# Phenolic compounds as chemical markers of low taxonomic levels in the family *Poaceae*

**V. Míka[1], V. Kubáň[2], B. Klejdus[2], V. Odstrčilová[1], P. Nerušil[1]**

[1]*Research Institute of Crop Production, Prague-Ruzyně, Research Station of Grassland Ecosystems, Jevíčko, Czech Republic*
[2]*Mendel University of Agriculture and Forestry in Brno, Czech Republic*

**ABSTRACT**

The spectra of non-structural phenolics in herbage were used to study genetic affinity in polyploid complex of *Dactylis* L., next in complex of octoploid brome species, and in red fescue cultivar collection. The diploid ($2n$ = 14) subspecies of *Dactylis* revealed distinct differences from the tetraploid ($2n$ = 28) ones as a clearly specialized group. In the genus *Bromus* the affinity bounds of the species inside the section (e.g. *Ceratochloa*) were not very tight in comparison to those between the species from various sections. Also remarkable differences in affinity bonds among red fescue cultivars were determined. Although the conclusions are only based on the results from one year and two sites, they bring significant information on exploitation of phenolic compounds. They demonstrate that phenolic profiles could be used in chemical taxonomy of grasses also at low taxonomic levels as a useful marker. As they are environmentally not as stable as the markers of primary metabolism, plant material for analyses should be standardized as to growing condition, growth stages of plants etc.

**Keywords:** phytochemistry; non-structural phenolic compounds; grasses; chemotaxonomy; plant taxonomy; secondary metabolites

The development of DNA-based technology allows versatile research into genetic diversity of plants. It is based on the polymorphism of markers of primary metabolism, esp. enzyme proteins. These compounds are found in all plants and perform metabolic roles that are essential and usually evident.

Beside this, various products of secondary metabolism possessing extreme diversity might be also considered. However, (1) they are often differentially distributed among limited taxonomic groups within the plant kingdom and (2) their functions, many of which remain unknown, are being elucidated with increasing frequency (Croteau et al. 2000). Chemical markers, such as phenolic compounds, have been still extensively used in botanical chemosystematic studies. These have largely concerned the high or middle taxonomic levels: order, family, genus, section. As a consequence of new, more sensitive and automatic techniques, it is now possible to study phenolic profiles of low taxonomic levels, even of individual genotypes (Hubáček and Lachman 1977, Jay et al. 1996, Míka et al. 2004). The discussion of plant phenolics is a discussion of plant diversity itself. Although the bulk of these compounds assumed cell wall structural roles, a vast array of non-structural constituents was also formed (Croteau et al. 2000). They are essential for continued survival of all types of vascular plants. The widespread occurrence of this type of secondary metabolics in *Poaceae* and currently also in other plant families makes them useful markers for botanical and evolutionary relationships.

Being encouraged by the results of Jay et al. (1996), intraspecific variability and differences in profiles of phenolic compounds in grass subspecies and cultivars belonging to 3 genera, were examined. Using the cluster analysis the affinity bounds in plant systematic classification according to profiles of phenolic compounds were confronted and compared if they match the official taxonomic classification schema (Dostál 1989).

## MATERIAL AND METHODS

Affinity bounds in low taxonomic levels of grasses were considered in:

Supported by the Grant Agency of the Czech Republic, Project No. 521/02/1367.

Table 1. Content of phenolic compounds in herbage (mg/kg dry matter) of *Dactylis glomerata* subspecies

| | *Dactylis glomerata* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | ssp. *glomerata* ecotypes | | ssp. *galiciana* ecotypes | | ssp. *lusitanica* ecotypes | | ssp. *glomerata* cultivars | | ssp. *polygama* cultivar |
| In Figure 1 marked as | 1 | | 2 | | 3 | | 4 | | 5 |
| *n* | 35 | | 5 | | 3 | | 7 | | 1 |
| | mean | *SD* | mean | *SD* | mean | *SD* | mean | *SD* | mean |
| Protocatechuic acid | 12.4 | 3.0 | 13.6 | 2.8 | 12.9 | 3.2 | 12.5 | 3.1 | 14.7 |
| *p*-Hydroxybenzoic acid | 10.3 | 2.6 | 9.9 | 1.9 | 10.0 | 2.1 | 10.4 | 2.5 | 12.4 |
| Vanillic acid | 9.2 | 3.1 | 6.7 | 3.1 | 5.3 | 0.7 | 8.1 | 2.9 | 14.4 |
| Vanilline | 1.8 | 0.9 | 2.2 | 1.0 | 1.5 | 0.2 | 2.3 | 0.9 | 6.6 |
| Chlorogenic acid | 11.5 | 5.3 | 11.1 | 8.4 | 14.7 | 2.9 | 14.7 | 6.6 | 56.0 |
| Caffeic acid | 203.4 | 103.6 | 133.8 | 37.5 | 192.8 | 50.0 | 163.4 | 69.7 | 228.1 |
| *p*-Coumaric acid | 37.8 | 20.7 | 25.1 | 25.5 | 17.3 | 3.5 | 46.5 | 22.2 | 86.6 |
| Ferulic acid | 110.2 | 64.5 | 69.9 | 86.4 | 52.5 | 6.4 | 144.9 | 75.9 | 300.7 |
| Rosmarinic acid | 7.2 | 3.6 | 6.2 | 3.1 | 10.8 | 4.4 | 11.3 | 6.2 | 29.3 |

1. *Dactylis* (cocksfoot) subspecies (Table 1, Figure 1)
2. *Festuca rubra* (red fescue) fodder cultivars (Table 2, Figure 2)
3. *Bromus* (brome) species and cultivars (Table 3, Figure 3)

Herbage samples were collected from field trials at 2 sites (Jevíčko, Tábor-Červený Dvůr) in first cut (heading stage) in case of ad 1, and in 3 terms in case of ad 2, 3 (Table 2, Figures 2, 3) in 2002 and desiccated at 55°C.

The content of 11 non-structural phenolic acids was determined after herbage extraction with a 2M HCl an the fexIKA-Werke 50® extractor, reverse phase on Hypersil BDS C18 sorbent and gradient eluce of mobile phases on the HPLC HP 1100 with DAD detection. The identification of compounds was carried out by the comparison of retention times of standards and by comparison with library of spectra. The data measured (content of phenolic acids in mg/kg dry matter) were standardised, Euclidean measure used and processed by hierarchical cluster analysis according to Ward method.

## RESULTS

Caffeic acid dominated among non-structural phenolic acids in cocksfoot and red fescue, followed by ferulic and *p*-coumaric acids. However, in brome species the caffeic acid took the 3rd position after chlorogenic and ferulic acids. The presence of rosmarinic acid (Tables 1–3) is of interest. Plentiful are also flavonoid compounds like kvercetin, rhamnetin, isorhamnetin and others.

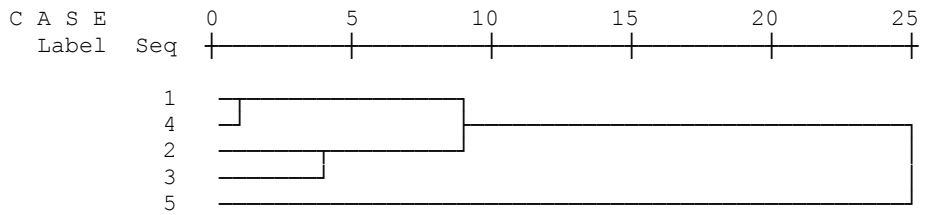


Figure 1. Hierarchical cluster analysis of *Dactylis glomerata* subspecies (for codes 1–5 see Table 1); dendrogram using Ward method; euclidean measure was used; agglomeration schedule using Ward method

Table 2. Content of phenolic compounds in herbage (mg/kg dry matter) of *Festuca rubra* fodder cultivars ($n$ = 4) at 3 subsequent sampling stages

| Phenolic compound | Code | 1st cut, heading | | 1st cut, flowering | | 2nd cut | |
|---|---|---|---|---|---|---|---|
| | | mean | *SD* | mean | *SD* | mean | *SD* |
| Protocatechuic acid | PRO | 15.4 | 2.8 | 17.9 | 3.8 | 24.1 | 3.4 |
| *p*-Hydroxybenzoic acid | POM | 10.7 | 2.8 | 15.1 | 4.3 | 17.0 | 1.5 |
| *p*-Hydroxybenzaldehyde | BALD | 2.7 | 0.2 | 3.6 | 0.8 | 2.4 | 1.1 |
| Vanillic acid | VAN | 27.0 | 2.8 | 32.7 | 2.0 | 15.1 | 3.0 |
| Vanilline | VANL | 27.6 | 7.3 | 38.9 | 6.3 | 19.3 | 6.9 |
| Chlorogenic acid | CHLOR | 172.2 | 71.0 | 130.6 | 46.9 | 172.0 | 61.8 |
| Caffeic acid | CAF | 303.2 | 75.7 | 237.1 | 41.6 | 311.5 | 66.0 |
| *p*-Coumaric acid | PCOUM | 193.1 | 40.3 | 217.2 | 17.8 | 170.7 | 39.1 |
| Ferulic acid | FER | 273.3 | 39.4 | 316.5 | 34.8 | 251.8 | 46.0 |
| Sinapic acid | SIN | 10.6 | 0.2 | 16.8 | 0.3 | 8.7 | 0.1 |
| Rosmarinic acid | ROSM | 5.2 | 2.5 | 5.2 | 2.3 | 10.0 | 5.0 |

But, as to factor loadings, over 3 grass genera studied, ferulic acids provided most information, followed (in descending order) by *p*-coumaric, vanilic acids, vanillin, *p*-hydroxybenzoic, caffeic, chlorogenic, rosmarinic, and protocatechuic acids, *p*-hydroxybenzaldehyde, and finally sinapic acid (Table 4).

Phenolic profiles are sufficiently specific for single groups of *Dactylis*, so according to Euclidean measure, the affinity from taxonomy point of view (Table 1, Figure 1) can be distinctively determined. *Dactylis glomerata* ssp. *polygama* separates in dendrogram from the other tested diploid and tetraploid subspecies. Out of them *D. g.* ssp. *glomerata* ecotypes and cultivars are very close. Relatively close are also diploids of *D. g.* ssp. *galiciana* and *D. g.* ssp. *lusitanica*.

Figure 2 shows the differences between fodder cultivars of red fescue, as to phenolic profiles. Small, rescaled distance of cultivars Tradice and Tagera in dendrogram fully agrees with reality, since the former one was derived using the latter one as main component in the breeding program.

In Figure 3 three discreet groups of bromes were singled out according to the dates of the samples collection (cut). Inside each of them the species belonging to the section *Ceratochloa* (*Bromus catharticus*, *B. sitchensis*, *B. marginatus*, *B. stamineus*) show a smaller distance among them, whereas *B. inermis* (section *Pnigma*) joins them in dendrogram as the farthest.

## DISCUSSION

Out of 11 (resp. 9) phenolic acids over 3 grass genera studied most information was provided by ferulic acid, followed by *p*-coumaric acid, as supports with evidence factor loading (in PCA). Both acids also demonstrate the highest content in

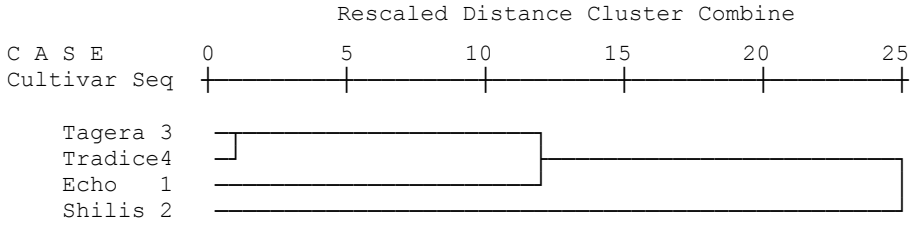


Figure 2. Hierarchical cluster analysis of *Festuca rubra* fodder cultivars; dendrogram using Ward method; euclidean measure was used; agglomeration schedule using Ward method

Table 3. Content of phenolic compounds in herbage (mg/kg dry matter) of *Bromus* species and cultivars ($n$ = 8) at 3 subsequent sampling stages

| Phenolic compound | 1st cut, heading | | 1st cut, flowering | | 2nd cut | |
|---|---|---|---|---|---|---|
| | mean | *SD* | mean | *SD* | mean | *SD* |
| Protocatechuic acid | 7.1 | 3.8 | 6.4 | 1.7 | 11.2 | 2.2 |
| *p*-Hydroxybenzoic acid | 4.8 | 1.4 | 9.6 | 3.2 | 6.4 | 1.2 |
| Vanillic acid | 6.4 | 2.2 | 10.3 | 4.1 | 3.6 | 0.8 |
| Vanilline | 4.3 | 1.7 | 5.9 | 3.8 | 4.7 | 2.5 |
| Chlorogenic acid | 96.2 | 35.8 | 73.3 | 30.8 | 126.2 | 50.0 |
| Caffeic acid | 74.5 | 29.2 | 58.7 | 17.4 | 77.7 | 18.0 |
| *p*-Coumaric acid | 32.4 | 12.5 | 43.4 | 17.4 | 26.7 | 10.7 |
| Ferulic acid | 94.2 | 38.9 | 144.7 | 47.2 | 74.3 | 27.1 |
| Rosmarinic acid | 3.4 | 1.2 | 2.9 | 1.3 | 4.2 | 2.1 |

them (Table 1) and they are known for their high biological activity (Chesson et al. 1982, Míka et al. 2001). Their high content probably goes back to the enzyme phenyl (thyrosine) ammonia-lyase, which abundantly occurs in grasses. It is a central enzyme in phenylpropanoid metabolism (Croteau et al. 2000).

*Dactylis glomerata* is very differentiated species in its both morphological and physiological traits. It creates more subspecies possessing different economic values. Principally it is a complex polyploid with a diploid cytotype. The diploid subspecies separate from the tetraploid ones not only according to isoenzyme and flavonoid polymorphism (Casler et al. 1996), but also according to phenolic profiles (Figure 1). The diploid subspecies prove distinct differences as a clearly differentiated and specialized group (similarly Jay et al. 1996). This picture fully corresponds to the position of tested diploid and tetraploid subspecies in the frame of Eurasian morphogeographical group enunciated recently according to cytogenetic, morphological, geographic and ecological criteria (Lumaret 1988).

The phenolic profiles of red fescue fodder cultivars indicated that they might reveal a degree of genetic affinity. Although cultivar Tradice in DUS tests demonstrated different morphological traits from cultivar Tagera, from which it was bred, their phenolic profiles were highly similar (Figure 2). However, it must be added that molecular technique DNA, which studied compounds of primary metabolism in plants, reacted at least as sensitively in previous tests. The volume of our data is too limited to allow formulation of general recommendation of phenolic profiles as a marker in breeding programmes. The possibility to use phenolic compounds as chemical markers was among the first mentioned by Hubáček and Lachman (1977). They came to significant conclusions for taxonomy of cultivars of spring and winter barley (*Hordeum vulgare* L.) on the basis of the content and proportion of phenolic compounds. The representation of phenolic compounds was also used in taxonomy of genus *Trisetum* (Frey 1996), *Cuscuta* (Loffler et al. 1997), for description of cultivars of grapevine (Di Stefano 1996) and others. Further study of phenolic profiles seems to be then needful and useful, not only for the family *Poaceae.*

Phenolic profiles in genus *Bromus* L. specifically demonstrate genetic affinities among species inside the section *Ceratochloa* (Figure 3). Stebbins (1981) already expressed the idea that all species of North-American octoploid population ($2n$ = 56) should merge in one species regardless of the barriers made by hydrides sterility, which separates many of them. On the other hand, phenolic profiles of both examined Czech brome cultivars (Tacit/*B. marginatus*/belonging to the section *Ceratochloa*, and Tabrom/*B. inermis*/belonging to the section *Pnigma*) demonstrated significant polymorphism and thus justified classification into distinct sections, as stated before.

The relatively biggest difference referring to diversity in phenolic spectra demonstrated herbage samples was in first cut at the stage of heading, smaller in the stage of flowering and smallest in the second cut, in which some brome species do not grow blades, just leafy regrowth. Hence, phenolic spectra recorded at the stage of heading (first cut) in chemotaxonomy research should be preferred (Table 5). To use phenolic profiles more widely as genetic markers, these would have to be not only universal and abundant, but also environmentally stable and convenient to determine.

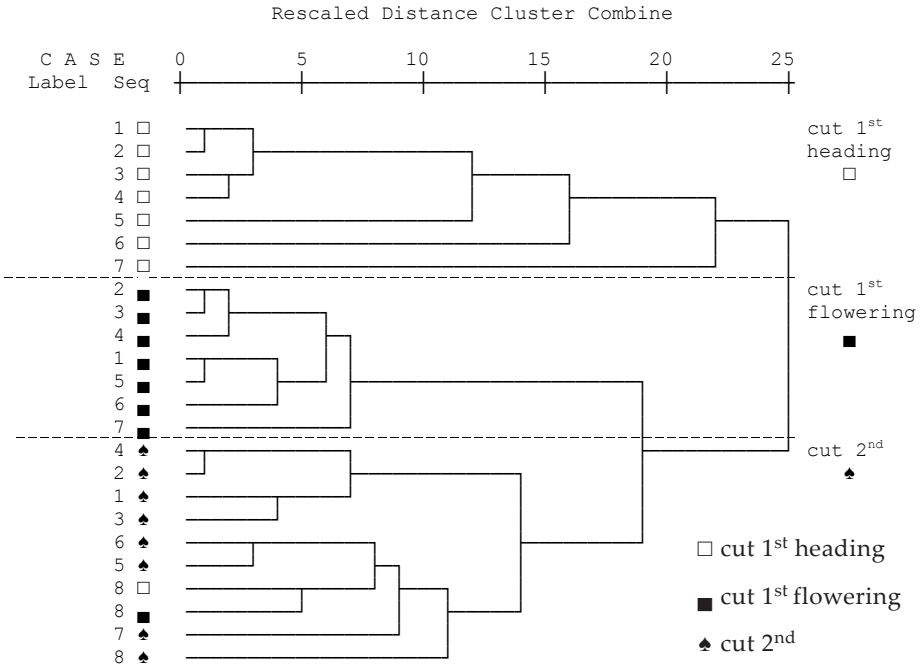


Figure 3. Hierarchical cluster analysis of *Bromus* sp.; dendrogram using Ward method; euclidean measure was used; agglomeration schedule using Ward method

| | | | |
|---|---|---|---|
| 1 | *Bromus catharticus* Grasslands Matua | (NZ) | sect. *Ceratochloa* |
| 2 | *Bromus catharticus* Anabel | (F) | sect. *Ceratochloa* |
| 3 | *Bromus sitchensis* Boris | (F) | sect. *Ceratochloa* |
| 4 | *Bromus sitchensis* Lubro | (F) | sect. *Ceratochloa* |
| 5 | *Bromus marginatus* Tacit | (CZ) | sect. *Ceratochloa* |
| 6 | *Bromus stamineus* Grasslands Gala | (NZ) | sect. *Ceratochloa* |
| 7 | *Bromus stamineus* Grasslands Tiki | (NZ) | sect. *Ceratochloa* |
| 8 | *Bromus inermis* Tabrom | (CZ) | sect. *Pnigma* |

Table 4. Principal component record; factor loadings; for codes of phenolic compounds see Table 2

| *Dactylis* ssp. | | *Festuca rubra* cultivars | | *Bromus* sp. | |
|---|---|---|---|---|---|
| phenolic compound codes | factor loading | phenolic compound codes | factor loading | phenolic compound codes | factor loading |
| VANL | –0.79 | PCOUM | –0.98 | VAN | –0.99 |
| POM | –0.78 | POM | –0.96 | FER | –0.95 |
| PCOUM | –0.77 | CHLOR | –0.90 | VANL | –0.95 |
| VAN | –0.71 | PRO | –0.80 | PCOUM | 0.95 |
| FER | –0.70 | CAF | –0.80 | ROSM | –0.84 |
| PRO | –0.68 | FER | –0.77 | POM | 0.82 |
| CAF | –0.55 | ROSM | –0.47 | CHLOR | 0.73 |
| CHLOR | –0.51 | VAN | –0.38 | PRO | –0.71 |
| ROSM | –0.03 | BALD | –0.29 | CAF | 0.41 |
| | | VANL | 0.10 | | |
| | | SIN | 0.02 | | |

Table 5. Simultaneous tests of significance of simple contrasts for 3 sampling stages of *Bromus* species (1 = cut 1st heading, 2 = cut 1st flowering, 3 = cut 2nd)

| Sampling stage No. | Sampling stage No. | *F* | Significance of *F* |
|---|---|---|---|
| 1 | 2 | 1.80 | ns |
| 1 | 3 | 4.33 | ** |
| 2 | 3 | 2.74 | * |

Significance of *F*: ** at $P_{0.01}$, * at $P_{0.05}$, ns = not significant

Phenolics as a chemical marker can be used as one of the substantial criteria for decisions in plant taxonomy. Rigorously, the expression marker with products of secondary metabolism is not quite correct. The molecules of phenolic compounds are there namely in kind of a balance for a given plant, and they are not biogenetically bound (Jay et al. 1996). The balance is directed by metabolic processes (genetically fixed) as an adaptation reaction of a plant in its long-term history to selection pressure of the environment (Míka et al. 2002). Each individual organism produces a highly integrated image of its micro world (Jay et al. 1996). To evaluate the function values of given molecules or structures it is necessary to make a synthetic analysis of the interaction between genotype and environment and to specify the function of a filter. From this point of view phenolic spectra seem to be somewhat less sensitive compared to markers bound to products of primary metabolism.

On the other hand, introduction of sophisticated laboratory techniques has brought not only a significant increase of detection sensitivity of phenolics, but also due to automation of analytical procedure, a radical increase in the number of analyses. It allows starting intensive research into the use of these markers for lower taxonomic units, for the sake of plant taxonomy as well as other branches.

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Received on January 17, 2005

**ABSTRAKT**

**Fenolové látky jako chemické markery nižších taxonomických jednotek čeledi *Poaceae***

Ke studiu genetické příbuznosti v polyploidním komplexu *Dactylis* L., dále v komplexu oktoploidních druhů *Bromus* a v kolekci kultivarů *Festuca rubra* byla využita spektra nestrukturálních fenolových látek v nadzemní hmotě. Diploidní subspecies *Dactylis* (2*n* = 14) se odlišovaly od tetraploidů (2*n* = 28) jako zřetelně specializovaná skupina. V rámci rodu *Bromus* vykazovaly druhy uvnitř sekce (např. *Ceratochloa*) těsnější příbuzenské vazby než druhy náležející do různých sekcí. Byly stanoveny také rozdíly v příbuzenských vazbách mezi kultivary *Festuca rubra*. Ačkoli se výsledky opírají o jednoleté výsledky ze dvou pokusných míst, přinášejí významné poznatky o možnosti využití fenolových látek. Dokládají, že fenolové profily lze použít v chemické taxonomii trav jako užitečné markery rovněž i u nižších taxonomických jednotek. Jelikož tyto profily nejsou v různých prostředích tak stabilní jako markery primárního metabolismu, rostlinný materiál pro analýzy je třeba připravit standardním způsobem, pokud jde o podmínky růstu, růstovou fázi rostlin apod.

**Klíčová slova:** fytochemie; nestrukturální fenolové látky; trávy; chemotaxonomie; systematika rostlin; sekundární metabolity

*Corresponding author:*

Ing. Václav Míka, DrSc., Výzkumný ústav rostlinné výroby, Praha-Ruzyně, Výzkumná stanice travních ekosystémů, Jevíčko, K. H. Borovského 461, 569 43 Jevíčko, Česká republika
phone: + 420 462 327 814, fax: + 420 462 327 814, e-mail: vste@seznam.cz