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THE COMPOSITION OF ALYSSUM BERTOLONII DESV. (CRUCIFERAE): FURTHER CONTRIBUTION (***)

Riassunto — *La composizione di Alyssum bertolonii Desv. (Cruciferae): ulteriore contributo*. Il metabolismo di *A. bertolonii*, specie endemica dei serpentini toscani e capace di accumulare eccezionali quantità di nichel, è stato ulteriormente studiato in modo da avere un quadro più completo della sua composizione in macro e microelementi (alcuni dei quali presi in considerazione per la prima volta) e della presenza e concentrazione degli acidi organici, aminoacidi e fenoli. I campioni di foglie analizzati provenivano sia dall'affioramento ultrabasico dell'Alta Val Tiberina, sia da coltivazioni in vaso su terreno di giardino realizzate nell'Orto Botanico dell'Università di Firenze. I risultati mostrano il complesso metabolismo di questa pianta influenzato dalle particolari condizioni del serpentino e dall'eccezionale arricchimento di nichel delle foglie.

Abstract — The metabolism of *Alyssum bertolonii* Desv., a nickel accumulating species endemic to Tuscan serpentines, has been further investigated, assessing its elemental composition, organic acid, aminoacid and phenol presence and concentration in leaves collected from plants grown on a serpentine outcrop in the Upper Tiber Valley and from plants cultivated in garden soil in the Botanical Garden of Florence University. The results show the complex metabolism of *A. bertolonii*, which is affected by the presence of high nickel levels and by the peculiarities of serpentine soil composition.

Key words — *Alyssum bertolonii* - composition - serpentine.

Alyssum bertolonii Desv. (Cruciferae), an endemic plant of the serpentine outcrops of Tuscany, has been the object, since the discovery of its exceptional nickel accumulating capacity (MINGUZZI and VERGNANO, 1948) of several investigations on its mineral composition and nickel accumulation during growth (cfr. VERGNANO *et al.*, 1977;

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1979) and on the identification of the compounds with which nickel is associated in this plant (PELOSI *et al.*, 1974, 1976; PANCARO *et al.*, 1978). Particular interest was focused on organic acids, because organic acid concentration does not only show marked alterations in the presence of heavy metals (DE KOCK and MORRISON, 1958; LAULHÈRE et ALQUIER-BOUFFARD, 1969; MATHYS, 1977; THURMAN and RANKIN, 1982; etc.) but it is often related with nickel accumulation (LEE *et al.*, 1977), citric acid being most frequently involved in complexing nickel (LEE *et al.*, 1978). In the leaf extracts of *Alyssum bertolonii* the accumulation of nickel along with malic and malonic acids has been observed (PELOSI *et al.*, 1976; PANCARO *et al.*, 1978; LEE *et al.*, 1978).

These previous investigations were carried out on material collected in different serpentine areas and not always at the same developmental stage, therefore this research aimed at producing a more detailed information on the composition of plants grown on a single serpentine outcrop and at comparing the composition of these plants with that of specimens grown in pots with garden soil, in order to obtain specimens in which, exhausted of the nickel supply of the seeds, such element would be brought to very low levels.

Particular attention was also given to other metabolic products which might be involved in the tolerance mechanisms to heavy metals, such as phenols, chlorogenic acid, and some aminoacids as methionine and cystine.

In addition to the leaves, seeds have been also examined as data on this material are particularly scarce.

MATERIAL AND METHODS

Leaves of *A. bertolonii* were collected from plants growing on the ultrabasic outcrop of the Upper Tiber Valley, at Monte Murlo (near Pieve S. Stefano), from February to October. They were placed in plastic bags, carried to the laboratory, washed in distilled water and freeze-dried. The seeds of these plants, collected in August, were sown in pots with garden soil in the following January and germinated in March: when the plants were two years old, leaves were collected, washed and freeze-dried. No seeds were produced by these plants.

All the leaf analyses were carried out on freeze-dried material. The seeds were dried at 80°C.

On this material the organic acid composition was determined by HPLC, as previous determinations using gas chromatography or colorimetric methods gave contrasting results. For this analysis the aqueous extract of the freeze-dried material was passed through a Dowex 50 column and injected into a Perkin Elmer ser. 10 HPLC instrument. Separation of organic acids was carried out with a Lichrosorb C-18 5 μm column, eluated with a solution of triethylamine 0.01N and phosphoric acid (pH 2.0). Oxalic acid was estimated according to the method of COOKE and SANSUM (1976) using an azo-dye (3,4-dihydroxyzobenzene-2-carboxylic acid). The extraction procedure for the determination of the free aminoacids has been described elsewhere (cfr. BICK *et al.*, 1982). Total phenols were determined using the method described by PIRIE and MULLINS (1976), chlorogenic acid by BASTIN method (1968); chloride by electrometric titration (SHONE, 1968). The elemental analysis was carried out by atomic absorption spectrometry. Sulphur was determined by the method of BUTTERS and CHENERY (1959). Trace element composition was assessed by spark source mass spectrometry.

The analyses were carried out either at the Macaulay Institute for Soil Research, Aberdeen, in the Plant Physiology, Spectrochemistry and Organic Chemistry Departments or in the Plant Physiology Laboratory, Department of Plant Biology, Florence University.

The results are reported in tables 1-4.

RESULTS

Organic acids

The outstanding feature of the organic acid composition is the remarkable amount of malic and malonic acids found in all the leaf samples collected on serpentine (Tab. 1).

The level of these acids does not show a clear relationship with nickel concentration: actually both acids are far in excess in comparison to the nickel concentration of the sample. Malic acid reaches slightly higher values than those previously observed by PANCARO *et al.* (1978), ranging from 218 to 350 μM; the same occurs also with malonic acid, which reaches in the sample collected in September 300 μM. Of the other acids detected, only citric and tartaric reach remarkable levels, oxalic acid, measured only in three samples, shows in the October sample an increase which is not related to a higher calcium concentration. Low levels of fumaric acid are shown by all samples. The total organic acid concentration reaches in June the

TABLE 1 - *Organic acid composition and nickel concentration ($\mu M\ g^{-1}$ f-d tissue) of leaves and seeds of* Alyssum bertolonii.

	Malic	Malonic	Citric	Tartaric	Fumaric	Oxalic	Ni
Serpentine							
leaves Febr.	350	230	55	n.d.	2	46	205
April	338	280	65	44	0.5		192
May	380	230	30	29	0.5		137
June	260	200	32	n.d.	n.d.	35	141
July	218	276	37	49	2		195
Sept.	275	302	38	30	0.2		250
Oct.	265	215	31	29	1	54	177
seeds	107	n.d.	26	33	0.7	131	120
Garden soil leaves	105	n.d.	38	n.d.	5	32	0.32

n.d. = not detected.

lowest level, and is possibly related to the leaf mineral depletion of some elements during the production of flowers and fruits, and to the shedding of the older leaves (cfr. Tab. 3).

In the seeds malonic acid was not detected and this confirms the low values (1 $\mu M\ g^{-1}$ d.m.) of this acid found also by PANCARO *et al.* (1978) in the seeds of *A. bertolonii* collected at Pomaia (Pisa). All the other acids (citric, tartaric, oxalic and fumaric) and particularly malic are instead well represented.

In the sample of leaves collected on garden soil the total organic acid concentration is much lower, particularly due to the absence of any consistent quantity of malonic acid, which was detected in relatively small amounts in the samples examined by PANCARO *et al.* (1978), and also of tartaric acid. Oxalic and citric acids have almost the same values shown by the serpentine samples. Malic acid is present in relatively lower concentrations.

Total phenols, chlorogenic acid and chloride

The prominent feature in distribution of total phenols (Tab. 2) is the low value in the nickel-depleted plants, in which the concentration is reduced to almost 50% compared to the serpentine samples, which show an almost constant phenol content, no increase being shown in the February sample, which has also the lowest chlo-

TABLE 2 - *Total phenols, chlorogenic acid and chloride composition of* A. bertolonii *leaves and seeds (µM g^{-1} f-d tissue).*

	Phenols	CGA	Cl
Serpentine			
leaves Febr.	43.20	0.20	315.5
» June	51.75	1.42	281.0
» Oct.	48.90	1.80	253.5
seeds	55.30		
Garden Soil leaves	25.50		521.3

TABLE 3 - *Elemental composition of* A. bertolonii *leaves and seeds.*

	N	Ca	Mg	K	P	S	Fe	Ni	Na	P/Fe
			%					µg g^{-1}		
Serpentine										
leaves Febr.	2.60	3.80	0.43	1.04	0.17	0.34	369	12090	1127	4.6
» June	2.19	2.70	0.61	1.13	0.11	0.28	257	8340	644	4.3
» Oct.	1.76	2.98	0.67	0.95	0.07	0.30	324	10450	598	2.2
seeds	3.06	0.70	0.18	1.05	0.50	0.20	45	6990		
Garden Soil leaves	3.80	4.16	0.20	0.27	0.44	1.05	592	19	1816	7.4

rogenic acid concentration. In the garden soil plants chlorogenic acid was not detected.

Rather high chloride values have been found in all samples, this clearly being a specific feature of the plant.

Mineral composition

It is interesting to note that *Alyssum bertolonii* can produce well developed plants also on a non serpentine substrate and at very low internal nickel concentrations: the only difference that we have been able to detect is the scarce production of fruits with viable seeds in the garden soil plants.

Comparing the leaf composition of the serpentine samples with that of the garden soil plants (Tab. 3), the most striking difference is the high nitrogen, phosphorus and sulphur concentration of the garden plants as the garden soil is obviously richer than serpentine in these elements. The calcium level is higher in the plants not grown on serpentine, but also on this substrate *A. bertolonii* can reach remarkable calcium concentrations, as it often occurs in other serpentine species (PROCTOR and WOODELL, 1975). The Ca:Mg ratio is more than four times higher in the garden soil plants, as they have a very scarce magnesium concentration. Also potassium has lower values in these plants: possibily also the high organic acid concentration of serpentine plants accounts for their higher potassium uptake. The P:Fe ratio shows greater values in the garden plants, as phosphorus uptake is particularly enhanced in the garden soil.

All the essential microelements (Tab. 4) boron, manganese, copper and zinc, with the exception of molybdenum, show generally higher concentrations in the serpentine samples. Copper and zinc are particularly scarce in seeds.

TABLE 4 - *Microelemental composition of* A. bertolonii *leaves and seeds* (*μg* g^{-1} *f-d tissue*).

	B	Ti	V	Mn	Cu	Zn	Mo	Ag
Serpentine								
leaves Febr.	13.4	5.1	1.3	190	3.8	29	0.6	0.2
» June	33.0	6.1	n.d.	360	5.1	70	0.4	0.1
» Oct.	36.0	9.7	0.9	380	14.0	100	0.8	2.0
seeds	8.8	0.6	0.3	40	2.0	22	0.9	0.1
Garden Soil leaves	12.0	4.4	0.5	36	4.6	55	6.3	0.1

Free aminoacids

The free aminoacids composition of *A. bertolonii* has been treated separately (BICK *et al.*, 1982), so that a short survey of the main results is given here. The aqueous acetone extracts of freeze-dried leaves of *A. bertolonii* show that the differences between the serpentine and the garden plants are only quantitative: the free aminoacid concentration is much higher in the garden plants (3402 μg g^{-1}), as is their total nitrogen (3.80%). Within the serpentine samples, the one collected in February shows the highest (2166 μg g^{-1}), and the June sample the lowest (881 μg g^{-1}) aminoacid level.

The S-containing aminoacids, cystine and methionine, do not reach higher levels in the serpentine plants; actually methionine was clearly detected only in the garden sample (27.8 μg g^{-1}). Threonine, aspartic acid, glycine, alanine, valine, isoleucine and leucine show much stronger values in the samples collected on normal soil.

Proline, which is the major aminoacid of the leaves of *A. bertolonii*, reaches high levels in the serpentine plants, particularly in February (899 μg g^{-1}) and in October (561 μg g^{-1}); but high proline levels seem a characteristic of this species, as also the garden plants are rich in this aminoacid (906 μg g^{-1}).

Of the aminoacids involved in mustard oil synthesis, as glutamate, aspartate and alanine, only glutamate shows high values (180 μg g^{-1}) in the sample collected in February; in the June sample these aminoacids, although less abundant, still account for almost 50% of the total free aminoacids.

In the seeds proline (15 μg g^{-1}) and arginine (9 μg g^{-1}) are very scarce, quite high histidine (131 μg g^{-1}) and homoserine (83 μg g^{-1}). This aminoacid, which is generally not found in dormant or dry seeds, is one of the main aminoacids of *Alyssum* seeds, and was not detected in the leaves. Alanine, glycine, serine and glutamine, main constituents of seeds, are present also here at remarkable levels.

CONCLUSIONS

This research has put into evidence how the development of high nickel tolerance affects several metabolic aspects of *A. bertolonii* and even if heavy metal tolerance can be ascribed to many factors, it is probable that heavy metals affect also the redox potential of the cell. If we assume that the redox potential is measured by the ratio of ferric iron to ferrous iron, it follows that the heavy metals must influence the oxidation state of iron. This is affected by the concentration of phosphorus and the active iron fraction, i.e. the fraction involved in enzymic reactions which is linearly related to the ratio of total phosphorus to total iron (DEKOCK, 1964; DEKOCK and HALL, 1955; DEKOCK *et al.*, 1974, 1979): a low P:Fe ratio suggests a greater amount of iron available for such reactions. Therefore one of the characteristics of a tolerant species is the low amount of phosphate in the tissues and a relatively high amount of iron. *A. bertolonii* shows a P:Fe ratio always lower on serpentine, where probably nickel interferes with iron metabolism, so that a lower P:Fe ratio allows greater iron availability. Also related to a low phosphorus concentration is the organic acid concentration of this species. Although citric acid frequently occurs as chelant for heavy metals (LEE *et al.*, 1977; 1978), the main acids implicated with complexing nickel in *A. bertolonii* seem malic and malonic acids (PELOSI *et al.*, 1976; PANCARO *et al.*, 1978), but while malic acid is always connected with the nickel fraction, this is not always the case of malonic acid. In addition this acid is not always clearly detectable and sometimes it might be totally absent, while high malic acid concentration is a constant feature of *Alyssum bertolonii* plants. Therefore it is more probable that nickel is mainly bound to malic acid, although other acids might also be involved in complexing nickel.

In the serpentine plants also the phenolic acids level is markedly increased: these compounds are often involved in complexing heavy metals (GOMAH and DAVIES, 1974; FOY *et al.*, 1978) and it is not surprising their increase under stress conditions. These compounds take part in a number of fundamental cellular processes and are involved in anthocyanin synthesis, often stimulated in plants under heavy metal stress. In this specific case an increase in red pigmentation is typical of the leaves and stems of *A. bertolonii*, particularly when the plants are grown in water culture with the addition of nickel. An increase in red pigmentation, due to high anthocyanin concentration, is found in many serpentine plants, and actually it is considered a typical morphological alteration caused by this substrate (RITTER-STUDNIČKA, 1971). As the phenolic acids are built into polymers in lignin, heavy metals could be chelated also in this way and held in an insoluble form in the conducting tissues.

Whilst most trace elements range within normal values, in the serpentine samples molybdenum concentration is rather low: scarce molybdenum availability has been considered to contribute to the infertility of serpentine soils (WALKER, 1954; PROCTOR and WOODELL, 1975) and this condition is confirmed for the first time also for these Italian plants. Instead the possibility of boron toxicity, suggested by SHKOLNIK and SMIRNOV (1970) for some serpentine areas, has to be excluded in our case.

A characteristic of *A. bertolonii* is also its high concentration of proline (BICK *et al.*, 1982), which in February and October accounts for more than 40% of the total free aminoacids. A high proline level was noticed by FARAGO (1981) in the roots of a copper tolerant strain of *Armeria maritima* and the presence of this aminoacid could be an indication of stress conditions.

Cystine and methionine, which are often reported to be involved in complexing heavy metals, do not show higher values in the samples collected on serpentine and from a quantitative standpoint do not seem to play any role in nickel tolerance.

The aminoacids involved in the synthesis of mustard oil glycosides, which are present in high amounts in the Cruciferae and in *A. bertolonii*, and which could complex nickel (SASSE, 1976), do not show an increase in the serpentine plants except for glutamate, which has high values in the sample collected in February, when nickel level is one of the highest.

In conclusion it is evident that *A. bertolonii* is adapted to serpentine soils through a complex metabolism resulting from the interaction of various factors which make ultramafic soils infertile. Therefore the high nickel tolerance of this plant cannot be ascribed to a single mechanism but to a series of mechanisms which operate at different biochemical and physiological steps and which make possible also its high nickel accumulating capacity.

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