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An Investigation of the Protein Characters of Four *Phaseolus* Species with Special Reference to the Question of their Phylogenesis

JOSEF KLOZ

Institute of Experimental Botany of the Czechoslovak Academy of Sciences, Praha*

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Studie bílkovinných znaků čtyř druhů rodu Phaseolus se zřetelem k otázce jejich fylogenese

Byla provedena kvantitativní a kvalitativní analysa bílkovinných znaků z děloh v semenech a z hypokotylů + kořínků (primárního kořene) naklíčených semen těchto druhů: *Phaseolus vulgaris* L., *Phaseolus coccineus* L., *Phaseolus lunatus* L., *Phaseolus aureus* ROXB. s těmito výsledky:

1. Fazeolin téměř shodný je v dělohách druhů *Phaseolus vulgaris* L a *Phaseolus coccines* L.; u druhů *Phaseolus lunatus* L. a *Phaseolus aureus* ROXB. chybí.
2. Byla stanovena bílkovinná složka „Phaseolus protein III", který je obsažen v hypokotylu + kořínku druhů *Phaseolus vulgaris* L., *Phaseolus coccineus* L., *Phaseolus lunatus* L.; u druhu *Phaseolus aureus* ROXB. chybí.
3. Je znovu potvrzena skutečnost nestejné šíře taxonní (skupinové) specifity bílkovinných znaků. Tato skutečnost je uváděna v souvislosti s relativním fylogenetickým stářím znaků. Na základě toho bylo sestaveno vývojové schéma uvedených druhů.

Summary

The results are discussed of a quantitative and qualitative analysis of the protein characters of seed-enclosed cotyledons and of hypocotyls + roots (of the primary root) of germinated seeds of the following species: *Phaseolus vulgaris* L., *Phaseolus coccineus* L., *Phaseolus lunatus* L., *Phaseolus aureus* ROXB.

* *Address*: Ústav experimentáluí botaniky ČSAV, Na cvičišti 2, Praha-Dejvice.

1. A practically identical phaseolin was found in the cotyledons of *Phaseolus vulgaris* L. and *Phaseolus coccineus* L., being absent in *Phaseolus lunatus* L. and *Phaseolus aureus* ROXB.

2. A protein component described as Phaseolus protein III was found in the hypocotyl + root of *Phaseolus vulgaris* L., *Phaseolus coccineus* L. and *Phaseolus lunatus* L.; it was absent in *Phaseolus aureus* ROXB.

3. The inequality of breadth of taxonic (group) specificity of the protein characters has been confirmed anew. This fact is discussed in connection with the relative phylogenetic age of the characters. On the basis of the results obtained a developmental scheme of the studied species is proposed.

Introduction

The present paper contains a unilateral analysis of the protein components (in comparison with *Phaseolus vulgaris* L. only) of four species of the genus Phaseolus: *P. vulgaris* L., *P. coccineus* L., *P. lunatus* L. and *P. aureus* ROXB. The protein characters of the cotyledons and of the hypocotyl + root part of germinated seeds were investigated.

Methods

Qualitative (immunoelectrophoresis — GRABAR, WILLIAMS 1953 in ŠKVAŘIL and co-workers modification 1958) and quantitative (quantitative ring precipitation — KLOZ 1960b, 1961a) serological methods were used.

Protein antigens for testing as well as for immunization were prepared in the following two ways.

1. From dry, non-germinated seed cotyledons freed of germ and seed coat (reserve proteins): The cotyledons were ground to a fine powder and deprived of fat by rinsing twice in acetone (1 : 10) and twice in ether for 3 min. with thorough stirring and brief centrifugation afterwards. The whole operation took place below 0° C.

2. From hypocotyl + root, i.e. from the subcotyledonous parts of seedlings (4 to 5 days of germination at c. 23° C). The isolated subcotyledonous parts were pulverized by homogenization in a knife homogenizer with cooled acetone passing through a Buchner funnel, washing with cold ether and drying. The dry powdery preparations of both cotyledons and of the subcotyledonous parts were kept dry (in a desiccator over silica gel) and in the cold at about 3° C.

Crystalline phaseolin was prepared from the seeds of *Phaseolus vulgaris* L. according to BOURDILLON (1949). Phaseolin from the seeds of *Phaseolus coccineus* L. was prepared in the same way.

Protein extracts were prepared from the powders by extracting with physiological saline (for immunization and quantitative ring precipitation) or with 0·5 M NaCl (for immunoelectrophoresis of cotyledon proteins.) The concentration of the extracted material in solvent was adjusted to contain about 10 mg. protein/ml. for immunization, about 3 to 4 mg. protein/ml. for the quantitative ring precipitation and about 50 mg. protein/ml. for immunoelectrophoresis. The extraction was carried out for several hours, the material pressed in a piece of silk cloth where necessary, the extracts centrifuged, and protein estimated by the biuret reaction. For quantitative ring precipitation the extracts were diluted exactly to 1·5 mg./ml.

In order to obtain specific antisera, rabbits were immunized in the following way:

1. Rabbits immunized with proteins of the cotyledons of *Phaseolus vulgaris* L. were given 11 injections intravenously, a total of 70 mg. protein per animal within 42 days.

2. Rabbits immunized with proteins of the hypocotyl + root received 20 injections (4 of them with an alum adjuvant) intravenously, a total of 300 mg. per animal within 66 days.

Antisera against crystalline phaseolin were prepared by immunization of rabbits with 11 injections of a total of 94 mg. phaseolin within 31 days. The antisera from four rabbits after immunization were combined. They were treated in the usual way, freeze-dried and kept in the cold and dry condition.

For immunoelectrophoresis the antisera (7%) were repeatedly added to grooves in an agar plate (3 times at 40 min. intervals) in order to reinforce the effect.

Quantitative ring precipitation was carried out in several repetitions (with 4 antigen samples and with 4 antisera, the figures shown in Table 1 representing means of measured values. Both immunoelectrophoresis and quantitative ring precipitation were carried out according to the references mentioned above. For further details of methods see KLOZ 1960a, 1961b.

Results and Discussion

It may be stated that the immunoelectrophoretic pattern of the protein characters is typical for the given species and organ (HALL 1959, KLOZ 1961b).

Phaseolus vulgaris and *Phaseolus coccineus* contain phaseolin in their cotyledons as the main protein character (Fig. 1, 2, 3 — see also KLOZ, TURKOVÁ, KLOZOVÁ 1961). The phaseolins of the two species are not immunochemically distinguishable (see Fig. 1 and 2 and Table 2). They differ only in some physicochemical properties (e.g. phaseolin from *Phaseolus vulgaris* can be prepared in the crystalline state by the above method which does not give a crystalline preparation with *Phaseolus coccineus*; they also differ in their solubility at low temperatures. The two phaseolins may thus be assumed to be at the beginning of their differentiation. Since it is absent in *Phaseolus lunatus* and *P. aureus*, it seems probable that phaseolin is a protein character relatively young in phylogenesis. It may therefore be assumed that the species *Phaseolus vulgaris* and *Phaseolus coccineus* are relatively closely related. This seems to be sufficiently founded in spite of the fact that TAUBERT (ENGLER and PRANTL 1891) place *Phaseolus vulgaris* and *Phaseolus coccineus* into different groups. By the specificity of its protein characters, however (see Table 1 and the immunoelectrophoretograms in Fig. 3) *Phaseolus vulgaris* is closer to *Phaseolus coccineus* while *Phaseolus lunatus* and *Phaseolus aureus* are farther apart. This is in agreement with the crossing capacity. It is possible to cross only *Phaseolus coccineus* with *Phaseolus vulgaris*.

Table 1. Intensity of quantitative ring precipitation in testing proteins of different *Phaseolus* species with an antiserum against proteins of *Phaseolus vulgaris* L.

	I cotyledons	II hypocotyl + root
Phaseolus vulgaris L.	100 %	100 %
Phaseolus coccineus L.	87·8	97·2
Phaseolus lunatus L.	5·1	79·0
Phaseolus aureus ROXB.	3·2	65·1

Table 2 Intensity of quantitative ring precipitation in testing phaseolins isolated from seeds of *Phaseolus vulgaris* L. and *Phaseolus coccineus* L. with an antiserum against phaseolin from seeds of *Phaseolus vulgaris* L.

Antiserum against phaseolin from seeds of *Phaseolus vulgaris* L.	Intensity of reaction
× phaseolin from seeds of *Phaseolus vulgaris* L.	12·06 = 100%
× phaseolin from seeds of *Phaseolus coccineus* L.	12·35 = 102·35%

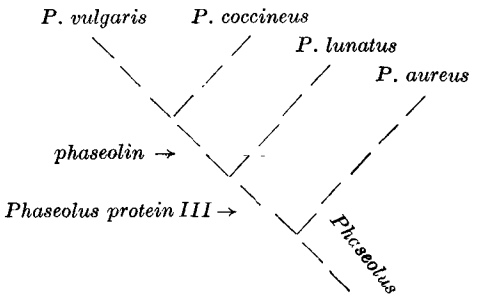
These and further experiences, among them the disagreement between the original classification of the studied species by taxonomists, on one hand, and their genetic characters and the results of the investigation of their protein characters on the other deserve further consideration.

The immunoelectrophoretograms reveal no significant differences between *Phaseolus vulgaris* and *P. coccineus* (Fig. 3 and 4); by the quantitative method, however, the two species can be distinguished with certainty (Table 1).

Differences in the reserve proteins between *Phaseolus vulgaris* and *Phaseolus coccineus* and between *P. lunatus* and *P. aureus* are very striking when using either immunoelectrophoresis or the quantitative method (Fig. 3 and Table 1).

Striking differences were observed between the reserve and the structural proteins from the hypocotyl + root; these differences exist over an inequal breadth of taxonic specificity. In this case the reserve proteins possess a narrower taxonic specificity (they occur in a smaller range of related taxons), the structural proteins of the hypocotyl + root a broader one (they occur over a broader range of related taxons — see also KLOZ 1961b; KLOZ, TURKOVÁ, KLOZOVÁ 1959).

We shall now consider the protein component of the hypocotyl + root which moves farthest toward the cathode (marked with an arrow in Fig. 4). It is a protein character which is clearly present in *Phaseolus vulgaris*, *P. coccineus* and *P. lunatus* while it is absent in *P. aureus*. We shall designate it tentatively Phaseolus protein III (it may be concluded that the Phaseolus protein III has a broader taxonic specificity than phaseolin). Since the presence of a certain protein character in a certain range of related taxons is taken as evidence that the taxons possess a common phylogenetic ancestor (see also KLOZ 1961b), the following evolutionary scheme of these four species may be suggested:



In our limited selection of species the Phaseolus protein III is characteristic for American species (*P. vulgaris, P. coccineus* and *P. lunatus*) while both the Phaseolus protein III and the phaseolin are absent in the Asiatic one (*P. aureus*). It can be assumed that phaseolin, too, is in close connection with the geographic origin of the given species. It will be shown by further experiments when more material is available, to what extent the present conclusions are applicable to other species of Phaseolus (there are 190 of them, two-thirds being of American origin — cf. IVANOV 1960). They might be considered to be of general validity, however, as far as no convergence of the protein characters and no loss of any protein character by mutation occurs. Convergence in protein characters probably represents a very rare case as, for example, in the partial agreement of the protein characters in some cases between host and its parasite (FEDOTOVA 1944, DOUBLY et al. 1960). The question of the loss mutations can be evaluated only when more material is available.

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Й. Клоз (Институт экспериментальной ботаники ЧСАН, Прага)

Белковые признаки четырех видов рода *Phaseolus* в связи с вопросом их филогенезиса

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Проведен количественный и качественный анализ белковых признаков из семядолей в семенах и из гипокотилей + корешков (первичного корня) прорастающих семян следующих видов: *Phaseolus vulgaris* L., *Phaseolus coccineus* L., *Phaseolus lunatus* L,. *Phaseolus aureus* Roxb. с нижеизложенными результатами:

1. Фазеолин почти сходен в семядолях видов *Phaseolus vulgaris* L. и *Phaseolus coccineus* L.; у вида *Phaseolus lunatus* L. и *Phaseolus aureus* Roxb. отсутствует.

2. Определен белковый компонент протеин III, который содержится в гипокотиле + корешках видов *Phaseolus vulgaris* L., *Phaseolus lunatus* L., *Phaseolus coccineus* L.; у вида *Phaseolus aureus* Roxb. отсутствует.

3. Снова подтвержден факт неодинаковой ширины таксонной (групповой) специфичности белковых признаков. Этот факт приводится в связь с относительным филогенетическим возрастом признаков. На основе этого составлена схема развития приведенных видов.