

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

Yano-Melo A. M.¹; Vicente, C. ²; Xavier- Filho L.³

¹Dept^o Micologia , C.C.B., Universidade Federal de Pernambuco, Recife, 50670-710, Brazil

²Laboratory of Plant Physiology, Faculty of Biology, Complutense University of Madrid, Spain

³Centro de Ciências Biológicas e da Saúde–Instituto de Tecnologia e Pesquisa – 49015-070 , Aracaju-Sergipe-Brazil

Summary: The allelopathic activity of the different type of *Cladonia verticillaris* lichen extracts and fumarprotocetraric acid on the early growth of *A. cepa* (IPA 6) germinated 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 high level of fumarprotocetraric, acid confirmed by HPLC – technique. In addition, it was found, that the phosphate buffer extract, which contained high level of methy β -orcinol carboxilate measured by HPLC, significantly reduced the length of the hypocotyls. Under our experimental conditions there was no influence of different type of extract and fumarprotocetraric acid on the seed germination ratio of *A. cepa*, in relation to control. From the study of HPLC it was found that fumarprotocetraric acid and methy β -orcinol carboxilate were present in all extracts at different concentrations, according to the method of extraction.

Abbreviations: HPCL = high performance liquid chromatographic, i.d. = inner diameter, UV= ultraviolet, FUM= fumarprotocetraric acid.

Introduction

The lichens, as a group of the symbiotic organisms, composed by the association of seaweed (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 symbiosis, there are depsides, depsidones, dibenzofurans and usnic acid. Some of these products originated from the secondary metabolism of lichens, possibly play an importance role in its physiology, besides defense characteristic. The fumarprotocetraric acid is an aldehyde derivative form of depsidones, of the series meta of the type β -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 being one the lichenic acids more soluble (Iskandar and Syers, 1971), it is leached in the soil in little amount 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), on the ecology field, (Reutimann and Scheidegger 1987) found that the lichens which produce the fumarprotocetraric acid were rejected by mites on the alimentary diet. Furthermore, 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), declare that this effect can be stimulatory, inhibitory on the concentration. Up to now, few works were carried out showing 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, works about fumarprotocetraric acid and allelopathy are few. The goal of the present work was to study the allelopathy influence of different extracts obtained from *C. verticillaris* and of the 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°C) were pulverised and utilised for preparation of extracts. The procedures for obtaining the aqueous and buffered extracts were similar, differing only in the solvents utilised, constituted, respectively, of distilled water and phosphate buffer 50mM in pH 7.0. For the total extract, the following solvents were used: ethylic ether @acetone@ethanol: water (8:2v/v). All solvents remained in shaker 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 the polarity for a better extraction of the substances present in the thallus.

Germination tests

The following concentrations of fumarprotocetraric acid were used: 72.6 mM, 290.6mM and 726.7mM. The concentration of extracts was 127 mg/l, corresponding to 290.6 mM 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°C, diffused natural light). The evolutions were performed on the sixth and tenth day after the start of the experiment, being the following parameters evaluated: germination rate, and radicle and hypocotyl length. The experimental design was completely randomised, with four replications.

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

TREATMENTS	GERMINATION RATE (%)
1. FUM - 290.6mM	96.0 a
2. FUM – 72.6mM	95.75 a
3. Phosphate buffer	95.25 a b
4. Total extractl	94.75 a b
5. FUM – 726.7mM	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).

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 (80:20v/v): water (98:2 v/v), flux of 0.3cm/ min., 26°C of temperature, 84 atm of pressure and UV detector at 280 nm.

Results

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 Turkey test (Table 1).

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

Hypocotyl length – The results obtained in this parameter were not similar to those obtained in the radicle length. The evaluation of hypocotyl lenght showed in the treatment with phosphate buffer extract, since it exhibited value significantly lower than the control. On the contrary, however, there is no evident response of stimulating effect of fumarprotocetraric acid. Although the concentrations of 290.6µM AND 726.7µM have shown higher values than the control, they were not statistically significant (Table 2)

Chemical composition of the extracts

Aqueous extract – The aqueous extract was basically composed of two substances: 58.1% of

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 μ M	3.90	a
2. FUM – 290.6 μ M	3.41	a b
3. Total extract	2.95	b c
4. Aqueous extract	2.75	b c d
5. FUM – 72.6 μ M	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 μ M	5.38	a
2. FUM – 290.6 μ M	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 μ M	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).

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

EXTRACTS	SUBSTANCES	RETENTION TIME (min)	ÁREA (%)
* Aqueous	menthyl β -orcinol carboxilate	0.93	41.9
	fumarprotocetraric acid	1.47	58.1
* Phosphate buffer	menthyl β -orcinol carboxilate	0.89	67.39
	fumarprotocetraric acid	1.29	30.28
	no identified	1.94	1.09
	protocetraric acid	2.75	0.057
* Total	menthyl β -orcinol carboxilate	0.76	5.86
	fumarprotocetraric acid	1.39	78.57
	protocetraric acid	2.87	13.29
	atranorin	5.56	2.18

fumarprotocetraric acid and 41.9% of methyl β -orcinol carboxylate (Table 4).

Phosphate buffer extract – In the phosphate buffer extract it was recorded 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%. In this extract, methyl β -orcinol carboxylate increases its concentration to 67.39% while fumarprotocetraric acid decreases, remaining with 30.28% (Table 4).

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

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 biological response of the lichen.

In our investigation significant differences were found by HPLC-analyses among the three type 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 much 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 carboxylate, since it is the precursor of fumarprotocetraric acid (Xavier-Filho et al., 1985). In the total extract, Asahina (1943) and Culbertson (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) show the presence of the orcinol, methyl β -orcinol carboxylate, atranorin and evernic acid, and (Vicente and Xavier-Filho 1979); Huovinen et al. (1990) and (Ahti et al. 1993), who found fumarprotocetraric acid and other substance also, named Cph 2.

In our investigation, the pure fumarprotocetraric acid at 290.6 μ M and 72.6 μ M shows the greatest germination rates, though not statistically different. Comparing the fumarprotocetraric acid concentration in the aqueous extract with the 72.6 μ M concentration (Table 4), they show near values, but the responses shown in germination were different. Due to this fact, it can be suggested that methyl β -orcinol carboxylate, with approximately 41.9% of the aqueous extract, presents an antagonistic effect to fumarprotocetraric acid. There are works indicating antiherbivorous (Reutimann and Scheidegger, 1987) and antineoplasm (Lima et al., 1990) activities of the fumarprotocetraric acid. Results obtained in the present work suggest that the decrease in cellular proliferation found in animal 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 increase in cell division and/or elongation. As previously discussed, the methyl β -orcinol carboxylate seems to neutralise the effect of fumarprotocetraric acid, what can be observed in the treatment with the phosphate buffer extract regarding the length of the hypocotyl. In this extract, methyl β -orcinol carboxylate is in higher concentration, with approximately 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 β -orcinol carboxylate an antimicrobial activity. If this activity includes antimetabolic activity as previously discussed, the results found in the present work are according to what was expected. It is emphasizing that the phosphate buffer extract showed a significant inhibition on the hypocotyl length (Table 3), probably relates to 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.

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Bibliography

- Ahti, T.; Stenroos, S.; Xavier-Filho, L.** 1993. The lichen family Cladoniaceae in Paraíba, Pernambuco and Sergipe, northeast Brazil. *Tropical Bryology*, 7: 55-70.
- Asahina, Y.** 1943. Chemismus der Cladonien unter besonderer Berücksichtigung der Japanischen Arten (Fortsetzung). *Journal of Japanese Botany*, 49: 227-244.
- Asahina, Y.; Shibata, S.** 1954. Chemistry of lichen substances. Tokyo: Japan Society for the Promotion of Science, 240p.
- Ascaso, C.; Galvan, J.** 1986. Studies on the pedogenetic action of lichen acids. *Pedobiologia*, 16(5): 321-331
- Brown, R.T. & Mikola, P.** 1974. The influence of fruticose soil lichens upon the mycorrhizae and seedling growth of forest trees. *Acta Forestalia Fennica*, 191: 5-23.
- Caccamese, S.; Compagnini, A.; Toscano, R. M.; Cascio, O.** 1986. Methyl β -orcinol carboxylate and atranol from the lichen *Sterocaulon vesuvianum*. *Journal of Natural Products*, 49(6): 1159-1160.
- Culbertson, C.F.** 1969. Chemical and botanical guide to lichen products. University North of Carolina Press: Chapel Hill 628p.
- Dauriac, H.; Rondon, Y.** 1976. Quelques aspects des propriétés d'un lichen, le *Letharia vulpina* (L.) Hue, en biologie végétale expérimentale. I. Action sur la germination du blé et le développement de l'ali. *Bulletin de la Société Botanique de France*, 123: 235-241.
- Fisher, R.F.** 1979. Possible allelopathic effects of reindeer-moss (*Cladonia*) on jack pine and white spruce. *Forest Science*, 25(2), 256-260.
- Garcia-Junceda, E.; Xavier-Filho, L.** 1986. Solubilization of lichen phenolics from *Cladonia sprucei* by simulated rainfall. *Lichen. Physiol. Biochem.*, 01:61-69.
- Geyer, M.; Feige, G. B.** 1987. Analysis of depsides, depsidones, and other lichen specific aromatics by high performance liquid chromatography. *Bibliotheca Lichenologica*, 25: 467-468.
- Giez, I; Lange, O.L.; Proksch, P.** 1994. Growth retarding activity of lichen substances against the polyphagous herbivorous insect *Spodoptera littoralis*. *Biochemical Systematic Ecology*, 22(2): 113-120.
- Goldner, W.R.; Hoffman, F.M.; Medve, R. J.** 1986. Allelopathic effects of *Cladonia cristatella* on ectomycorrhizal fungi common to bituminous strip-mine spoils. *Canadian Journal of Botany*, 64: 1586-1590.
- Huneck, S.** 1973. Nature of lichen substances. In: AHMADJIAN, V.; HALE, M. E. (eds.) **The lichen**. London: Academic Press. 697p.
- Huovinen, K.; Hilten, R. Schantz, M.** 1983. Analysis of lichen acids from extracts of *Cetraria islandica* by high performance liquid chromatography. In: Gesamtkongress der Pharmazeutischen Wissenschaften Die in der Arzneimittelforschung. Abstracts, p. 294.
- Huovinen, K. Ahti, T.; Stenroos, S.** 1990. The composition and contents of aromatic lichen substances in *Cladonia* section *Cladonia* and group *Furcatae*. *Bibliotheca Lichenologica*, 38: 209-241.
- IsKandar, I.K.; Syers, J.K.** 1971. Solubility of lichen compounds in water: pedogenetic implication. *Lichenologist*, 05: 45-50
- Lima, R.C.; Nascimento, S. C.; Pereira, E. C. G.; Campos-Takaki, G. M.** 1990. Atividade citotóxica e antitumoral de extratos líquênicos. *Boletim da Sociedade Broteriana*, 63(2): 339-348.

- Pyatt, F.B.** 1967. The inhibitory influence of *Peltigera canina* on the germination of graminaceous seeds and the subsequent growth of the seedlings. *The Bryologist*, 70(3): 326-329.
- Putnam, A.R.** 1985. Allelopathic research in agriculture: past highlights and potential. In: Thompson, A.C. (ed) *The chemistry of allelopathy biochemical interaction among plants*. Washington: American Chemical Society. Cap.01, 470p.
- Reutimann, P.; Scheidegger, C.** 1987. Importance of lichen secondary products in food choice of two oribatid mites (Acari) in an alpine meadow ecosystem. *Journal of Chemical Ecology*, 13(2): 363-369.
- Roger, M.J.R.; Ocaña, A C.** 1992. La alelopatía y su papel en las comunidades vegetales. Revisión crítica, metodológica y bibliográfica. Santiago de Compostela: Universidade. Serviço de Publicacions e intercambio científico, 109p.
- Rondon, Y.** 1996 Action inhibitrice de l' extrait du lichen *Roccella fucooides* (Dicks) Vain sur la germination. *Bulletim de la Societe Botanique de France*, 113: 01-02
- Rundel, P.W.** 1978. The ecological role of secondary lichen substances. *Biochemical Systematics and Ecology*, 06: 157-170.
- Schatz, A.** 1963. Soil microorganisms and soil chelation. The pedogenic action of lichens and lichen acids. *Agricultural and Food Chemistry*, 11(2): 112-118.
- Syers, J.K.** 1969. Chelating ability of fumarprotocetraric acid and *Parmelia conspersa*. *Plant and Soil*, 31(1): 205-208.
- Tolpysheva, T.Yu.** 1984a Effects of lichen extracts on fungi. I. Effects of aqueous extracts of *Clandina stellaris* and *C. rangiferina* on growing soil fungi. *Mikologia i Fitopatologiya*, 18(4): 287-293.
- Tolpysheva, T.Yu.** 1984b Effects of lichen extracts on fungi. II. Effects of joint preparation obtained from *Clandina stellaris* and *C. rangiferina* on growing soil fungi. *Mikologia i Fitopatologiya*, 18(5): 384-388.
- Vainshtein,; Tolpysheva, T. Yu.** 1992. The effects of lichen extract of *Hypogymia physodes* (L.) Nyl. And pure lichen acids on wood destroying fungi. *Mikologia i Fitopatologiya*, 26(6): 448-455.
- Vicente, C.** 1988. Polissacarídeos carcinostáticos y antibióticos procedentes de líquenes. IN: MEZO, M.ºG. (Coord.) *Curso de investigación de fármacos*. Granada: Universidade de Granada, Instituto de Ciências de la Educación. p. 153-189.
- Vicente, C.; Xavier-Filho, L.** 1979. Urease regulation in *Cladonia verticillaris* (Raddi) Fr. *Phyton* (Buenos Aires), 37: 137-144.
- Xavier-Filho, L.; Arruda, E. C.; Paulo, M. Q.; Legaz, M. E.; Vicente, C.** 1984. Chemotaxonomical study on *Cladonia verticillaris* (Raddi) Fr. And *Cladonia calycantha* (Del) Nyl. *Phyton* (Buenos Aires), 44(2): 115-119.
- Xavier-Filho, L. Paulo, M.Q.; Vicente, C. Legaz, M. E.** 1985. Phenols from *Cladonia sandstedei* analyzed by high performance liquid chromatography. *Criptogamie, Bryologie et Lichenologie*, 6(2): 143-149.
- Whiton, J.C. & Lawrey, J.D.** 1982. Inhibition of *Cladonia cristatella* and *Sordaria fimicola* ascospore germination by lichen acids. *The Bryologist*, 85(2): 222-226.
- Whiton, J.C. & Lawrey, J.D.** 1984. Inhibition of crustose lichen spore germination by lichen acids. *The Bryologist*, 87(1): 42-43.

