STUDIA GEOBOTANICA
13: 299-312. 1993

ARISTOLOCHIC ACIDS VARIATION AND DISTRIBUTION IN SOME *ARISTOLOCHIA CLEMATITIS* L. POPULATIONS

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Keywords: *Aristolochia clematitis*, aristolochic acids, geographic differentiation, Friuli-Venezia Giulia Region (North-Eastern Italy).

Abstract: Seven wild populations (hypogean and aerial parts) of *Aristolochia clematitis*, sampling in the North-East Italy , were studied with regard to its aristolochic acids content. Two compounds (aristolochic acids I and II) were identified and quantified by HPLC. The hypogean organs were the most productive parts. Significant quantitative variation was found among populations: two chemical types were identified. The quantitative variability of the two secondary metabolites was correlated with seasonal and sinanthropical factors but not with the climatic variables. The compounds can play a defence role against phytophagous insects.

Introduction

The genus *Aristolochia* L. (*Aristolochiaceae*) is distributed principally in tropical, sub-tropical and temperate areas of the Boreal hemisphere (Rechinger 1958).

In Italy, the genus consists of ten species (Nardi 1984), four of them occur in the Friuli-Venezia Giulia Region (NE-Italy) (*A. clematitis* L., *A. rotunda* L., *A. pallida* Desf., *A. lutea* Willd.) (Gortani 1906; Poldini 1980 and 1991; Martini 1990).

In this paper we investigated *A. clematitis* L. It is widespread in large part of the Continental Europe (Jalas & Suominen 1976) from Caucasus to Northern Minor Asia; within Italy it is uncommon only in the Southern area (Nardi 1984). Frequently cultivated, it is widely naturalized (Hultén & Fries 1986).

The species is nitrophylous, ruderal and primarly restricted to moist and aired land habitats, along water-courses, drains, cultivated lands, hedges, bush and coasting sands.

The hypogean parts have been used as a tonic, emmenagogue, expectorant, uterocinetic and antispasmodic drug (Horrisberger 1971). Since they represent a natural source of aristolochic acids, they show pharmaceutical interest.

These compounds are well known for their antibacterial, fagocitary, cytotoxic and antitumor effects. Some Authors, however, report a carcinogenic activity (Mengs & Klein 1988). In the hypogean parts other substances with biological activity are also produced (Munavalli & Viel 1969; Slavik *et al.* 1987).

Aristolochic acids I and II constitute the main natural products in *A. clematitis* L. (Slavik *et al.* 1987).

Although these natural products have been reported in other species of the genus and in *A. clematitis* L. of different origin, neither phytochemical study was hitherto conducted on the *A. clematitis* of Friuli-Venezia Giulia.

The present paper reports the investigation on the aristolochic acid quantitative variation in different organs of seven populations, one of which was analyzed during the seasonal stages.

The correlations between the chemical variability and the climatic factors were also examined.

Quantitative studies of secondary metabolites, regardless of application level, almost uniformly lack statistical comparisons for interpreting significance of variation. However, with the availability of HPLC detection technology and easy access to statistical software (methods), it is possible to design and conduct appropriate quantitative studies.

This report shows also that statistical analysis of quantitative data reflect the true probabilistic variation due to seasonal influences.

Materials and methods

The sampling has concerned six populations homogeneously distributed in the *A. clematitis* L. areal in Friuli-Venezia Giulia Region. The plants were collected, during one week in may 1989 (incipient-anthesis) to examine the quantitative variability in the different organs of the plants, in the following localities:

1 - Gorizia, loc. Mainizza (40 m);
2 - Cividale (UD), loc. Rualis (150 m);
3 - Monfalcone (GO), loc. Alberoni (2 m);
4 - Basaldella (UD), loc. S. Daniele (85 m);
5 - Polcenigo (PN), loc. S. Giovanni (60 m);
6,7 - Sgonico (TS) (255 m).

Another sampling has concerned one population of *A. clematitis* L. located on the Trieste Karst and collected in the following periods:

April 1990 (pre-anthesis)
May 1990 (incipient-anthesis)
July 1990 (full-anthesis)
September 1990 (post-anthesis)

to compare the quantitative variability during the whole ontogenic cycle.

In each population three samples of ten plants were collected. Plant specimens are deposited in the Herbarium Universitatis Tergestinae (TSB 2174).

The plant parts (hypogean, aerial) were treated with liquid N_2, finely ground (60 mesh/mm) and weighted (1 g dry weight estimated on another sample). The extraction was carried out with 150 ml of methanol in Soxhlet to exhaustion.

The extracts were evaporated *in vacuo* to small volume. The residue was redissolved in defined volumes (10 ml methanol); they were analyzed by high performance liquid chromatography (HPLC) as described in a previous paper (Cateni *et al.* 1992). HPLC analyses were performed using a Perkin Elmer ODS RP-column filled with bonded octadecylsilane on silica (10 μm) with the flow rate 1,8 ml/min, detection 310 nm (detector sensitivity 0,01 a.u.f.s.) and methanol/water/acetic acid (80:20:1) as mobile phase. All the determination were performed at room temperature (22 °C) and at isocratic condition of elution.

The analytical data were submitted to elaboration with statistical methods (ANOVA Jerrold 1984; ELLIPLOT, REGLIN and PROBR Lagonegro & Feoli 1985a, 1985b).

Results and discussion

Aristolochic acids I and II were identified on the basis of their retention times and spectrophotometric characteristics (in respect to standards) and quantitatively evalued (% dry weight) (Cateni *et al.* 1992).

The acid distribution differs in the various organs. In the populations 2, 4 and 6 aristolochic acid I is the major constituent in the hypogean parts of the plant, while aristolochic acid II predominates in the hypogean parts of the remaining populations, besides in the aerial parts of all the examined populations. In the populations 6 and 7 aristolochic acids I and II are present in similar amounts in the hypogean parts (Tab. 1).

Since the seven populations show interesting differences in the aristolochic acid contents, they can be divided in two chemical groups. The first group includes the more productive populations (1, 2 and 3), while the second includes the less productive ones (4, 5, 6 and 7).

In order to examine the differences in aristolochic acid compositions, between the examined populations, we used a nested (ANOVA). Results showed that highly significant differences existed in the content of the two acids between the populations (Tabs. 2, 3).

Three samples of ten plants were sampled at random in the population 7 in some ontogenetic cycle phases (pre-, incipient-, full- and post-anthesis, corresponding at April, May, July and September) over a one year period.

Quantitative variation was found among the two acids (Tab. 4).

In hypogean parts of the plant the quantities of aristolochic acids were at

Tab. 1 - Variation in aristolochic acids I and II (% dry weight) in populations of *Aristolochia clematitis*.

Population	Organ	Aristolochic acids I (%)	II (%)
1	Root	0.561	0.604
	Stem	0.020	0.046
	Leaf	0.062	0.199
2	Root	0.455	0.387
	Stem	0.015	0.021
	Leaf	0.044	0.127
3	Root	0.425	0.541
	Stem	0.034	0.056
	Leaf	0.085	0.101
4	Root	0.330	0.260
	Stem	0.017	0.033
	Leaf	0.048	0.113
5	Root	0.268	0.284
	Stem	0.006	0.029
	Leaf	0.025	0.088
6	Root	0.250	0.244
	Stem	0.021	0.033
	Leaf	0.043	0.120
7	Root	0.256	0.259
	Stem	0.011	0.020
	Leaf	0.026	0.073

Tab. 2 - Analysis of variance of aristolochic acid I based on % dry weight in *Aristolochia clematitis*.

Organ	Source of variation	Sum of squares	D.F.	Mean square	F
Root	Treatment	0.258	6	0.043	8815.854
	Error	0	14	0	
	Total	0.258	20		
Stem	Treatment	0.001	6	0	256.156
	Error	0	14	0	
	Total	0.001	20		
Leaf	Treatment	0.008	6	0.001	405.925
	Error	0	14	0	
	Total	0.008	20		
	F 0.05 (6.14) = 2.85				

Tab. 3 - Analysis of variance of aristolochic acid II based on % dry weight in *Aristolochia clematitis*.

Organ	Source of variation	Sum of squares	D.F.	Mean square	F
Root	Treatment	0.369	6	0.066	16510.34
	Error	0	14	0	
	Total	0.369	20		
Stem	Treatment	0.003	6	0.001	174.606
	Error	0	14	0	
	Total	0.003	20		
Leaf	Treatment	0.03	6	0.005	450.315
	Error	0	14	0	
	Total	0.03	20		
	F 0.05 (6.14) = 2.85				

their highest levels (0.256 and 0.259 % dry weight respectively) in May (incipient-anthesis) and at their lowest levels (0.199 and 0.186 % dry weight respectively) in autumn; the two acids in the hypogean parts show similar patterns of variation with highest and lowest yields in the same ontogenic phases (Fig. 1). On the contrary, aristolochic acid I in the stems and leaves is more or less constant, whereas aristolochic acid II has a maximum in July (full-anthesis) and a minimum in May (Figs. 2 and 3). Generally, the highest quantities occured during the annual period of maximal vegetative growth. The amount of acids regularly decrease throught the fall flowering season. Analysis of variance of quantitative data revealed significant seasonal variation among the % composition (Tabs. 5, 6).

The two acids significatively characterize the single organs of *A. clematitis*, independently of geographical origins. Each organ is chemically distinguishable from the other on the basis of regression lines and centroid values (Fig. 4) and is positively identifiable.

The results also indicate that among the organs the aerial parts are more similar on the chemical point of view to respect of hypogean parts, which appear clearly separate.

To determine whether the two chemical groups of populations are the result

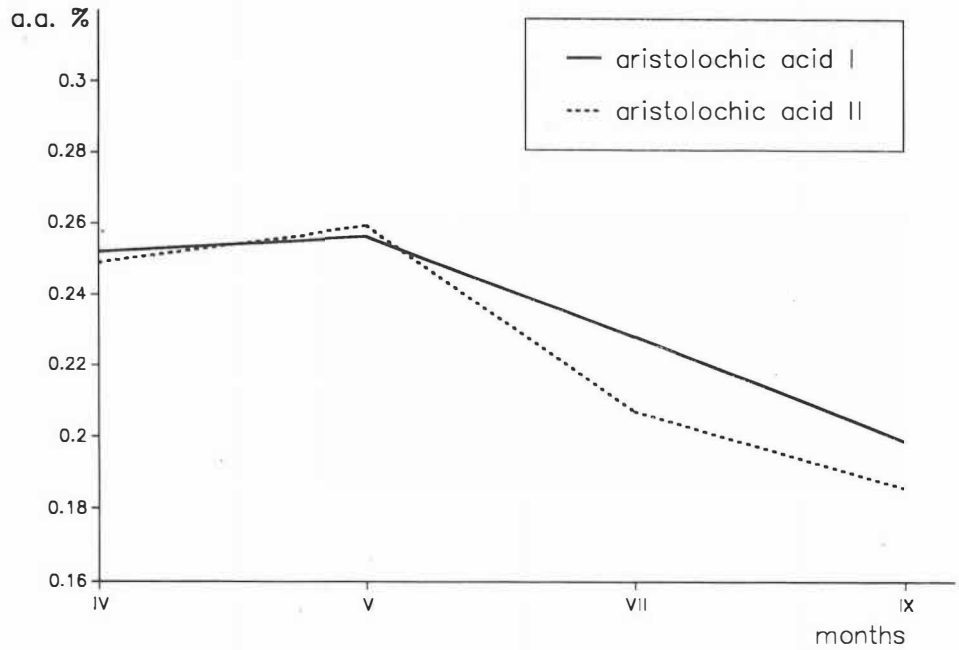

Fig. 1 - Seasonal variation of aristolochic acids as % dry weight in roots of *Aristolochia clematitis*.

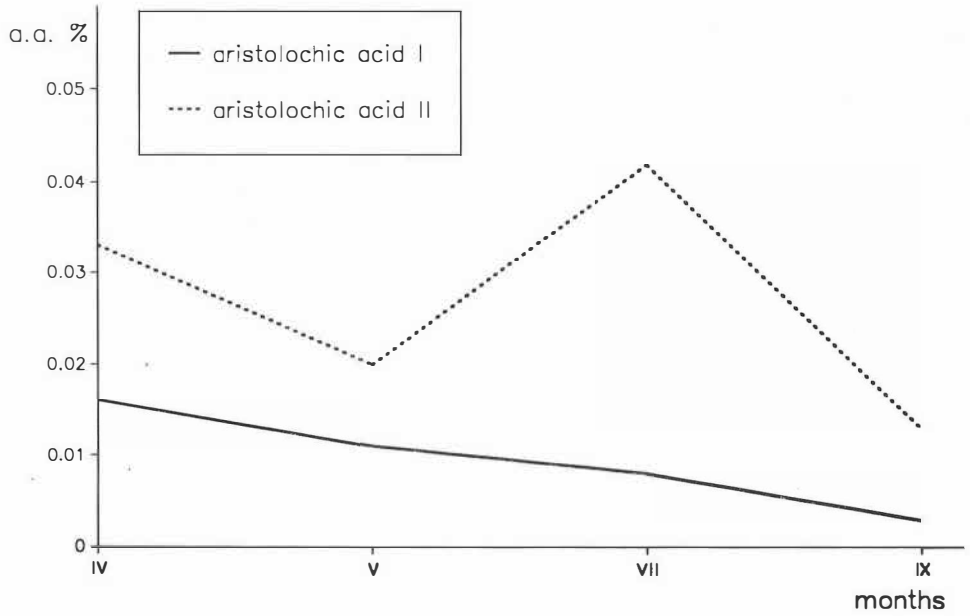

Fig. 2 - Seasonal variation of aristolochic acids as % dry weight in stems of *Aristolochia clematitis*.

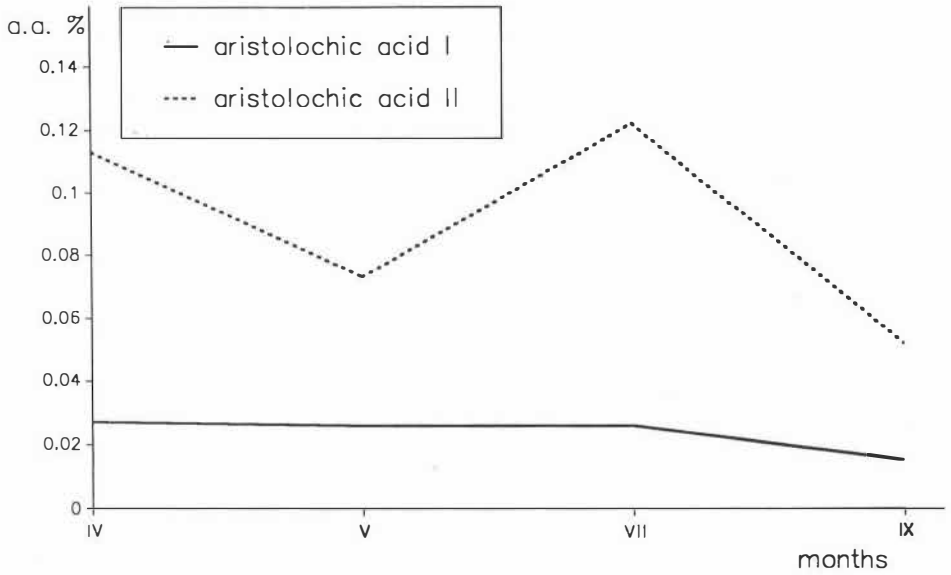

Fig. 3 - Seasonal variation of aristolochic acids as % dry weight in leaves of *Aristolochia clematitis*.

of selection or of other factors, we started to investigate the impact of the geographic distribution and the climatic conditions. No comparable geographic distribution can be found in the quantitative data. The population sample localities cover a broad range of climatic conditions so that we would expected a correlation with some factors. Just the opposite was observed.

Through elaboration of the theoretical regression lines (REGLIN), there was not correlation between the different amounts of the two acids and mean annual temperatures, precipitations and altitudes (Tabs. 7, 8, 9). Therefore, different yields were not closely correlated with any of the climatic variables considered.

These results have suggested that the difference observed in quantitative composition of the seven populations may depend on growing conditions

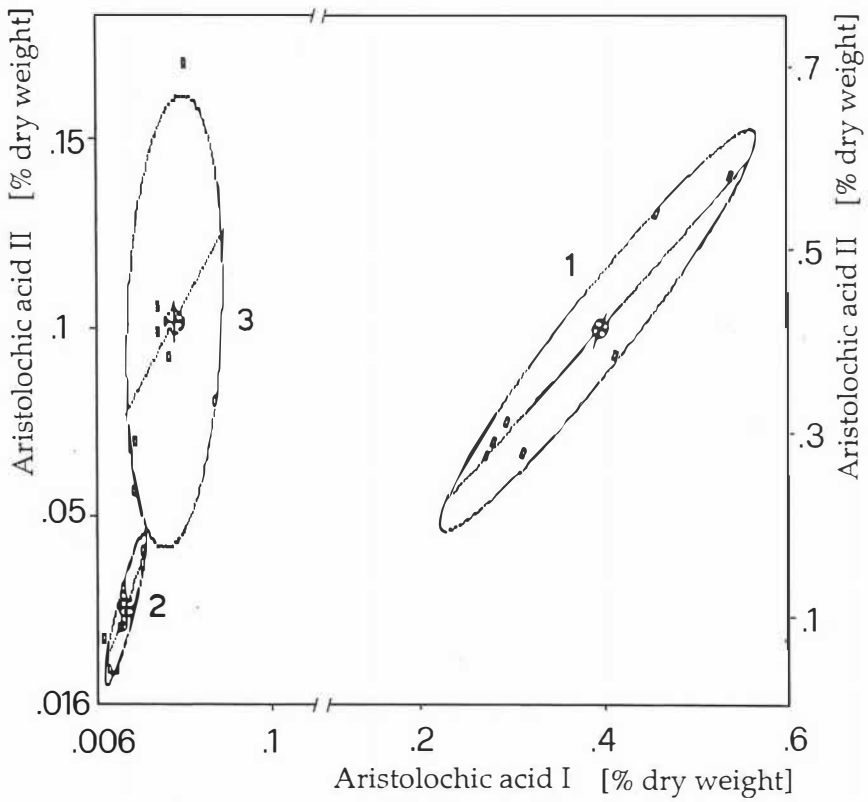

Fig. 4 - Ellipses of equal concentration, regression lines and centroids for three plant organs at the 5 % level of confidence. The slopes and intercepts of the regression lines are significantly different ($p < 0.05$). 1 Roots (centroid: x = 0,397301, y = 0,4063143; regression line: $y = 1{,}123222x - 3{,}994298 \cdot 10^{-2}$); 2 Stems (centroid: $x = 2{,}151613 \cdot 10^{-2}$, $y = 3{,}849194 \cdot 10^{-2}$; regression line: $y = 1{,}181518x + 1{,}307025 \cdot 10^{-2}$); 3 Leaves (centroid: $x = 5{,}510179 \cdot 10^{-2}$, y = 0,1233503; regression line: $y = 0{,}8209238x + 7{,}811593 \cdot 10^{-2}$).

Tab. 4 - Variation in aristolochic acids I and II (% dry weight) over the phenological stages in population 7.

Phenological stage	Month	Organ	Aristolochic acids I (%)	Aristolochic acids II (%)
Pre-anthesis	IV	Root	0.252	0.249
		Stem	0.016	0.033
		Leaf	0.027	0.113
Incipient-anthesis	V	Root	0.256	0.259
		Stem	0.012	0.02
		Leaf	0.026	0.073
Full-anthesis	VII	Root	0.228	0.207
		Stem	0.008	0.043
		Leaf	0.026	0.122
Post-anthesis	IX	Root	0.199	0.185
		Stem	0.004	0.014
		Leaf	0.015	0.052

rather than other factors. The 1, 2 and 3 population sampling near cultivated and abundantly fertilized fields (anthropization prevail), were more productive; on the contrary, in the other populations, sampling along the borders of roads (ruderalization prevail), the aristolochic acids were less present. Probably the acid variabilities are the results of different grade of anthropization and/or ruderalization of the habitat in which they growth.

Because of its variable production and other characteristics including the variations in the acid quantity, produced by different populations, *Aristolochia clematitis* would serve as an excellent subject for the study of the genetics and inheritance of acid compositions. It is obvious from these points that populations must be grown in a controlled environment in order to be able to detect if the acid variation is a result of sinanthropic influence.

The aristolochic acids are secondary metabolites, ubiquitous in plants and quantitatively diverse in the various organs. Some physiological and ecological hypotheses about the functional role of the secondary metabolites have been proposed, one of which regards the plant chemical defence (*i.e.* molluscicidal, insecticidal, antifungal, anti-herbivore etc.) (Chew & Rodman 1979).

In our case, the major concentration of aristolochic acids trough the growth season support the hypothesis that they play a chemical role in reducing damage

Tab. 5 - Analysis of variance of aristolochic acid I based on % dry weight of phenological stages in *Aristolochia clematitis*.

Organ	Source of variation	Sum of squares	D.F.	Mean square	F
Root	Treatment	0.006	3	0.002	547.693
	Error	0	8	0	
	Total	0.006	11		
Stem	Treatment	0	3	0	76.084
	Error	0	8	0	
	Total	0	11		
Leaf	Treatment	0	3	0	161.59
	Error	0	8	0	
	Total	0	11		
	F 0.05 (3.8) = 4.07				

Tab. 6 - Analysis of variance of aristolochic acid II based on % dry weight of phenological stages in *Aristolochia clematitis*.

Organ	Source of variation	Sum of squares	D.F.	Mean square	F
Root	Treatment	0.011	3	0.004	2756.479
	Error	0	8	0	
	Total	0.011	11		
Stem	Treatment	0.001	3	0	423.332
	Error	0	8	0	
	Total	0.001	11		
Leaf	Treatment	0.01	3	0.003	1204.167
	Error	0	8	0	
	Total	0.01	11		
	F 0.05 (3.8) = 4.07				

caused by herbivorous and phytophagous insects.

Aristolochic acids have been found also in some species of *Lepidoptera* since the larvae of these butterflies feed on the plants of the genus *Aristolochia* (Nishida & Fukami 1989; Urzua 1988; Urzua & Priestap 1985; Slavik *et al.* 1987). The aristolochic acids deterred feeding of tree sparrows, which suggested a defensive role against vertebrate predators (Nishida & Fukami 1989).

Tab. 7 - Values y(x) of aristolochic acids I and II (x = temperature); degrees of freedom = 4; significativity < 5%.

Organ	Aristolochic acids	Regression line	Corr. coeff.
Root	I	y = 0.3682x - 0.7935	0.38
	II	y = 0.6711x - 4.5333	0.55
Stem	I	y = 0.0427x - 0.3449	0.59
	II	y = 0.6711x - 4.5333	0.79
Leaf	I	y = 0.3682x - 0.7935	0.74
	II	y = 0.6711x - 4.5333	3.05
Plant in toto	I	y = 0.3682x - 0.7935	0.48
	II	y = 0.6711x - 4.5333	0.50

Tab. 8 - Values y(x) of aristolochic acids I and II (x = precipitation); degrees of freedom= 4; significativity < 5%.

Organ	Aristolochic acids	Regression line	Corr. coeff.
Roots	I	y = 8.6382E-5x + 3.699	1.81
	II	y = - 1.9313E-3x + 6.4611	- 0.31
Stems	I	y = - 3.1874E-4x + 0.6171	- 0.88
	II	y = - 4.2108E-4x + 0.9297	- 0.85
Leaves	I	y = - 6.0702E-4x + 1.3301	- 0.77
	II	y = 9.4323E-5x + 1.1187	6.1
Plants in toto	I	y = - 2.7978E-4x + 1.8820	- 0.15
	II	y = - 7.5275E-4x + 2.8366	- 0.30

Tab. 9 - Values y(x) of aristolochic acids I and II (x = altitude); degrees of freedom= 4; significativity < 5%.

Organ	Aristolochic acids	Regression line	Corr. coeff.
Root	I	y = - 6.4282E-3x + 4.4471	- 0.48
	II	y = - 1.0705E-2x + 4.9193	- 0.63
Stems	I	y = - 2.2145E-4x + 0.2107	- 0.22
	II	y = - 8.2171E-4x + 0.2107	- 0.60
Leaves	I	y = - 1.0104E-3x + 0.614	- 0.46
	II	y = - 2.6542E-4x + 1.2716	- 6.18
Plants in toto	I	y = - 2.5536E-3x + 1.7573	- 0.50
	II	y = - 3.9306E-3x + 2.2119	- 0.56

Concluding remarks

The results of this study on seven *Aristolochia clematitis* populations allow the following conclusions:

a) a decreasing gradient of aristolochic acid quantities from hypogean parts to leaves through the stems has been observed;

b) April and May are the best months for sampling, since they give the most acid amounts;

c) Aristolochic acids significatively discriminate the single organs of plant and may be used in further chemosystematic studies of genus;

d) none of the observed pattern of variation are highly correlated with climatic and geographic variables;

e) the seasonal and geographical variations seem to be determined by the different grade of anthropization and/or ruderalization.

Further experiments on the ecological and chemosystematic significance of aristolochic acids from *Aristolochia* L. are underway.

Riassunto. VARIAZIONE DEL CONTENUTO DI ACIDI ARISTOLOCHICI IN ALCUNE POPOLAZIONI DI *ARISTOLOCHIA CLEMATITIS* L. - Sette popolazioni spontanee di *A. clematitis* (parti ipogee ed aeree), raccolte nel Friuli-Venezia Giulia (Italia nordorientale), sono state studiate con particolare riguardo al contenuto in acidi aristolochici. Sono stati identificati e quantificati tramite analisi HPLC due composti (acidi aristolochici I e II). Gli organi ipogei sono risultati la parte più produttiva. Variazioni

quantitative significative sono state evidenziate nell'ambito delle popolazioni: sono stati identificati due tipi chimici. La variabilità quantitativa dei due metaboliti secondari appare correlata con fattori stagionali e sinantropici ma non con variabili climatiche. I composti possono assumere un ruolo di difesa contro gli insetti fitofagi.

Aknowledgements

Financial support was provided by the Italian Ministry of Public Instruction (MPI 40%), responsible Prof. L. Coassini Lokar.

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