290.6 μM	5.03	a b
3. Control	5.00	a b
4. Aqueous extract	4.27	b c
5. Total extract	4.19	c
6. FUM–72.6 μM	4.02	c
7. Phosphate buffer extract	3.55	c

means followed by the same letter do not differ statistically by the Tukey test (0.05)

Hypocotyl length – The results obtained in this parameter were not similar to those obtained in the radicle length. The evaluation of hypocotyl length showed inhibition in the treatment with phosphate buffer extract, since it exhibited values significantly lower than the control. On the contrary, however, there is no evident response of stimulating effect of fumarprotocetraric acid. Although the concentrations of 290.6 µM and 726.7 µM have shown higher values than the control, they were not significant, statistically (*Table 3*).

Chemical composition of the extracts

Aqueous extract – The aqueous extract was basically composed of two substances: 58.1% of fumarprotocetraric acid and 41.9% of methyl β-orcinol carboxilate (*Table 4*).

Phosphate buffer extract – In the phosphate buffer extract, the presence of protocetraric acid at very low concentration (0.057%) and of a non identified substance in the retention time of 1.94 min. with approximately 1.09% were recorded. The concentration of methyl β-orcinol carboxilate increases to 67.39% while fumarprotocetraric acid decreases, remaining with 30.28% (*Table 4*).

Total extract – The total extract, the appearance of protocetraric acid (13.29%) again, and the first record of atranorin with 2.18% of the composition, and 5.86% and 78.57% of methyl β-orcinol carboxilate and fumarprotocetraric acid, respectively (*Table 4*).

Table 4 – Data of HPLC from Cladonia verticillaris (Raddi) Fr. extracts.

EXTRACTS	SUBSTANCES	RETENTION TIME (min)	ÁREA (%)
* Aqueous			
	methyl β-orcinol carboxilate	0.93	41.9
	fumarprotocetraric acid	1.47	58.1
* Phosphate buffer			
	methyl β-orcinol carboxilate	0.89	67.39
	fumarprotocetraric acid	1.29	30.28
	not identified	1.94	1.09
	protocetraric acid	2.75	0.057
* Total			
	methyl β-orcinol carboxilate	0.76	5.86
	fumarprotocetraric acid	1.39	78.57
	protocetraric acid	2.87	13.29
	atranorin	5.56	2.18

Discussion

Although several former works with lichenic extracts (*Rondon*, 1966; *Dauriac* and *Rondon*, 1976; *Tolpysheva*, 1984a, 1984b and *Vainshtein* and *Tolpysheva*, 1992), including the phosphate buffer extract (*Vicente*, 1988) have produced biological activity, the absence of stimulatory or inhibitory effect of these extracts on the germination rate corroborate the idea that the chemical composition and the concentration are responsible for the allelopathic effect of the lichens.

In our investigation significant differences were found by HPLC-analyses among the three types of *C. verticillaris* thallus extracts. In the aqueous extract the presence of only

two substances demonstrated clearly, that the fumarprotocetraric acid, although a lichenic acid, show high solubility in water, in agreement with *Iskandar* and *Syers* (1971) and *Ascaso* et al. (1986). In the phosphate buffer extracts, the above mentioned substances were also present, but at different concentrations. In addition, the different chemical substances present in the extracts in small amounts, like protocetraric acid, non identified substances and atranorin, the latter present in the total extract, according to *Geyer* and *Feige* (1987) are biogenetically related. This fact can be evidenced in the total extract where protocetraric acid (13.29%) is likely to influence directly the concentration of fumarprotocetraric acid, since it is produced from its hydrolysis (*Huovinen* et al., 1983), and indirectly, the concentration of methyl β-orcinol carboxilate, since it is the precursor of fumarprotocetraric acid (*Xavier-Filho* et al., 1985). In the total extract, *Asahina* (1943) and *Culberson* (1969) recorded only the presence of fumarprotocetraric acid in *C. verticillaris*. However, our result show the presence of other substances (*Table 4*). Probably, the methodology employed in the extraction was responsible for the presence of other substances. This hypothesis was corroborated by the works of *Xavier-Filho* et al. (1984) showing the presence of orcinol, methyl β-orcinol carboxilate, atranorin and evernic acid, and *Vicente* and *Xavier-Filho* (1979); *Huovinen* et al. (1990) and *Ahti* et al. (1993), who found fumarprotocetraric and protocetraric acid and an other substance also, named Cph 2.

In our investigation, the pure fumarprotocetraric acid at 290.6 µM and 72.6 µM shows the greatest germination rates, though not proved statistically. Comparing the fumarprotocetraric acid concentration in the aqueous extract with the 72.6 µM concentration (*Table 4*), they show similar values, but the responses shown in germination were different. Due to this fact, it can be suggested that methyl b-orcinol carboxilate, with approximately 41.9% of the aqueous extract, has an antagonistic effect to fumarprotocetraric acid. There are works indicating anti-herbivorous (*Reutimann* and *Scheidegger*, 1987) and antineoplasm (*Lima* et al., 1990) activities of the fumarprotocetraric acid. Results obtained in the present work suggest that the reduced cellular proliferation found in animals cells by *Lima* et al. (1990) probably did not occur in this study, since the high means obtained in seedling length are possibly a result of enhanced cell division and/or elongation. As previously discussed, the methyl b-orcinol carboxilate seems to neutralise the effect of fumarprotocetraric acid, as being observed in the treatment with the phosphate buffer extract regarding the length of the hypocotyl. In this extract, methyl b-orcinol carboxilate is in higher concentration, with appoximately 67% of the extract composition and fumarprotocetraric acid with 30%, the lowest concentration of this acid compared to the other extracts. *Caccamese* et al. (1986) attribute to methyl b-orcinol carboxilate an antimicrobial activity. If this activity includes antimitotic activity as previously discussed, the results found in the present work are in accordance with our

expectations. It is emphasized that the phosphate buffer extract showed a significant inhibition on the hypocotyl length (*Table 3*), related probably, to the cell division and/or elongation. Our results can contribute for future studies on the action of these secondary substances, since the stimulatory and/or inhibitory effects obtained can be indirect, mediated by plant hormones, enzymes, protein synthesis and/or through the modification of the membrane permeability.

Acknowledgements

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