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REVUE ROUMAINE DE BIOLOGIE
SÉRIE DE BIOLOGIE VÉGÉTALE
Calea Victoriei 125
R-79 717, București, România
Téléphone 650 76 80

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Calea 13 Septembrie, nr. 13, sector 5
R-76 117, București, România
Tél. 410 32 00; 401-411 90 08
Tél./Fax: 401-410 39 83

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SOMMAIRE

THE STUDY OF ELECTRON TRANSPORT IN CYANOBACTERIA: PAST, PRESENT AND PERSPECTIVES

I. ARDELEAN, G. ŻARNEA

This minireview is focussed on the main steps involved in the development of concepts concerning respiratory and photosynthetic electron transport in living matter, with emphasis on cyanobacteria. Special attention is devoted to the advantages of using cyanobacteria to study the above processes, mainly after the development of molecular biology. The most important results as well as the perspectives are briefly highlighted, together with our few contributions to this growing field.

Cyanobacteria are the largest and most diversified, ecologically most successful and evolutionarily most important group of procaryotes (Peschek, 1994) defined by the ability to carry out oxygenic photosynthesis, aerobic respiration and nitrogen fixation, the main processes in which electron transport is involved (reviews: Aoki and Katoh; 1983, Babu and Mohanty; 1996; Tli et al. 1996).

The development of photosynthetic research was achieved mainly on chloroplasts and green algae and respiration was copiously studied in mitochondria. The truth is that before 70's cyanobacteria were rather neglected in studies concerning photosynthesis and respiration.

The initial developments of concepts on electron transport (ET) were based on whole cell approaches before more precise experiments become available by the use of cell-free preparations. The shift around 40's from whole cells studies to cell-free system was a major step forward in understanding metabolic processes including ET, as nowadays is the "return" to whole cell studies by the profitable use of specific mutants.

Nowadays cyanobacteria are largely used in studies on respiratory electron transport (RET) and photosynthetic electron transport (PET) mainly because of the availability of specific mutants obtained by molecular techniques ranging from random mutagenesis to site directed mutagenesis (Dzelzkans et al. 1984; Joset, 1988; Hagemann and Zuther1992; Jeanjean et al. 1990; Gabbay-Azarya et al. 1992; Geerts et al. 1994; Yu et al. 1993., Vermaas, 1994, 1996; Manna and Vermaas, 1997) These enable the scientists to perform *in vivo* experiments on whole cells, more informative than whole-cell experiments done 50 years ago. This clarity comes from precise knowledge of genetic differences between the mutant and the wild type.

Nowadays the molecular genetics of bacteria, including cyanobacteria, has greatly advanced with beneficial effects on our knowledge concerning their biol-

ogy. The use of mutants is under increase in studying electron transport processes in (cyano) bacteria and we assume that it will further grow with beneficial effects both for fundamental (Fujita, 1996; Dzelzkans et al. 1984; Hagemann and Zuther, 1992; Jeanjean et al. 1990; Gabbay-Azarya et al. 1992; Geerts et al. 1994; Yu et al. 1993; Vermaas, 1994, Manna and Vermaas, 1997) and applicative oriented research (Waismann and Benemann, 1977, Weaver et al. 1980; Ochiai et al. 1980; Rao and Hall, 1983; Rawson et al. 1987, 1989; Tanaka et al. 1988; Rawson 1988, 1993; de Philippis et al. 1992; Subramanian, 1993, Markwell et al. 1993; Subramanian et al. 1994; Subramanian and Uma, 1995, Patterson, 1996, Malliga et al. 1996; Vermaas, 1996).

The study of the interplay between respiration and photosynthesis as well as the biological significance of respiratory activity under light in intact wild type and transgenic cyanobacteria are constant aims of our research in the last five years (Ardelean 1992; Ardelean et al 1992, 1994 a, b, 1995; Ardelean 1997; Ardelean and Zarnea, 1997 a, b)

The aim of this paper is to briefly summarize the main aspects of fundamental aspects and concepts concerning RET and PET in cyanobacteria. Special attention is devoted to the search of new paradigms of this domain, taking into account its history as well as the evolution of the related domains.

RESPIRATORY ELECTRON TRANSPORT (RET)

This aspect was scarcely studied during the first decade after the first reports on respiration in *Anabaena* sp. (Webster and Frenkel, 1953; Brown and Webster, 1953; Padan et al. 1953; Jones and Myers, 1963). These papers are also important because they put forward the hypothesis concerning the interplay between respiration and photosynthesis in cyanobacteria, a domain rather neglected till 80's (Fay, 1965; Carr, 1969; Leach and Carr, 1970; Biggins 1971; Pelroy et al. 1972; Trebst, 1985).

The study of respiratory activity (RA) in cyanobacteria was very much delayed as compared with similar studies both in eucaryotes (tissues, cells and mitochondria) and heterotrophic bacteria as well. The most important results obtained on RA were obtained till 60s (almost) exclusively on eucaryotic systems (see Table 1).

The beginning of 60s is extremely important for our subject because:

i) in 1961 Mitchell published the first paper on his hypothesis concerning the chemioosmotic mechanism of coupling between electron transport and phosphorylation of ADP via the proton motive force;

ii) in 1962 Stanier and Van Niel published the first paper on the concept of bacterium, further development of which produced the division of life in Procaryotes including cyanobacteria (1977) (Rippka et al., 1979) and Eucaryotes.

The study of bacterial RA and RET (see Table 1), greatly increased around 60's and nowdays is under flourish (Spiller, 1980; Scherer and Boger, 1982, Koike

Table 1

Main important steps in evolution of the concept of respiratory electron transport (after Lundegardh, 1960; Leroy, 1976; Beinert, 1992)

Year	Procaryotes	Eucaryotes
1886		Mac Munn: Discovered histohematines, later called (1925) cytochromes
1912		Warburg: Put forward the idea concerning the existence of a respiratory enzyme able to activate O_2
1922	Wieland: Acetic bacteria can oxidize ethanol in the absence of oxygen, providing that artificial oxidant (e.g. methylene blue) is present	Wieland: Put forward the idea that in tissues there are enzymes called dehydrogenases which activate the bound hydrogen
1925		Keylin: Rediscovered cytochromes in animals, higher plants, fungi
1932		Engelhardt: Discovered that oxidative phosphorylation is coupled with respiration
1933	Gaffron: O_2 consumption by purple sulfur bacteria	Keylin: The isolation of cyt.*c* used to reconstitute electron transport in cell-free preparations.
1934	Keylin: Cytochromes in bacteria	
1937	Nakamura: Light inhibition of O_2 consumption in *Rh. palustris*	
1938–1941	1939 – Lipmann: oxidation of pyruvate supports phosphorylation in *Lactobacillus delbrueckii*	Keiylin: Cyt. oxidase is the respiratory enzyme of Warburg (1912) Kalckar: Quantitative studies on oxidative phosphorylation
1941		Ochoa: Oxidative phosphorylation in cell-free preparation, with P/O > 1
1945		Lundegardth: Theory of anionic respiration linking electron transport to the transport of anions
1948		Robertson and Walkins: Four electrons are required for complete reduction of O_2
1951		Kielly and Kielly: Oxidative phosphorylation is associated with mitochondria
		Lehninger: Oxidative phosphorylation is based on electron transfer from NADH to O_2

Table 1 (continued)

Year	Procaryotes	Eucaryotes
1952		Lardy and Weillman: Respiratory control in mitochondria
1953	Webster and Frenkel: Respiration in *Anabaena*; interaction between respiration and photosynthesis	
1955	Kratz and Myers: The inhibition of respiration by light	
1956		Chance and Wiliams: Spectroscopic definition of components of mitochondrial respiratory chain
1955–1960	Identification in heterotrophic and anoxygenic phototropic bacteria of the main components of the respiratory chain	1957 – Crane et al., Ubiquinone is a component of mitochondria; 1960 – Beinert and Sands: Additional components of the respiratory chain 1960 – Lundegardth: Electrochemical model of cytochrome activity within a biologic membrane
1961	Mitchell: Chemiosmotic hypothesis to explain the mechanism of ATP synthesis coupled to electron transport	
1963	James and Myers: A common link between respiration and photosynthesis	
1968	Biggins and Dietrich: terminal oxidases	
	Horton: NADH oxidases	
1969	Biggins: *In vivo* and *in vitro* assay of respiration using inhibitors active against mitochondrial respiration	
1970–1980	Few important contributions focused mainly on ATP synthesis and respiration chain	
1976	Imafuku and Katoh: The problem of cellular localization of respiration	
1981–1990	The interplay between respiration and photosynthesis is fully recognized	
1986	Peschek et al.: The occurrence of cytochrome oxidase a_3 in plasma membrane and its increase activity under salt stress	
1987	Peschek and Kuentner: Phyloquinone is electron carrier in respiration and photosynthesis	

Year	Procaryotes	Eucaryotes
1990	Jeanjean et al.: The use of mutants impaired both in respiration and in halotolerance	
1992	Ardelean et al.: Aerobic respiration in light as an electron sink for electrons generated by water photolysis	
1994	Peschek et al.: Imunocytochemical localization of cyt. *c* oxidase in cyanobacteria	
1997	Ardelean and Zarnea: Experimental evidence for the increase in respiratory activity in light under salt stress	

and Satoh, 1986; Erdman et al 1989; Nicholls et al. 1992; Jeanjean et al., 1993; Matthjis et al 1984 a, b; Fujita, 1996; Joset et al., 1996; Peschek et al. 1984, 1988, 1994, Peschek, 1996, Wastyn, 1988; Ardelean, 1997; Ardelean and Zarnea 1997 a, b).

Warburg (1912) assumed that during respiration the change of Fe valence within a respiratory enzyme is important. Moreover, Wieland and Thunberg (1922) developed the theory of respiration as dehydrogenation of organic substrates catalyzed by enzymes called dehydrogenases.

In 1932, Engelhardt showed that respiration is coupled with oxidative phosphorylation; quantitative studies on the correlation between the number of atoms of P consumed for phosphorylation and atoms of oxygen consumed within this process were carried out. In 1933, Gaffron found that under aerobic conditions sulfur purple bacteria exhibit the ability to take up O_2.

Lundegardth and Burstrom (1933; 1935) and Lundegardth (1945) elaborated the theory of anionic respiration, arguing that the active transport of anions is casually linked to the transfer of electrons, thereby anticipating one aspect of chemiosmotic hypothesis (1961). Moreover, Lundegardth (1960) elaborated the first electrochemical model of cytochrome systems acting within a biologic membrane.

In bacteria the identification of the main components of the respiratory chain started in 1934 with the discovery of cytochromes, arrested till 50s when, using the same conceptual design and instruments, and even scientists, the components of respiratory chain in few bacteria e.g. (*E. coli* and *Rhodospirillum rubrum)* become known (Jones and Readfour, 1967, Baccarini-Meleandri et al. 1973; Niven et al. 1975; Haddock and Jones, 1977; Gest 1981).

In 1939, Keiylin and Hartree found that the cytochrome a_3 is indeed the respiratory ferment of Warburg, and called it cytochrome oxidase.

In cyanobacteria it appears, up to our best knowledge, that as late as in 1953 the scientific community realized that cyanobacteria do respire; within the next 10 years there were published three papers only on this topic!

The chemiosmotic theory itself did not increase the interest to study RET in cyanobacteria at the beginning (1961), when the main important subjects to study were the less complicated organelle mitochondria and chloroplast.

Around 70s, the main components of the respiratory chain have been recognized in cyanobacteria by taking advantage from both conceptual and experimental achievements accumulated along the previous 50 years of research. Within this period cell-free systems become to be often studied even in cyanobacteriology.

The interest in RET in cyanobacteria (as well as in PET, see next chapter) increased significantly in and after 80's (Spiller, 1980; Scherer and Boger, 1982; Koike and Satoh, 1986; Mullineaux and Allen, 1986; Erdman et al. 1989; Nicholls et al. 1992; Jeanjean et al. 1993; Matthjis et al. 1984 a, b; Fujita, 1996; Joset et al. 1996; Peschek et al. 1984, 1988, 1994; Peschek, 1996; Wastyn, 1988, Ardelean, 1997; Ardelean and Zarnea, 1997 a).

Once the occurrence of RA and RET in strictly autotrophic as well as in facultative heterotrophic cyanobacteria has been well documented, together with the occurrence of common electron carriers active both in RET and in PET (Hoch and Owens, 1963; Hirano et al 1980; Valiente et al. 1992; Hi et al. 1992) a new major scientific problem raised: the cellular localization of respiration. This problem was first evoked by Imafuku and Katoh (1977), but it came up to the interest of scientific community few years later.

Beside RET in bacteria is well known to be located at the cellular membrane (CM) (Haddock and Jones, 1977) some scientists (Omata and Murata, 1985,) argued that in cyanobacteria RET is located exclusively at the thylacoidal level (intracellular membrane-ICM). A very recent review on cyanobacterial RET (1996) recognizing that RET also occurs at the plasma membrane, focused exclusively on RET within ICM, leaving beyond its aim the RET active at the CM.

The occurrence of RET at CM sustained a very vivid dispute between reputed scientists (Omata and Murata, 1985; Peschek et al. 1984, 1988, 1994; Peschek 1996; Wastyn, 1988; Hinterstoisser et al. 1991). Almost 20 years after the problem was raised by Imafuku and Katoh (1977) it is nowdays fully accepted by the scientific community that RET occurs in cyanobacteria both at ICM and CM (Fujita 1996, Peschek 1996). The existence within the same cell of two distinct cellular localizations for RET is unique for cyanobacteria; moreover they are unique organisms to study the interplay between respiration and oxygen photosynthesis (this interplay occurs in anoxygenic phototrophic bacteria too, but they do not evolve O_2).

Here we put forward the idea concerning the possibility that the interplay between photosynthesis and respiration in cyanobacteria and the occurrence of a common electron carrier active in both processes could be only a particular case of the so-called branched electron pathways in bacteria (review Haddock and Jones, 1977; Thauer et al. 1977). Aerobic respiratory pathways branched at the terminal oxidase enable the cells to adapt to different O_2 tensions and/or the presence of

poisons (for example KCN), besides the fact that sometimes one chain is not coupled with phosphorylation (Jones and Redfearn, 1967; Niven et al. 1975).

In anaerobic clostridia branched pathways led to the possibility to generate end products of fermentation in different ratios, ATP being also synthesized in different ratios against substrate. In unbranched catabolic pathways end products and ATP are generated in fixed ratios (Thauer et al. 1977).

Branched electron transport pathways from PSII to PSI and cytochrome oxidase could be a way to maximize PET, as originally suggested by Ardelean et al (1992, 1994, 1995) and further sustained byArdelean (1977); Ardelean and Zarnea (1997 a), Peschek et al. 1995, Geerts (1995).

PHOTOSYNTHETIC ELECTRON TRANSPORT

The sequence of PET in cyanobacteria was assumed (Krogmann 1973) to be described by the scheme of Hill and Bendal (1960); as late as in 1977 in a major collective volume on PET, from around 600 pages only 12 are devoted to cyanobacterial PET. The full extent of these relationships has been appreciated much later (Bricker et al. 1986).

The most important results obtained on the emergence of PET concept were obtained till 60s (almost) exclusively on eucaryotic systems (see Table 2).

Table 2

Major steps in the development of the concept of photosynthetic electron transport (after Clendenning 1960, Leroy 1976, Arnon 1991, modified).

Year	Procaryotes	Eucaryotes
1881		Engelmann: Isolated chloropasts evolve O_2
1906		Tvet: Chromatographic separation of chlorophyll
1913		Wilstater and Stoll: Structure of chl a and b
1922		Warburg and Negelein: Quantum yield of photosynthesis
1931	Van Niel: Unitary concept of photosynthesis in plants and bacteria, as oxidoreduction reaction	
1937		Hill: Found the oxidant dependent – O_2 evolution by isolated chloroplast
1944		Warburg and Luttgens: Hill reaction with *Chlorella* using BQ
1949		Kratz: The ability of chl to lose electrons

Table 2 (continued)

Year	Procaryotes	Eucaryotes
1953		Gilmour et al.: showed that isolated chloroplasts act as electron donors in light Lewitt: Photosynthesis as a flow of electrons induced by light
1951		photoreduction of NAD or NADP by isolated chloroplasts (3 scientific groups)
1953	Webster and Brown – O_2 measurements	
1954	Arnon; Freckel: Noncyclic photophosphorylation in isolated reaction centers (bacteria) and isolated chloroplast	
1960	Hill and Bendall: "Z scheme" to explain the arrangements of electron carriers in photosynthesis	
1961	Mitchell: Chemiosmotic theory to relate electron transport to ATP synthesis	
1964	Susor and Krogman: Hill reaction on cell-free preparation	
1965	Carr and Holway: Hill reaction by intact *Anabaena variabilis*	
1970–1980	PET in different cyanobacteria is studied by many groups following the concepts and methodology developed for chloroplasts	
1981–1990	PET and the interplay with RET become largely studied	
1985	Deisenhofer, Hueber and Michel – the structure of reaction center of purple bacteria	
1993	Jeanjean et al.: Electron flow around PSI is involved in salt tolerance of cyanobacteria	
1990–	Vermaas et al.: Mutants to study PSI and PSII	

The ability of isolated chloroplasts to evolve O_2 under light was discovered long time ago by Engelmann (1881) by using the chemotactic response of motile bacteria to oxygen in anaerobic chloroplast suspension. The "bacterial method", although qualitative, was used as late as 40s to follow O_2.

However, the concept of PET developed steadily the main achievements being presented in Table 2.

1) In 1931 Van Niel proposed a unified conceptual model for both bacteria and plants, photosynthesis being a special case of light induced oxidation-reduction

processes in which the hydrogen donor becomes oxidized and CO_2 becomes reduced.

2) In 1937, 1939 Hill discovered the ability of isolated chloroplast to evolve O_2 coupled to the reduction of electron- (e.g. ferric oxalate or potassium ferricyanide) or hydrogen- acceptor (benzoquinone) substances.

3) Katz (1949) suggested that the absorption of light by chlorophyll enables it to lose electrons that can be transferred to an electron acceptor which is effectively reduced.

4) In 1951 three groups independently and simultaneously found that the isolated chloroplasts can photoreduce NADP and NAD (Vishniac and Ochoa; Tolmach; Arnon) a very important step forward to the concept of PET.

5) The experiments of Gilmour et al. (1953) provided evidence that chloroplasts serve as electron donors when illuminated under suitable conditions by replacing the artificial oxidant with platinum electrode. Upon illumination they generated a photoreductive action on the electrode; this photopotential is not measured when the electrode is loaded with collodium. Further addition of a water soluble electron carrier induced the photopotential. This is the first reported experiment of the use of electrochemical devices to study PET.

6) Lewitt (1953, 1954) proposed that the entire process may thus be visualized as a flow of electrons activated by light.

7) The discovery of cyclic photophosphorylation by Arnon et al. (1954) in isolated chloroplasts and anoxygenic bacteria by Frenkel et al. (1954).

8) In 1958 Arnon et al. discovered non cyclic photophosphorylation ATP being synthesized simultaneously with the electron flow from water to $NADP^+$.

9) In 1960 Hill and Bendal proposed the "Z scheme" concerning the arrangements of electron carriers between the two photosystems.

10) In 1961 Mitchell put forward the hypothesis of chemiosmotic mechanism linking oxidative- and photosynthetic- phosphorylation with electron transport within biologic membranes. This was a great conceptual advance in modern biology (Nobel Prize 1977) to which experiments on chloroplasts and chloroplasts particles were very fruitful.

In between 1965–1990, PET cyanobacteria became largely studied in many laboratories following concepts and experimental designs already developed on chloroplasts, thereby abolishing the delay in cyanobacterial studies.(Rao and Hall 1994 Spiller 1980, Scherer and Boger 1982, Koike and Satoh 1986, Erdman et al. 1989, Mi et al. 1992, Nicholls et al. 1992, Jeanjean et al, 1993, Matthjis et al. 1984 a+b, Fujita 1996, Joset et al. 1996, Peschek et al. 1984, 1988, 1994, Peschek 1996, Wastyn 1988, Ardelean and Zarnea 1997).

Furthermore, toward the end of this period the improvements in molecular biology of cyanobacteria enable them to became favorite materials to study photosynthesis (Dzelzkans et al. 1984; Joset, 1988; Hagemann and Zuther, 1992; Jeanjean et al. 1990; Gabbay-Azarya et al. 1992; Geerts et al. 1994; Yu et al. 1993; Vermaas 1994, 1996).

The most important achievement in 80s was the crystallization of reaction center from anoxygenic purple bacteria followed by the determination of its structure by X-ray analysis (Deisenhoferm Hueber and Michel 1985, Nobel Prize 1988). This advancement is also important because it was the first time when bacteria became leader materials in photosynthetic research.

MUTANTS

The emergence of molecular biology by the achievements in bacterial genetics led to utmost discoveries in the knowledge of metabolism, including electron transport processes in bacteria. As an example, in a review on bacterial respiration, Haddock and Jones (1977) listed 13 mutants of *Escherichia coli* deficient in electron transport processes that were studied in about 30 scientific papers.

The increasing use of cyanobacteria as model systems to study PET and RET is based on their capacity to be modified. Thus the ability to use molecular techniques for genetic modification of electron transfer processes within intact cyanobacteria provides the opportunity to study a given step in far more detail than it was previously possible by managing the growth conditions or / and by random mutagenesis. The delay in knowledge of molecular genetics of cyanobacteria as compared with *E. coli* is the main cause of the corresponding delay in the use of cyanobacterial mutants for metabolic studies.

The cyanobacterium *Synechocystis* PCC 6803 is widely used nowadays because (Vermaas 1994) it is:

i) spontaneously transformable with linear or circular DNA;

ii) integrates foreign DNA into its genome by homologous recombination and

iii) it is able to grow (photo)heterotrophically with glucose as (supplementary) carbon source, so it can be used to study photosystem II (PSII) mutants and/ or photosystem I (PSI) mutants, unable to grow at the expense of light.

Furthermore, in 1996 its entire genomic sequence has been determined (Kaneko et al. 1966 a, b), thus opening avenues toward modification and engineering of biochemical pathways in this cyanobacterium (Vermaas, 1996).

In Table 3 there are presented a few mutants both respiratory and photosynthetic largely used to study RET or PET in cyanobacteria.

RESPIRATORY MUTANTS

In 1990, Jeanjean et al. selected spontaneous mutants (Na^s 43; Na^s 431) of *Synechocystis* PCC 6803 unable to grow under salt stress conditions (0.5M NaCl). These mutants are impaired in respiration as shown by both *in vivo* (O_2 consump-

Table 3

Cyanobacterial mutants used to study electron transport processes

Site of mutation	Encoding	Mutant phenotype	Used so far to study
*psa*AB gene	Core protein of PSI	PSI-less	PSII-structure and function; PSII/PSI relationship
*apc*E gene	Structural and functional protein of PSII	PSII mutants: PBS are not functionally attached to PSII	Energy transmission from PBS to PSII, the functionally assembly of antenna pigments to PSII
*psa*AB/ *apc*E	"	"	Respiration is the electron sink for PSII generated electrons; the mechanism of light sensitivity of PSI-less mutants
psaAB/ psv V	" cytochrome c_{550}	Decreased oxygen evolution and respiratory activities	The involvement of cytochrome c_{550} in the transfer of electrons from photosynthetic to the respiratory electron transport chain in the absence of PSI
*psb*D gene	D2 protein of PSII	Loss of photo-autotrophic growth	PSI and related processes
*psb*C gene	CP43-core protein of PSII	PSII-less mutants	PSII assembly; PSI structure-function
*psb*C gene	Tyr-160 in CP43	PSII-less mutants	Tyr 160 from CP43 is the accessory PSII donor
?	cytochrome oxidase	Loss of salt tolerance	Role of RA and cyt.c oxidase in salt tolerance
*stp*A	45KD protein	Pleiotropic effects	Role of RA, osmoregulators accumulation in salt tolerance
?	?	Fox-	The role of RA in protection and sustaining nitrogenase activity in light
	Plastocyanin	Oversynthesizing plastocyanin	Protein synthesis and sorting; electron flow around PSI/ exogenous PC is active in PET within thylakoids
	"	"	Exogenous PC is active in electron transport at the plasma membrane as well

tion by whole cells) and *in vitro* (cytochrome oxidase specific activity in CM and ICM as well) experiments.

Further analysis of mutant Na^s 431 showed that the locus *stp* a (salt-tolerance protein A)coded for a 45 KD protein, and the pleiotropic phenotype associated with *stp*A-allele includes: sensitivity to several ions and impairment of the capacity to increase the activity of thylacoid-located cytochrome *c* oxidase and PSI electron transfer (Onana et al. 1994).

Reddy et al. (1994) isolated a fast growing unicellular cyanobacterium, *Cyanothece* sp. 68K able to fix N_2 even under continuous light. Under nitrogen fixing

conditions the peak in nitrogenase activity corresponds to that of respiratory activity and fall in photosynthetic oxygen production. As in other unicellular cyanobacteria (review Fay 1992) RA seems to be involved in protection of nitrogenase against O_2 and in providing the energy to carry out its function. Furthermore, in *Cyanothece* sp. 68K the increase in respiratory activity is linked to nitrogenase function because cultures with low or no nitrogenase activity failed to show an increase in RA.

In order to deeper understand the mechanism(s) by which nitrogenase functions under aerobic conditions in light, Reddy et al (1995) used diethyl sulphonate mutagenesis and ampicillin enrichments to isolate:

i) Fox^- mutants able to fix N_2 only under anaerobic conditions;

ii) Fix^- mutants unable to fix N_2 in aerobic and anaerobic conditions either;

iii) Imp^- mutants generally expressed very low nitrogenase activity under either conditions. Fox mutants may contain mutants in RET, including cytochrome *c* oxidase; thus it seems that they are very good candidates to study the interplay between PET, RET and nitrogen fixation (Reddy et al. 1995). The use of respiratory mutants is still in its infancy with cyanobacteria but it seems to be a fortunate topic for cyanobacteria as it started since 60s with non-photosynthetic bacteria, mainly *E. coli*.

PHOTOSYNTHETIC MUTANTS

Following the achievements and improvements in recombinant DNA technology for generation of specific mutants, along with the realization that there are functional and structural homologies between core protein of the two photosystems in oxygenic photosynthesis and reaction centers of anoxygenic bacteria, cyanobacteria become in the first line of photosynthetic research for understanding structure function relationship at the molecular level. (Carpentier and Vermaas, 1989; Yu et al. 1993, Vermaas 1994, 1996), (see Table 3).

The core proteins of PSI are encoded by the *psa*AB operon; deletion within this operon led to PSI-less mutants which are extremely light sensitive. This kind of mutation can be used to study structure-function relationship in PSII without the need to separate membrane fragments enriched in PSII, which is an important improvement for biophysical characterization of electron transport processes (Vermaas, 1994).

Moreover PSI-less mutants are extremely light sensitive which can be alleviated by genetic reduction of PSII antenna size, by deletion of *apc* E gene, coding for a protein anchoring phycobilisomes (PBS) to thylacoids and establishing the functional connection between PBS and PSII.

From experiments on PSI-less mutants *apc* E^- it was concluded that the main reason of the light sensitivity phenotype of PSI-less mutant is the high electron

flux generated by PSII, because the double mutant (*psa*AB⁻*apc*E⁻) exhibits no longer extremely light sensitivity (Vermaas, 1994). Further studies of Vermaas' group have shown that PSII-generated electrons are utilized efficiently by terminal oxidase of the respiratory chain that strongly argues that RET and PET are closely linked in thylacoids of *Synechocystis* PCC 6803, mutants and wild type as well (Vermaas 1994).

By inactivation of *psb*C gene results in decreased amounts of some core proteins (CP47, D1 and D2) of PSII which resulted in loss of assembly of PSII.

Moreover, using site-directed mutagenesis, it was shown that in D2 protein, the mutants obtained by the replacement of histidine residues by tyrosine (His-197-Tyr) or asparagine (His-214-Asp) no longer contained structurally and functionally intact PSII. These mutants are very useful to study molecular events associated with the assembly of functional PSII as well as PSI. The molecular biology techniques provide available approach toward understanding the many complicated structural and functional interactions within PSII and PSI (Vermaas, 1994). It is thus expected that the impact of molecular biology on this topic will significantly increase in the (near) future.

The study of PET and RET could lead to improvements in apparently unrelated domains such us the theory of evolution (see review Broda and Peschek, 1979) and practical applications (Weismann and Benemann, 1977; Weaver et al. 1980; Ochiai et al 1980; Rao and Hall, 1983; Rawson et al. 1987, 1989; Tanaka et al. 1988; Rawson, 1988, 1993; de Philippis et al. 1992; Subramanian, 1993; Markwell et al. 1993; Subramanian et al. 1994; Subramanian and Uma, 1995; Patterson 1996; Malliga et al 1996).

A) Cyanobacteria are important for evolutionary theory because:

i) ancient cyanobacteria were the first cells able to carry out oxygenic photosynthesis; the shift from anoxic to oxic primitive atmosphere was due to cyanobacteria;

ii) ancient cyanobacteria were the first organisms pressed to solve the problem of oxygen toxicity against anoxic metabolism; this struggle is clearly visible in e.g. how nitrogen fixing cyanobacteria manage the photosynthesis and RA to sustain nitrogenase activity, an extremely oxygen-sensitive enzymatic complex;

iii) cyanobacteria have two types of bioenergetic processes/ membrane: a) thylacoidal (ICM) the site of photosynthesis and respiration and b) cellular membrane (CM) the site of respiratory activity;

iv) Some cyanobacteria can perform anoxygenic photosynthesis by using hydrogen sulfide or thiosulphate as primary electron donor instead of water;

v) Many cyanobacteria exhibit fermentative metabolism.

The progress in molecular biology of PET and RET including structure-function relationships in proteins related to these processes could contribute toward a better knowledge of the evolution of bioenergetic processes and of the evolution of procaryotic and eucaryotic kingdoms.

B) Following fundamental achievements concerning their biology, cyanobacteria are subject of intense research for applications (solar energy conversion and storage in useful forms H_2, NH_4, biomass and related compounds, vaste water treatment, environmental protection etc.).

PERSPECTIVES

There are a few main directions in ET studies on cyanobacteria which seem important for the development of cyanobacteriology both fundamental and applicative as well:

1. Molecular modification of PET in order to deeper understand photosynthetic processes at: electron-, molecular-, subcellular- and cellular level;

2. Molecular modification of RET occurring at ICM and /or at CM to better understand the biological significance of respiration in cyanobacteria;

3. The interplay between respiration and photosynthesis in wilde-type cells including extremophilic, symbiotic cyanobacteria, as well as in mutants (1) + (2);

4. The interplay between RET and PET with other processes: nitrogen fixation, transport of solutes (ions, CO_2 etc.) across the biologic membrane;

5. ET in cyanobacteria populations as a part of electron flux through the ecosystem, and related biogeochemical cycles.

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Institute of Biology,
Splaiul Independenței 296
Bucharest 79651
P.O.Box 56–53
Romania

PURIFICATION OF A BACTERIOCIN PRODUCED BY *LACTOBACILLUS ACIDOPHILUS* IB801

MEDANA ZAMFIR, CĂLINA PETRUȚA CORNEA, ION VĂTAFU, LORAND SAVU

Lactobacillus acidophilus IB801 produces a heat stable, strongly hydrophobic bacteriocin, with a narrow inhibitory spectrum. The inhibitory substance produced by this strain was concentrated and purified using different methods. The best results were obtained using a protocol with four steps: removal of the producer cells, ammonium sulfate precipitation, chloroform/methanol extraction/precipitation and reversed phase FPLC. The bacteriocin was isolated as a floating pellicle from the cell free supernatant with 40% of ammonium sulfate; it was precipitated with the organic mixture and eluted from the reversed phase column at 55% of 2-propanol. The estimated molecular weight after Tricine-SDS-PAGE was about 6000 Da.

The production of bacteriocins by lactic bacteria has been known for many years. The potential value of this property in the preservation of fermented food products has been recognized and has prompted many research groups to direct their studies to an investigation of these antagonistic proteinaceous compounds.

Bacteriocins can be defined as biologically active proteins or protein complexes exhibiting antimicrobial properties against bacteria which are usually closely related to the producer organism (5) and they are of great interest to the food fermentation industry because they may inhibit the growth of many food spoiling and pathogenic bacteria. For some bacteriocins, their activity at low pH and low temperature allows their use in acid foods and cold-processed and -stored food products. Therefore, an investigation of bacteriocins in lactic acid bacteria may offer potential applicability in food preservation.

Whereas most bacteriocins produced by Gram-negative bacteria only act on very closely related species, most bacteriocins of Gram-positive bacteria exhibit activity towards a wide range of Gram-positive species. A widely accepted hypothesis for their mode of action is that the bacteriocin acts in two steps, involving its adsorption to specific or non-specific receptors on the surface resulting in cell death (10).

Based on their primary structure, molecular mass and heat stability, bacteriocins produced by lactic acid bacteria can be subdivided into three classes: lantibiotic, small, heat stable bacteriocins (class I), non lantibiotic, small, heat, stable bacteriocins (class II) and non lantibiotic, large, heat sensitive bacteriocins (class III) (3).

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Several bacteriocins have been identified and characterized, but relatively few have been purified. The purpose of this paper is to try different protocols in order to concentrate and purify the bacteriocin produced by the strain *Lactobacillus acidophilus* IB801.

MATERIALS AND METHODS

1. *Bacterial strains and culture media.* The bacterial strains used in this study are described in Table 1. Lactic acid bacteria were maintained in liophilised form or in sterile skim milk supplemented with 0.1% yeast extract. Before experimental use, the cultures were propagated twice in MRS medium (2). The pathogenic test strains were maintained on nutrient agar slants and were activated in Luria Bertani broth.

Table 1

Bacterial strains used in this study

Indicator strain	Source	Growth conditions	Sensitivity*
Lb. delbrueckii subsp. *bulgaricus* 509	IFRB	MRS, 37°C	+
Lb. plantarum ATCC 8014	ICB	MRS, 37°C	–
Lb. lactis	IFRB	MRS, 37°C	–
Lb. casei ATCC 7469	ICB	MRS, 37°C	–
Lb. helveticus 102	IFRB	MRS, 37°C	+
B. subtilis ATCC 6633	IBB	LB, 37°C	–
E. coli Row	IBB	LB, 37°C	+
Pseudomonas aeruginosa IP 5858	ICB	LB, 37°C	+ / –
Staphylococcus aureus ATCC 6538	ICB	LB, 37°C	–

IFRB = Institute for Food Research, Bucharest
ICB = Institute "I. Cantacuzino", Bucharest
IBB = Institute of Biology, Bucharest
* = sensitivity to the action of the bacteriocin produced by *Lb. acidophilus* IB 801
\+ = clear inhibition zone
– = no inhibition zone
+ / – = variability

The bacteriocin producing strain, *Lb. acidophilus* IB801, was obtained from the Culture Collection of the Institute for Food Research, Bucharest and for bacteriocin isolation it was grown in MRS medium for 12h, at 37°C. The best inhibitory activity was observed against *Lb. helveticus* 102. For this reason, in our further studies we used this strain as indicator for the detection and quantitative determination of the bacteriocin activity. As solid media for these experiments we used MACA (Difco) or MRS containing 1.5% agar. For the top layer MRS was supplemented with 0.7% agar.

2. *Detection and quantitative determination of inhibitory activity.* Bacteriocin activity was assayed by an adaptation of the critical dilution method (6). Serial twofold dilution of samples supposed to contain bacteriocin were spotted (10 µl) onto fresh indicator lawns of the indicator strain (*Lb. helveticus*). These lawns were prepared by propagating fresh cultures and adding 200 µl of the cell suspension to 3.5–4.0 ml of overlay agar (top layer). Overlaid agar plates were incubated for 24h at 37°C. The activity was defined as the reciprocal of the highest dilution which demonstrated complete inhibition of the indicator lawn and was expressed in activity units (AU) per milliliter of sample.
3. *Bacteriocin purification.* For bacteriocin isolation, bacterial strain was cultivated for 12h in 2l of MRS broth, at 37°C (we observed that after 12h the inhibitory activity was the highest, after that it decreased slowly, maybe because of the adsorption of the active compound on the cell surface of the producer). After that, cells were removed by centrifugation and the pH of the supernatant was adjusted to 6.5.

   The inhibitory compound was concentrated by ammonium sulfate (at different final concentrations). Sometimes, after ammonium sulfate precipitation, lipid-like floating material could be observed. The precipitate or the floating pellicle obtained after this step was redissolved in 5 mM phosphate buffer, pH 6.5 and then extracted/precipitated with chloroform or with a mixture of chloroform/methanol (2/1).

   The concentrated sample was subjected to chromatography, using different types of columns: a gel filtration column with Sephadex G25 or Acrylex P10, a Bio-Scale Q2 column (Bio-Rad) containing an anion exchange material (Macro-Prep MP10 support derivatized with strongly basic groups) and a Pharmacia Pep RPC HR 5/5 column (reversed phase column).
4. *Tricine-SDS-PAGE.* In order to estimate the molecular weight of the bacteriocin, Tricine-SDS-polyacrylamide gel electrophoresis was carried out (according to H. Schagger and G. Von Jagow) (9). Polyacrylamide concentrations in the stacking gel and separating gel were 9.6% and 16.0% respectively. Electrophoresis was conducted at a constant voltage of 30V for 18h. After that, the gel was washed for 5h with sterile distilled water, replaced every hour and then the gel was transferred to a MACA plate and overlaid with top layer MRS containing the indicator strain, *Lb. helveticus* 102 (1). A part of the gel was stained with copper (II) chloride, as described by Tessmer and Dernuck (11), and another part of the gel, containing standard proteins, was stained with Coomassie Brilliant Blue.

RESULTS

In a first experiment we precipitated the cell free supernatant (with an inhibitory activity of about 800 AU/ml) with 80% of ammonium sulfate. The precipitate

obtained was resuspended in 5mM phosphate buffer, pH 6.5 and dialysed against the same buffer overnight. Two variants of MRS medium were used: with and without Tween 80. When MRS without Tween 80 was used, the inhibitory activity was lost upon dialysis. When Tween 80 was added to the medium, the inhibitory activity of the sample was maintained (about 3200 AU/ml). This fact suggests that bacteriocins are very small molecules, which can penetrate the dialysis bags and Tween 80 could from aggregates with these small molecules, aggregates that are retained in the dialysis bag.

In a second experiment the bacteriocin was concentrated with 60% of ammonium sulfate and we obtained a precipitate and a floating material. They were both collected by centrifugation, redissolved in 5 mM phosphate buffer, pH 6.5 and extracted with 5 volumes of chloroform. Two phases were obtained after this extraction: an aqueous and an organic phase. A fine precipitate was observed in the organic phase and it was collected either by centrifugation or by evaporation (Fig. 1). The inhibitory activity was measured in each fraction by the agar spot method and the results are presented in Table 2. As you can see, the highest activity was obtained in the organic phase, especially after evaporation.

Table 2

Inhibitory activity (AU/ml) of bacteriocin produced by *Lb. acidophilus* IB 801 in different samples

Sample	Activity (AU/ml)	Recovery (%)
Culture supernatant	400	100
Concentrated with ammonium sulfate		
– Aqueous supernatant	0	–
– Pellet (in phosphate buffer)	1000	8.82
– Pellicle (in phosphate buffer)	8000	29.4
Extraction with chloroform		
• pellet – aqueous phase	0	–
– organic phase, evaporated (in distilled water)	16000	11.76
• floating pellicle – aqueous phase	100	
– organic phase, evaporated (in distilled water)	9600	7.05

The concentrated samples were subjected to gel filtration. Two types of gel were used: Sephadex G25 and Acrylex P10. Both of them were equilibrated in 5mM phosphate buffer, pH 6.5 and the bacteriocins were eluted with the same buffer. Fractions of 2 ml were collected and tested for inhibitory activity towards sensitive strain. For the first gel, the activity was detected in some fractions (9–12), with a maximum in the fraction 11 (Fig. 2) and for the column with Acrylex P10, two active fractions (6 and 7) were detected. Nevertheless, using these columns we could not obtain a pure bacteriocin, it was still contaminated with other pro-

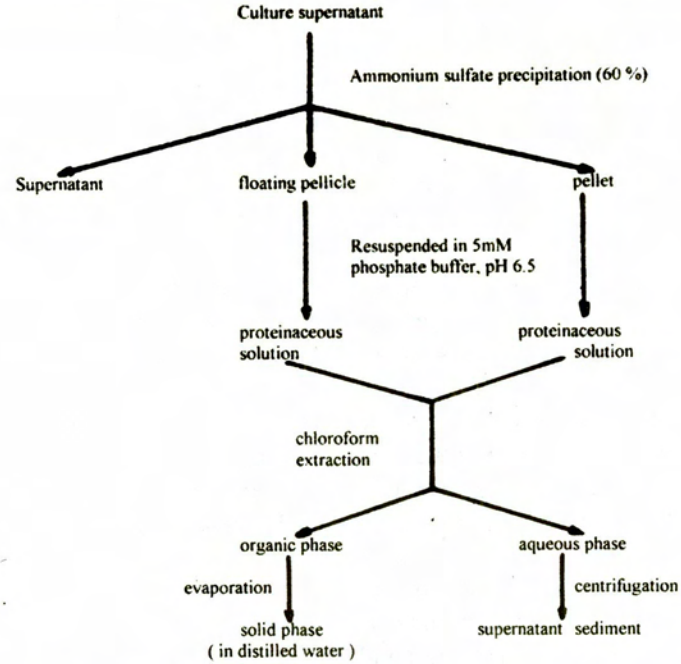

Fig. 1. – Steps in partial purification of bacteriocin produced by *Lb. acidophilus* IB 801.

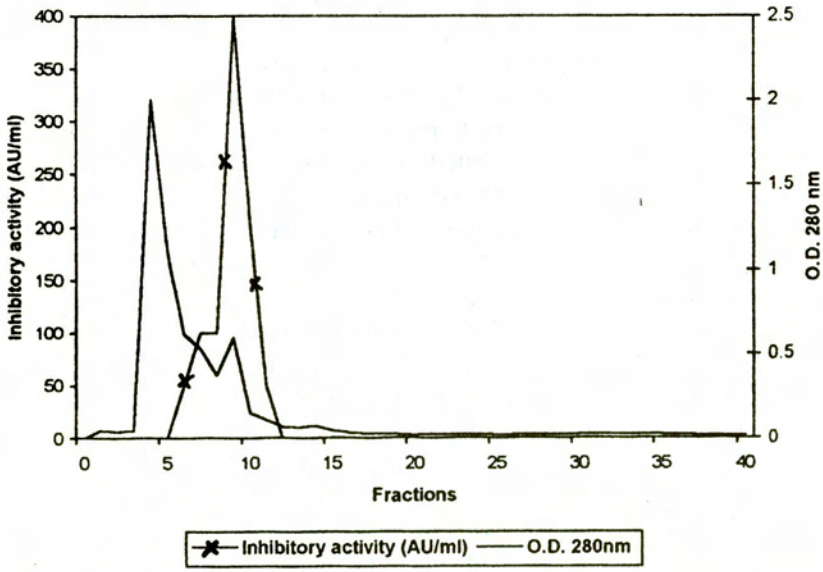

Fig. 2. – Elution profile of the bacteriocin produced by *Lb. acidophilus* IB 801, using a Sephadex G25 column.

teins eluted in front of it. In some experiments we could not detect any activity in the fractions, maybe the bacteriocins adhere to the columns wall because of their hydrophobicity. For these reasons, we had to find other gels or other techniques to purify this special protein.

The same concentrated samples were subjected also to ion exchange chromatography, using a BioSEC-125 HPLC column from BioRad. This Bio-Scale Q2 column contains an anion exchange material (Macro-Prep MP10 support derivatized with strongly basic groups). Elution was performed with a linear gradient of NaCl in 25 mM Tris buffer, pH 8.1. Fractions of 1 ml were collected and tested for inhibitory activity. Our results suggested that the bacteriocin did not bind to the column, inhibitory activity being detected only in the first fractions, before starting the elution with salt gradient.

In a third experiment we used an adaptation of a purification protocol described by De Vuyst et al. and by the research group of Nes (4, 7). This protocol has four steps: removal of the producer cells, ammonium sulfate precipitation (40%), chloroform/methanol extraction/precipitation and reversed phase FPLC.

The cell free supernatant, with the pH adjuster to 6.5, was precipitated with 40% ammonium sulfate at 4°C, during the night, with gentle stirring. In this case we obtained only a floating precipitate, which was collected after centrifugation and dissolved in 5mM phosphate buffer and then extracted/precipitated with 15 volumes of a mixture of chloroform/methanol (2/1), for 1h on ice. The white precipitate formed was centrifuged for 1h and resuspended in distilled water.

This partially purified bacteriocin was then injected to a Pharmacia Pep RPC HR 5/5 column. As mobile phases were used: 10% 2-propanol with 0.1% TFA (solvent 1) and 100% 2-propanol with 0.1% TFA (solvent B). The bacteriocin was eluted with a step gradient, from 10 to 100% of solvent B. The absorbance was measured at 210 and 280 nm. Fractions of 0.5 ml, at a rate of 0.5 ml/min were collected and their activity was tested against *Lb. helveticus* 102. The purified bacteriocin was eluted at about 55% of 2-propanol with a high absorption peak at 210 nm. Two fractions corresponding to this peak were active (Fig. 3).

Tricine-SDS-PAGE

The partially purified bacteriocin (after ammonium sulfate precipitation and chloroform/methanol extraction) was boiled for 5 minutes with an equal volume of sample buffer and then subjected to Tricine-SDS-PAGE for 18h, at 30 V. The gel was then cut in three parts.

A part of it, containing proteins with standard molecular weight (Low molecular weight range Sigma Marker protein standards) was stained with Coomassie Brilliant Blue. Another part was washed for 5h with sterile water and then transferred to a MACA plate and overlaid with the indicator strain. After 24h of incubation at 37°C, a clear inhibition zone corresponding to a molecular weight of about 6000 Da, could be observed (Fig. 4). The third part was stained with 0.3 M cop-

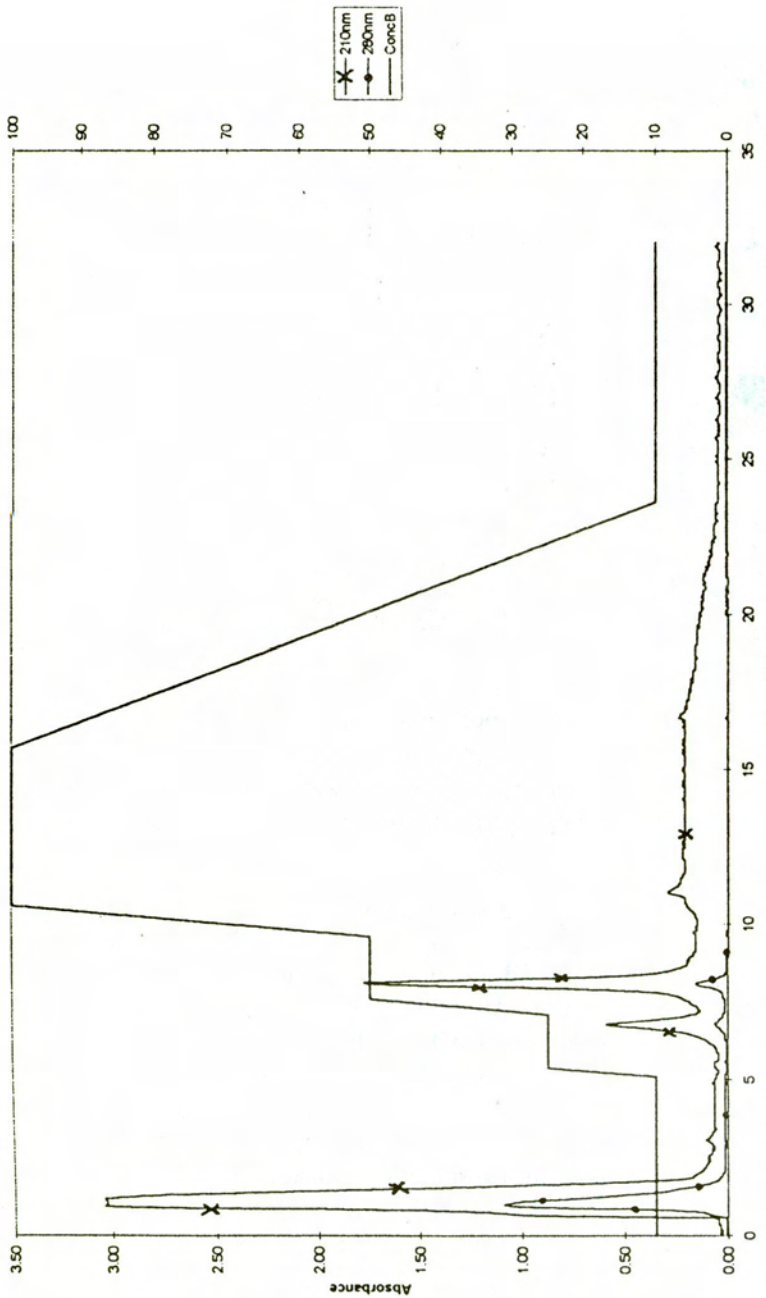

Fig. 3. – Elution profile of the bacteriocin produced by *Lb. acidophilus* IB 801, using a reversed phase column.

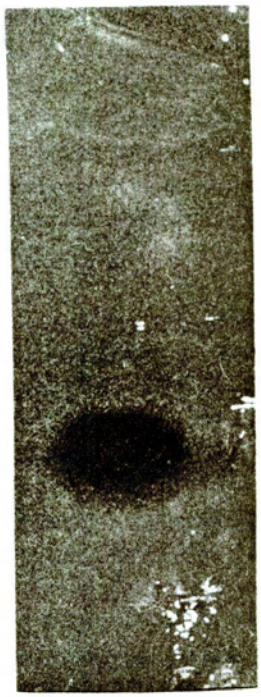

Fig. 4. – Detection of antimicrobial activity in a polyacrylamide gel after Tricine-SDS-PAGE followed by overlaying the gel with *Lb. helveticus* 102.

per (II) chloride for about 5 min at room temperature. Then it was washed 2–3 min in water to remove excess reagent and placed in water for storage. Protein appeared as a transparent band in semi-opaque gel. This band was similar in shape and location with the clear zone obtained by overlaying the gel with the indicator strain.

DISCUSSIONS

The strain *Lb. acidophilus* IB 801 produces a bacteriocin characterized by a quite limited inhibition spectrum. As we previously reported, this bacteriocin is a small, thermostable protein, strongly hydrophobic and stable at very different pH values (8).

Purification of these proteins has proved to be a very difficult task. Problems encountered during purification could be related to the tendency of such molecules to associate with other molecular substances, their hydrophobicity etc. additionally, because they form such an extremely heterogeneous group of substances, specific purification protocols generally need to be empirically designed for each bacteriocin.

Usually, after ammonium sulfate precipitation and centrifugation, lipid-like floating material can be observed. This hydrophobic material contains most of the

bacteriocin activity. The optimal concentration of ammonium sulfate used to precipitate the bacteriocin was 40%. Using larger amounts we could obtain larger amounts of bacteriocins, but they are more contaminated with other proteins which precipitate at these large concentrations of ammonium sulfate. The chloroform/methanol extraction step seems to be necessary when using complex media, such MRS containing Tween 80, which interfers with further purification (4, 7).

Sephadex G25 and Acrylex P10 could not separate our bacteriocin from other proteins and the ion-exchange chromatography was not effective, because the bacteriocin could not bind to the column. The reversed phase column, based on hydrophobic interactions, is appropriate because of the strong hydrophobic nature of the bacteriocins produced by lactic acid bacteria.

The molecular weight of bacteriocins may vary considerably, ranging from small peptides (e.g. lacticin, 1700 Da) to protein-protein and protein-lipid aggregates and macromolecules with a molecular weight of 200.000 Da (e.g. lactocin 27, lactacin B, helveticin J) (3). These aggregates may be disrupted by ultrafiltration or by treatment with detergents or urea. In the case of *Lb. acidophilus* IB 801, under denaturing conditions, a small bioactive protein (about 6000 Da) could be visualised by overlaying the gel obtained after Tricine-SDS-PAGE with the sensitive strain. We intend further to determine the amino acid sequence and the exact molecular mass of the purified bacteriocins and to determine its inhibitory spectrum because it could be changed from the initial spectrum.

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Institute of Biology
Spl. Independenței 296
Bucharest 79651
Romania

IMMUNOFLUORESCENCE STUDIES OF THE MITOTIC SPINDLE IN CIN8 CONDITIONAL DOMINANT LETHAL MUTANTS OF *SACCHAROMYCES CEREVISIAE* CELLS

LUMINIȚA PARAOAN

The present study furthered the characterization of conditional dominant lethal mutant alleles of CIN8 gene, with importance for the morphogenesis of the mitotic spindle in *Saccharomyces cerevisiae* cells. The morphology of cells and the structure of the mitotic spindle were analyzed in hydroxyurea synchronized *Saccharomyces cerevisiae* cells expressing the respective mutant cin8 alleles, following the release in fresh media and incubation at non-permissive temperature. All mutants showed difficulty in passing through mitosis; the phenotype of arrest was that of large-budded, mononucleate cells with short, but intact, preanaphase-like, mitotic spindle. Therefore, it can be implied that all these mutant forms of cin8p impede the action of both Cin8p and Kip1p in the process of transit of the cells from the hydroxyurea arrest point through mitosis. The active role of this kinesin-related microtubule-based motor protein in the relative sliding of the microtubules and a possible role in anaphase could be inferred.

The kinesin-related motor proteins Cin8p and Kip1p were shown previously to act redundantly in achieving an essential function for the morphogenesis of the mitotic spindle in *Saccharomyces cerevisiae* cells. The action of at least one of them is absolutely necessary for the assembly of a bipolar mitotic spindle (Hoyt et al., 1992). Their action is, also, further required for the maintenance of the structural integrity of the mitotic spindle; in their absence, the bipolar pre-anaphasic mitotic spindle, formed under permissive conditions, suffered a rapid collapse, after which the previously separated spindle pole bodies were found side by side (Saunders and Hoyt, 1992). Conditional dominant lethal mutant alleles of CIN8 gene were identified, isolated and characterized through an extensive screening of chemically induced mutants cin8 (Paraoan, 1994). Localization of the respective mutations in the motor domain of Cin8p helped then forward the investigation of structure-function relationship of this microtubule-based motor protein (Paraoan, 1995). Following this, cell biology specific studies based on immunofluorescence have naturally appeared required to further reveal details of the mechanism of action of these gene expression products.

The relevant cellular structures required to be analyzed by immunofluorescence were the intranuclear microtubules, spindle pole bodies and the mitotic spindle as a whole. The distribution and morphology of the mitotic spindle and its compo-

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nents were expected to be informative with respect to the function of wild type and mutant alleles of the gene CIN8 in different strains of *Saccharomyces cerevisiae* conveniently chosen. These strains have ensured the representation of different genetic backgrounds, such as CIN8 and/or KIP1 (wild type) and genomic or plasmidial multicopy alleles, in the presence of which the phenotype determined by the mutant cin8 alleles was investigated.

MATERIAL AND METHOD

The *Saccharomyces cerevisiae* strains used have been kindly provided by Dr. M. A. Hoyt, The Johns Hopkins University, Baltimore, USA. Their relevant genotypes and the main characteristics of the plasmids used are presented in Tables 1 and 2.

Rich (YEPD) and minimal (SD and SDC) media were prepared as described by Sherman (1983).

The *Saccharomyces cerevisiae* cultures with a cell density of around 10^7 cell/ml were either directly fixed or subject to change of their media conditions, i.e., change of incubation temperature and/or chemical treatment with hydroxyurea to synchronize the cells in the cell cycle (in S-phase of the cell cycle, as large budded, mononucleated cells). $1–5\times10^7$ cells were used per experiment.

Table 1

Saccharomyces cerevisiae strains used

Strain	Relevant genotype
MAY 591	α CIN8 KIP1 his3-Δ200 leu2-3112 lys2-801 ura3-52
MAY 2063	a cin8::URA3 KIP1 ade2-101 his3-Δ200 leu2-3112 lys2-801 ura3-52
MAY 2104	α CIN8 kip1::HIS3 ade2-101 his3-Δ200 leu2-3112 ura3-52 $cyh2^R$
MAY 2275	a cin8::URA3 kip1::HIS3 ade2-101 his3-Δ200 leu2-3112 lys2-801 ura3-52 $cyh2^R$ (pMA 1208)

Table 2

Recombinant plasmids used

Plasmid	Relevant characteristics	Size (kb)	Vector of origin (Cloning site)	Mutants analyzed
pMA1189	CIN8 LYS2(CEN)	12.7	pRS317(XbaI/SalI)	cin8-5c
pMA1208	CIN8 LEU2(CEN)	12.8	pRS318(BamHI/SalI)	cin8-201...206

The synchronization in the cell cycle was done with hydroxyurea (final concentration in media 0.1M) at 26°C. The DNA-specific staining with DAPI (4,6 diamidino-2 phenylindol) 1μg/μl was used to enable the subsequent analysis of size and distribution of the nuclei. When approximately 70% of *Saccharomyces cerevisiae* cells reached the morphology of cells with a large bud and one nucleus,

the cells were resuspended in fresh media lacking hydroxyurea and further incubated at 37°C. Samples of these cultures, at 30 minute intervals, were subsequently analyzed with regard to the distribution of cell morphology (presence or absence of a bud, size of the bud, mono- or binucleated cells). Samples from cultures of different phenotypes of interest were then fixed and subject to microtubule-specific immunofluorescence.

The indirect immunofluorescence technique used in this study was adapted after Pringle et al. (1981). Variations of different factors, as fixation of cells, permeabilization of cell wall, concentration of primary/secondary antibody were tested. The specific phenotype of the mitotic spindle observed for the strain MAY591 was used as a control for the study of the mutant cin8 phenotypes.

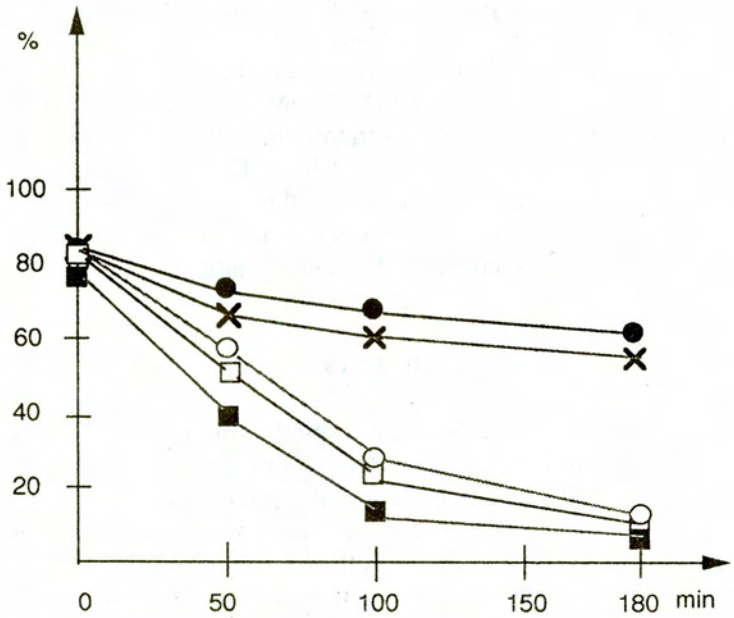
The cells were fixed in formaldehyde (final concentration 3.7%) in buffer solution (40mM K_2HPO_4, 10mM KH_2PO_4, 0.15M NaCl), pH 6.5, 1 hour at room temperature. The controlled permeabilization of the cell wall was achieved in the presence of zymolase (50µg/ml), 30 minutes at 30°C. The microtubules structure was analyzed using as a primary antibody the anti-tubulin monoclonal antibody YOL1/34 (Kilmartin et al., 1982; Bioproducts for Science, Inc.) in a dilution of 1:250 and the corresponding fluorescein-conjugated secondary antibody (Stearns et al., 1990).

RESULTS

The cell morphology and the mitotic spindle assembly and behavior were simultaneously monitored in *Saccharomyces cerevisiae* cells of the genotypes described which were firstly synchronized at 26°C and then released in fresh media at 37°C. Samples corresponding to cultures incubated for different times in the conditions described were fixed in 70% ethanol and DNA-specific stained with DAPI. About 500 to 800 cells were analyzed in each case. Cells were classified as unbudded, with small bud or with large bud (the size of the bud at least 50% of the size of the mother cell). The DAPI-stained chromosomal DNA mass revealed the number of nuclei per cell. The microtubules and mitotic spindle structure were analyzed by immunofluorescence performed as described using formaldehyde-fixed cells.

The loss of large-budded mononucleate cells from each culture and the consequent appearance of large-budded binucleate cells indicated the ability of cells to pass through mitosis. Cells with a wild type copy of CIN8 and/or KIP1 behaved likewise and were able to proceed successfully through mitosis under these conditions (Table 3, Fig. 1). The morphology of the mitotic spindle was the one characteristic for the phenotype of the wild type cells (Fig. 2 and Fig. 3 A–D). The cells did not present any difficulty in the process of assembly and/or elongation of the mitotic spindle. The majority of *Saccharomyces cerevisiae* cells with small but distinct bud had a short mitotic spindle, while practically all cells with a large bud and an enlarged mass of DNA or two distinct masses of DNA presented an elongated mitotic spindle, specific to the anaphase.

The analysis of the phenotypic changes induces by the mutant alleles cin8 in *Saccharomyces cerevisiae* cells, with regard to the morphology and the function of mitotic spindle, was performed in the case of all seven conditional dominant lethal cin8 alleles previously isolated through genetic tests (Paraoan, 1994 and 1995). Despite the similarity of the phenotypes induced, the mutants were still grouped in two distinct classes, based on their different abilities to sustain the viability of *Saccharomyces cerevisiae* cells in the absence of any copy of wild type alleles CIN8/KIP1, i.e. cin8-5c presented this ability, while cin8-201...cin8-206 did not.



Saccharomyces cerevisiae cells from hydroxyurea synchronized cultures at 26°C were released in fresh media and incubated at 37°C. The percentage of large-budded, mononucleate cells is represented as a function of the incubation time at 37°C.

□ Wild type CIN8/KIP1 (MAY591)

■ kip1-Δ (MAY2104)

× cin8-201...cin8-206 + CIN8
MAY2104 + pMA1208(cin8)

○ Multiple copies CIN8 (MAY2275)

● cin8-5c + CIN8
MAY2275 + pMA1189(cin8-5c)

Fig. 1. – Percentage of large-budded, mononucleate cells, following the transfer at non-permissive temperature (37°C).

The effect of mutant cin8 alleles was analyzed in different *Saccharomyces cerevisiae* strains with relevant genotypes: kip1-Δ; cin8-Δ kip1-Δ p(CIN8); CIN8 KIP1. Thus, the phenotype induced by the mutant alleles cin8-201...cin8-206 in the presence of wild type CIN8 was studied both in the presence and in the absence of the functionally redundant gene KIP1 and the phenotype characteristic of the cin8-5c mutant was studied both in the presence of multiple copies of CIN8 and in the absence of CIN8 and KIP1.

Firstly, the distribution of cellular morphology was observed at non-permissive temperatures. The structure of the bipolar mitotic spindle, assembled under permissive conditions of temperature, was then analyzed at different times following the shift to non-permissive temperature, when the phenotype induced by the mutant alleles became apparent.

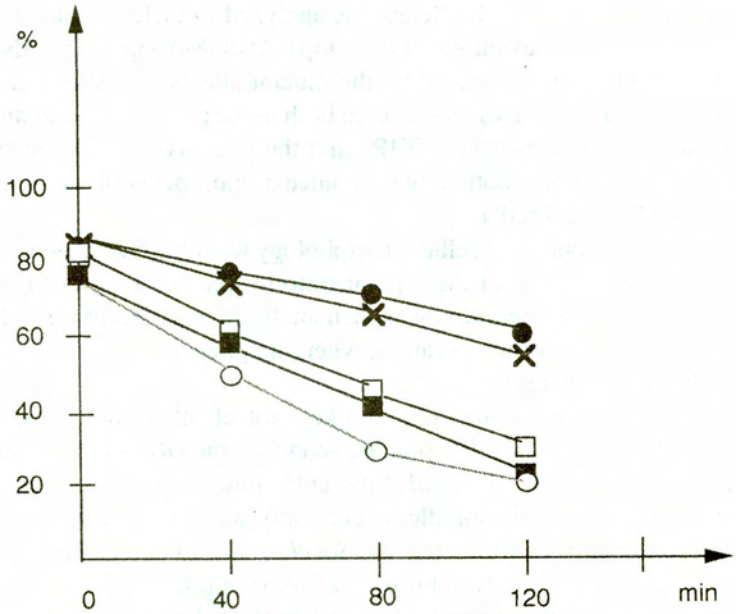
Saccharomyces cerevisiae cells, unlike a lot of other eukaryotic cells, are able to assemble a bipolar mitotic spindle even when the DNA replication is inhibited (Pringle and Hartwell, 1981). But the entry into anaphase, characterized by the elongation of the mitotic spindle, occurs only when the synthesis of DNA is complete. Consequently, as expected, the *Saccharomyces cerevisiae* cultures synchronized with hydroxyurea (inhibitor of DNA synthesis) at 26°C had approximately 80% of cells with a large bud, mono-nucleated and with a short, bipolar mitotic spindle in the nucleus (Table 3).

Following the shift to 37°C, all mutants determined similar phenotypes, characterized by a markedly different distribution of the cellular morphology com-

Table 3

Distribution of cellular morphology in synchronized cultures of mutant cin8 *Saccharomyces cerevisiae* cells. *(mean values characteristic of the mutants in each class)*

Mutant	Morphology (Type of cell)	Synchronized culture with HU, 26°C	Synchronized culture in fresh media, 37°C (3 hours)
CIN8/KIP1	unbudded	13%	42%
	small-budded	7%	45%
	large-budded, mononucleate	80%	13%
cin8-5c	unbudded	16%	21%
	small-budded	4%	18%
	large-budded, mononucleate	80%	61%
cin8-201-206	unbudded	15%	21%
	small-budded	6%	20%
	large-budded, mononucleate	79%	59%



The percentage of *Saccharomyces cerevisiae* cells with short spindle is represented against the incubation time at 37°C.

- □ Wild type CIN8 KIP1 (MAY591)
- ■ kip1-Δ (MAY2104)
- × cin8-201...cin8-206 + CIN8 MAY2104 + pMA1208(cin8)
- ○ Multiple copies CIN8 (MAY2275)
- ● cin8-5c + CIN8 MAY2275 + pMA1189(cin8-5c)

Fig. 2. – Percentage of cells with short mitotic spindle in synchronized cultures, following the release in fresh media at 37°C.

pared with the wild type CIN8/KIP1 strains. The most relevant of all was the percentage of large-budded, mono-nucleated cells of 59–61% in the case of mutant cells, 3 hours after the transfer at 37°C of the synchronized cultures, in fresh media without hydroxyurea (Table 3 and Fig. 1). The mutant cells were able to pass through mitosis after the arrest with hydroxyurea only when released in fresh media at 26°C.

The morphology of mitotic spindle was analyzed by tubulin-specific immunofluorescence in the case of all mutants arrested in the cell cycle at 37°C. With regard to this aspect, too, mutants from both classes presented a similar phenotype. After 2 hours of incubation in fresh media at 37°C, about 60% of cells presented a short, but intact mitotic spindle (Fig. 2 and Fig. 3 E–H). Previous studies

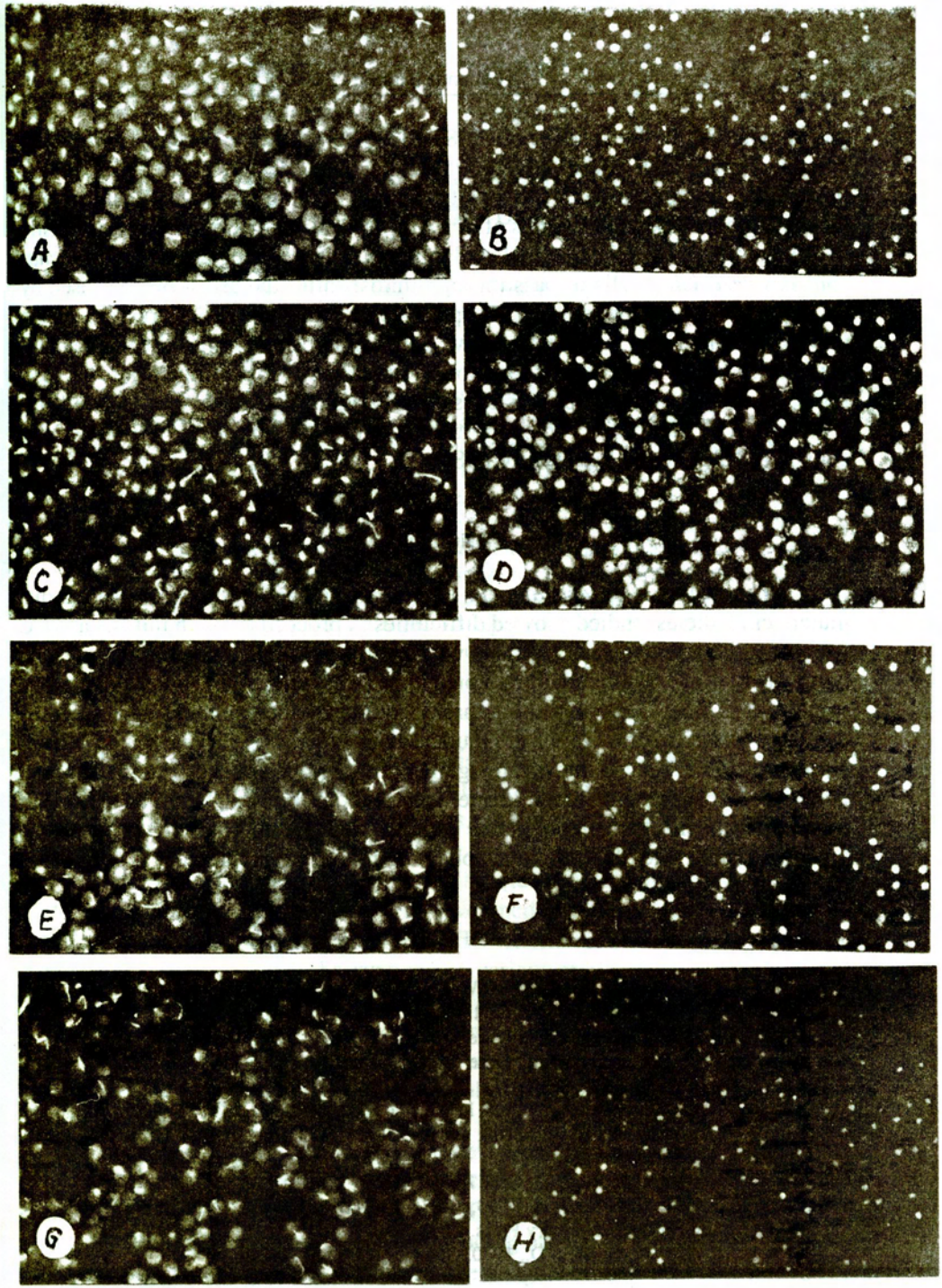


Fig. 3. – Immunofluorescence microscopy specific for tubulin. Synchronized cultures of wild type CIN8 and mutant cin8 *Saccharomyces cerevisiae* cells were released in fresh media and incubated at 37°C for 2 hours. Cells were fixed and stained by immunofluorescence as described. A, C, E and G – staining specific for tubulin; B, D, F and H – same field of cells, stained with DAPI; A and B – CIN8, hydroxyurea synchronized cells, 26°C; C, D, E, F, G and H – after 2 hours of incubation at 37°C in fresh media: C and D – CIN8; E and F – cin8 5c; G and H – cin8-201.

had shown that, in the absence of Cin8p/Kip1p function, the preanaphase mitotic spindle formed under permissive conditions collapsed, thus clearly indicating a role of this function also in the maintenance of spindle structure (Saunders and Hoyt). With this view, the results of this study revealed an important characteristic of the conditional dominant lethal cin8 mutants. These alleles stopped the *Saccharomyces cerevisiae* cells to pass through mitosis after the arrest point caused by hydroxyurea while maintaining the preanaphase-characteristic structure of the mitotic spindle. From this point of view, the phenotype determined by these alleles could be regarded as a "gain of function", rather than a "loss of function". Interestingly, this "rigor" phenotype was caused by two different types of mutant alleles, with regard to their ability to support the viability of *Saccharomyces cerevisiae* cells in the absence of wild type CIN8.

CONCLUSIONS

Saccharomyces cerevisiae cells expressing the conditional dominant lethal mutant cin8 alleles studied showed difficulties to proceed through mitosis at 37°C following the arrest in the cell cycle with hydroxyurea. The predominant phenotype was that of large-budded, mononucleate cells, with short, but intact mitotic spindle. It is very likely that the lethality caused by these mutant cin8 alleles is mediated by the interaction of the mutant gene expression products with the microtubules. This fact constitutes an argument for considering the protein Cin8p not only as a simple component of the inter-microtubules bridges, but as an active part involved in the mechanism of relative sliding of nuclear microtubules. At the same time, a possible role of Cin8p in anaphase could not be excluded.

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University of Bucharest
Department of Genetics
1–3 Aleea Portocalilor
Bucharest

MOLECULAR TAXONOMY STUDIES OF SOME LACTIC ACID BACTERIA STRAINS

ORTANSA CSUTAK, ILEANA STOICA, TATIANA VASSU, ELENA SASARMAN, RAMONA CERNAT, FLORIN MUȘAT

In the present paper the authors determined the molar percent of guanine and cytosine (% mol GC) of two lactic bacteria strains isolated from the intestinal tractus of pigs: *Lactobacillus sp.* and *Streptococcus sp.*, with the view to making a more accurate taxonomical classification of the two strains. First, the bacterial DNA was isolated and purified by the author's improved technique. The optimum concentration of lysozyme – 10 mg/ml^{-1} – and a 3h incubation time were established. The DNA samples were tested spectrophotometrically and electrophoretically and their integrity and purity were verified. The estimation of the GC content of DNA was achieved by the method of DNA thermal denaturation, the obtained values being recorded on the hyperchromic shift curves. The results obtained for *Lactobacillus sp.* % mol GC = 39.2 and for *Streptococcus sp.* % mol GC = 18.4 are equivalent to the values characteristic of the studied genera mentioned in the literature.

Due to their ability to perform lactic fermentation, lactic acid bacteria have been widely used since ancient times for food processing. Strains of *Lactobacillus* and *Streptococcus* are commonly used as starter cultures for meat, vegetables and bakery products (Chassy, 1985).

Given the wide range of genera and species generally named as "lactic bacteria", new molecular techniques were developed in order to provide a more accurate taxonomic characterisation. The classic taxonomic studies based on morphological and physiological observations of various strains provided only few data concerning the phylogeny of this group. Lactic acid bacteria are gram-positive, non-spore-forming cocci, coccobacilli or rods and they need a fermentable carbohydrate for growth: glucose is converted mainly to lactic acid (homofermentative strains) or to lactic acid, CO_2, and ethanol or acetic acid or both (heterofermentative strains).

In the last 10 years the spectacular development of molecular techniques that allowed the determination of DNA base ratio (usually expressed as the molecular % of guanine plus cytosine = % mol GC), the estimation of the homology level between two species or strains, the sequencing of total or mitochondrial DNA and genes coding for rRNA, revolutionized the insights of the bacterial phylogeny and taxonomy.

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The determination of genomic DNA guanine+cytosine molar percentage (%mol G+C) represents one of the most accessible and accurate techniques extensively used in molecular taxonomy which allows a better classification of microorganisms in new phylogenetic trees (Olsen and Woese, 1994).

MATERIALS AND METHODS

Bacterial strains, media and growth conditions. We studied two lactic bacteria strains: *Lactobacillus sp.* and *Streptococcus sp.* isolated from the intestinal tractus of pigs. The *Lactobacillus sp.* strain was cultivated on MRS liquid medium (DeMan, 1960) at 37°C, and the *Streptococcus sp.* strain was cultivated at 37°C on LIA liquid medium (lactose 1%; tryptone 1%; yeast extract 0.5%; gelatine 0.25%; NaCl 0.4%; ascorbic acid 0.05%) pH = 7.

Chromosomal DNA isolation and purification. We used the Ausubel technique (1995) as starting point and we modified it through successive testings for our strains, establishing the optimal conditions for bacterial chromosomal DNA isolation and purification. 1.5 ml of an overnight bacterial culture was centrifuged for 10 min. at 5000 rpm and the cellular pellet was resuspended in 1.5 ml TEG pH = 8 (Tris 25mM; EDTA 10 mM; glucose 50 mM) and lysozyme (10 mg ml^{-1}). After a new centrifugation the pellet was resuspended in 1 ml NAE (NaCl 0.15 M; EDTA 0.1 M), 0.2 ml proteinase K (1 mg ml^{-1}) and 0.1 ml SDS 20% and the mixture was incubated 3 hours at 37°C, then 20 min. at 60°C, and finally centrifuged for 20 min. at 10000 rpm. The supernatant was treated with an equal volume of chlorophorm:isoamilic (24:1) mixture. The upper layer was extracted with isopropanol at room temperature. Precipitated DNA was pelleted by centrifugation at 13000 rpm for 25 min. and resuspended in TE pH = 8 (Tris 10 mM; EDTA 1 mM).

DNA samples integrity and purity. An agarose gel electrophoresis of the DNA samples was performed in order to verify DNA integrity and low molecular weight RNA contamination (Provence 1994). The degree of proteic and sugar contamination was verified spectrophotometrically through the absorbance values at different wavelengths: $\lambda = 280$ nm for proteins, $\lambda = 300$ nm for sugars, and $\lambda = 260$ nm for DNA. A 1.8–2.0 value for the A_{260}/A_{280} ratio and a minimum value of A_{300} are considered to be optimal for proceeding to the next step of the experiment, e.g. the thermal denaturation of the DNA samples for % mol GC determination.

The % mol GC determination. The less time consuming method for determining the G+C content is based on the estimation of the melting temperature (Tm) of genomic DNA. The variation of the DNA samples absorbance at $\lambda = 260$ nm during the genomic DNA progressive thermal denaturation was registered and the distribution profiles of these values were plotted on a hyperchromic shift graph. The %mol G+C was calculated reported to T_m value using Owen's formula: % mol GC = $2.08 \times T_m - 106.4$.

RESULTS

For the isolation and purification of the DNA from the two lactic acid bacteria strains *Lactobacillus sp.* and *Streptococcus sp.* we used as starting point the Ausubel technique. After many experiments a lysozyme concentration of 10 mg/ml and a 3 hour time of incubation were established as being optimal.

The purity of the isolated DNA was spectrophotometrically verified, and as seen in Table 1, we found that the DNA samples showed a high level of purity, with minimal proteic and sugar contamination since an optimal A_{260}/A_{280} ratio and a minimal A_{300} value were obtained for both lactic bacteria strains.

Table 1

The spectrophotometric estimation of proteic and sugar contamination degree of the DNA samples using the A_{260}, A_{280} and A_{300} values

Bacterial strains	A_{260}	A_{280}	A_{260}/A_{280}	A_{300}
Lactobacillus sp.	0.85	0.415	1.975	0.054
Streptococcus sp.	0.77	0.383	1.970	0.020

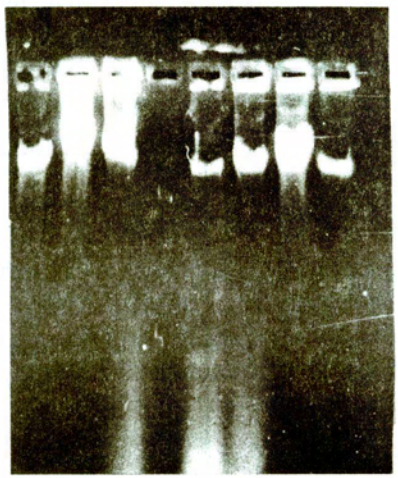

Fig. 1. – Agarose gel electrophoresis showing the integrity and minimal low weight RNA contamination of the chromosomal DNA samples [lanes 1, 2 – *Streptococcus sp.*; lane 3 – *Lactobacillus sp.*; lanes 5, 6 – *Lactobacillus sp. GM* (Genetic of Microorganisms Lab Collection); lanes 7, 8 – *Streptococcus sp. GM* (Genetic of Microorganisms Lab Collection)].

Agarose gel electrophoresis of the chromosomal DNA, (Fig. 1), allowed us to consider that the DNA was not hydrolysed during the procedure and appeared as a single band in gel. It is also evident the minimal RNA contamination of the DNA samples.

Finally, after performing all these tests, we concluded that the chromosomal DNA in our samples was ready for the next steps of % mol G+C estimation.

DETERMINATION OF GUANINE+CYTOSINE CONTENT OF THE CHROMOSOMAL DNA (% MOL GC)

As we have already mentioned the estimation of % mol GC was made by using the thermal DNA denaturation technique. The DNA samples were slowly heated from 20°C (room temperature) to 100°C, and the specific values of DNA absorbance at $\lambda = 260$ nm have been registered with an increment of 10°C.

The maximum A_{260} values for both *Lactobacillus sp.* and *Streptococus sp.* were registered at a temperature of 100°C (1.09 and, respectively, 0.985). The hyperchromic shift was observed to begin at 70°C for *Lactobacillus sp.*, when the A_{260} rose from 0.975 to 1.04, and at 60°C for *Streptococcus sp.*($A_{260} = 0.878$ at 60°C, $A_{260} = 0.924$ at 70°C). (Table 2)

Table 2

The hyperchromic effect of the DNA samples denaturation illustrated by the A_{260} values

Bacterial strain	30°C	40°C	50°C	60°C	70°C	80°C	90°C	100°C
Lactobacillus sp.	0.85	0.905	0.935	0.951	0.975	1.04	1.07	1.09
Streptococcus sp.	0.77	0.834	0.852	0.878	0.924	0.927	0.962	0.985

These data were plotted on hyperchromic shift graphs (Fig. 2, Fig. 3), and the T_m (melting temperature points) were found out by extrapolating the average absorbances (A_m).

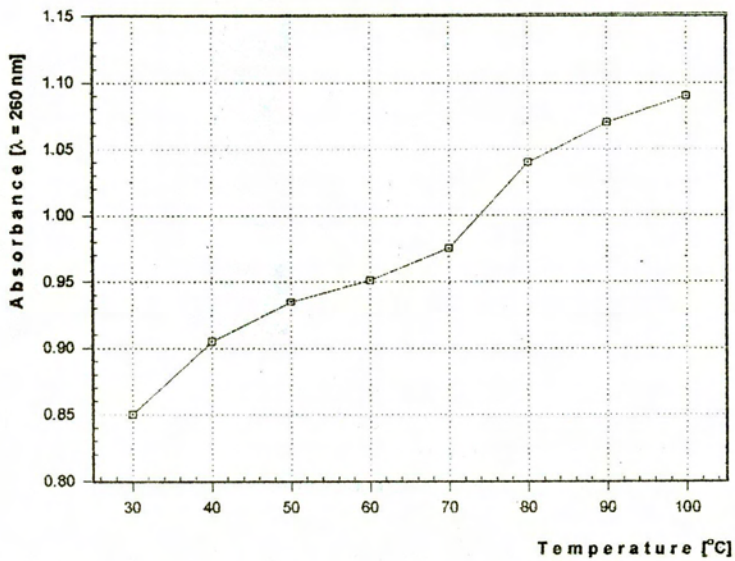

Fig. 2. – Hyperchromic shift of chromosomal DNA at *Lactobacillus sp.*

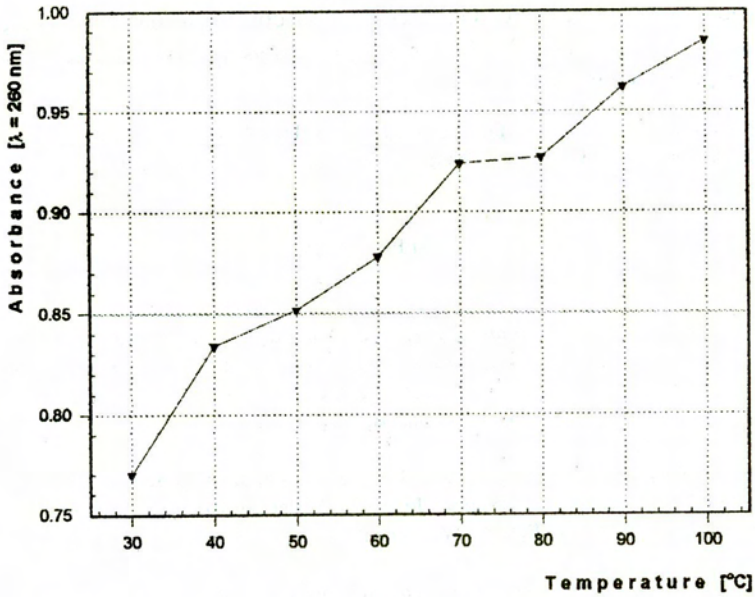

Fig. 3. – Hyperchromic shift of chromosomal DNA at *Streptococcus sp.*

Lactobacillus sp. $A_m = 0.97$ $T_m = 70°C$

Streptococcus sp. $A_m = 0.877$ $T_m = 60°C$

We estimated the guanine+cytosine percentage in the chromosomal DNA of our two lactic bacteria strains using Owen's formula: % mol GC = $2.08 \times T_m - 106.4$.

So, we obtained the values:

***Lactobacillus sp* % mol G+C = 39.2**

***Streptococcus sp.*% mol G+C = 18.4.**

It is very important to mention that these results range in those mentioned in literature as being characteristic for the studied genera. This conclusion allowed us to consider that the improved technique that we performed for the isolation and purification of bacterial DNA is very accurate, providing DNA samples with good integrity level and minimal proteic sugar and low weight RNA contamination degree.

CONCLUSIONS

The aim of our study was the determination of the guanine + cytosine content [% mol GC] of two lactic acid bacteria strains *Lactobacillus sp.* and *Streptococcus sp.* From our work we can conclude:

– our improved technique for chromosomal DNA isolation gave very good results, DNA in the samples being not sheared and having minimal proteic sugar and RNA contamination;

– the estimated values of GC% content of chromosomal DNA were **39.2** % for *Lactobacillus sp.* and **18.4** % for *Streptococcus sp.*, ranging in the literature values for the two genera.

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University of Bucharest
Faculty of Biology
Department of Genetics

MOLECULAR TAXONOMIC STUDIES OF *LISTERIA MONOCYTOGENES* WITH PRACTICAL APPLICATIONS IN CLINICAL DIAGNOSIS AND EPIDEMIOLOGY

VICTORIA HERLEA, DOINA BULAI, VERONICA LAZĂR

The R.F.L.P. technique was used to examine the genomic DNA restriction patterns of 14 strains of *Listeria monocytogenes* in order to differentiate the subtypes. Two restriction enzymes (*Hha* I and *Hae* III) were used and we consider that there are two different subtypes if we can distinguish only one band difference in the pattern.
The 14 analysed strains were previously enframed in 3 serotypes, but after technique RFLP they can be split in 12 genetic subtypes, half of them obtained by digestion with each of two endonucleases. This fact confirms the superior precision of the RFLP method as compared to the serological tests in clinical diagnosis and epidemiology.

In the latest decades, the extension and developments of the molecular biology techniques have lead to remarkable progresses in the identification (microbiological diagnostic) and the epidemiology of infectious diseases. These clinical fields apply the principles of the nucleic acid hybridisation with DNA probes for the infectious disease diagnosis and the identification of certain non-cultivable microorganisms, together with the principles of electrophoresis and chromatography with the aim to detect some cellular characteristic compounds for certain taxa, or for typing known taxa.

This study is focused on the analysis of some molecular techniques of subtyping of pathogenic bacteria. Several bacterial infections can be the result of the exposure of the organisms to a common source of etiologic agents. Generally, these pathogens, inside the species level, have enough large genetic diversity to permit the identification of many types inside the isolates belonging to a species and that were harvested from different sources and in different periods of time. So it is possible the examination of certain characteristics of the isolated strains which permit their differentiation at the infraspecific level, method called typing.

In the past, the typing was realised only by the examination of some phenotypic characters (biotyping), at which, there were further added serotyping techniques, phage typing, typing with bacteriocins. Once these techniques standardised, they became very simple, so that they are still used in clinical laboratories, but they cannot be used for all isolates. In the last years, the molecular typing methods revolutionised the epidemiology, because they are universally valuable (despite

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the different origins and the heterogeneity of the isolated strains) and are founded on the characterisation of some cellular molecules (Persing, 1993).

The most relevant molecular typing methods are based on the study of nucleic acids: the analysis of endonucleasic restriction fragments of plasmidial and genomic DNA, the fingerprinting with DNA probes and the fingerprinting based on Polymerase Chain Reaction.

The analysis of the chromosomal and extrachromosomal DNA restriction profiles is based on the comparison of the number and size of the fragments produced by the digestion with endonucleases, after their electrophoretic separation in agarose gel according to their length. Endonucleases are enzymes which cut the DNA molecule in constant positions, because they can recognise with high specificity nucleotide sequences built by 4–6 bp.. They are highly specific, so that the DNA belonging to a bacterial clone, which is cut with a restriction enzyme, always will form the same group of fragments. These fragments can be electrophoretically separated in agarose gel, by their molecular weight, and visualised by staining with ethidium bromide.

As a result of the DNA digestion with a specific endonuclease, variations can appear in the group of restriction fragments, especially concerning their sizes, due to genetic variations, phenomenon called RFLP (*restriction fragment length polymorphism*), which are very important for the typing studies (Hayes, 1989).

In the present study we tried to type by RFLP method 14 strains of *Listeria monocytogenes,* isolated from different sources (humans or animals with listeriosis, food), which were previously affiliated to three different serological types. *Listeria monocytogenes* is a Gram positive bacteria which is transmitted by contaminated food (especially with animal origins) and causes diseases with symptoms leading from digestive disorders to outbreak of the contaminated organism (Seeliger and Jones, 1986; Marth, 1988; Vadineanu *et al.*, 1992).

MATERIALS AND METHODS

Microbial strains: 14 strains of *Listeria monocytogenes* were examined. They were isolated from different sources. Seven of them belong to the serogroups 1/2, 3 and the other seven to the serogroup 4 (Table 1).

The growth conditions of the bacterial strains: The cultures were obtained by streaking the inoculum on blood-agar plates (nutrient agar with 5% horse blood), and incubation 24 hours at 37°C. For DNA extraction were used both cultures developed on agar plates and in gyratory shaken nutrient broth flasks.

The extraction, isolation and purification of genomic DNA: We used the alkaline lysis technique adapted by IshHorowitz (1981) after Birnboim and Dolly (1979 – as presented by Sambrook and Maniatis, 1986) and modified by Nocera *et al.* (1990), Gerner-Smidt (1992), Norrung and Gerner-Smidt (1993). The essential working steps are:

Table 1

The list of analysed strains of *Listeria monocytogenes*

Strain	Origin*	Serogroups 1/2, 3	Serogroup 4
Ts 4	F6854 (sporadic 1)	+	–
Ts 10	F8353 (sporadic 2)	–	+
Ts 13	F7440 (M/B)	–	+
Ts 15	F7272 (sporadic 7)	+	–
Ts 21	L4486j (Lausanne 1987)	–	+
Ts 24	F7596 (M/B)	+	–
Ts 29	F2365 (Los Angeles 1985)	–	+
Ts 31	L4491e (Anjou 1976)	–	+
Ts 42	G1092 (Boston 1983)	–	+
Ts 48	F7432 (patient 2)	+	–
Ts 52	F7125 (sporadic 1)	+	–
Ts 60	L4486b (Lausanne 1987)	–	+
Ts 68	F7295 (sporadic 7)	+	–
Ts 77	F6801 (sporadic 3)	+	–

*All the strains belong to the WHO Microbial Cultures Collection and were initially isolated from different countries, from humans, animals and food.

Isolates from the same outbreak are labelled with the location and the year of the outbreak.

Isolates from sporadic listeriosis are labelled as sporadic followed by a number.

Isolates from mother / baby pair are labelled as M/B.

Isolates from a patient are labelled with patient followed by a number.

1) the alkaline lysis of bacterial cells associated with the action of lysozyme (Sigma) and 10% S.D.S. (Gibco BRL);

2) the digestion of RNA and proteins with RN-ase (Sigma) and proteinase K (Sigma);

3) double extraction of DNA with phenol:chloroform:isoamylic alcohol = 25:24:1;

4) isolation and double purification of DNA with isopropanol, ammonia acetate 7.5 M and ethyl alcohol 96°.

The digestion of DNA molecules: There were used two restriction enzymes: *Hha* I and *Hae* III (Gibco BRL) for the cutting of DNA molecules; the mixture was incubated at 37°C for 2 hours.

The electrophoretic separation of restriction fragments in agarose gel: The migration of fragments was performed by horizontal electrophoresis in 0.7% agarose gel (Gibco BRL Ultrapure Agarose) in TBE buffer (90 mM Tris-90 mM borate-2 mM EDTA) with pH = 8.0, for 18–20 hours with a field strength of 3 mV/cm (using a submersed BRL Life Technologies Electrophoresis Bath and Sigma Power Supply).

The size markers: It was used a home-made marker which represents a mixture of restriction fragments of the λ phage genome digested with *Hind* III and *Sty* I and commercial marker X (Boehringer Mannheim) with the following sizes: 23.1; 19.3; 12.2; 11.2; 10.2, 9.4; 9.2; 8.1; 7.7; 7.1; 6.7; 6.2; 6.1; 5.1; 4.4; 4.3; 4.1; 3.5 kb.

The gel visualisation: It was realised by the staining of DNA with ethidium bromide 10 mg/ml and the photographing of the gel under UV light with a Polaroid Camera.

The tests reproducibility: Two of the strains were in duplicate, and the tests were performed several times with DNA repurified from the strains each time.

RESULTS AND DISCUSSIONS

A single band difference in the restricted DNA patterns was considered to be significant and used to distinguish between two subtypes. It is remarkable the fact that the separation of restriction bands was best for fragments larger than 6 kb. The electrophoretic patterns were examined with the naked eye.

The restriction enzymes that were used have recognition sites represented by 4 base pairs, for *Hha* I: GCGC and for *Hae* III: GGCC, and the mol G+C% content is approximately 38% for *Listeria monocytogeńes* (Seeliger and Jones, 1986), thus as were expected there were obtained numerous restricted fragments with small and medium sizes and a small number of large fragments, with dimensions > 6 kb which were separated with big resolution, easily to be interpreted.

Because the solving power of the gel allows the macroscopic examination for the large and medium fragments, the selection of the subtypes was made in dependence on the configuration of the restriction patterns.

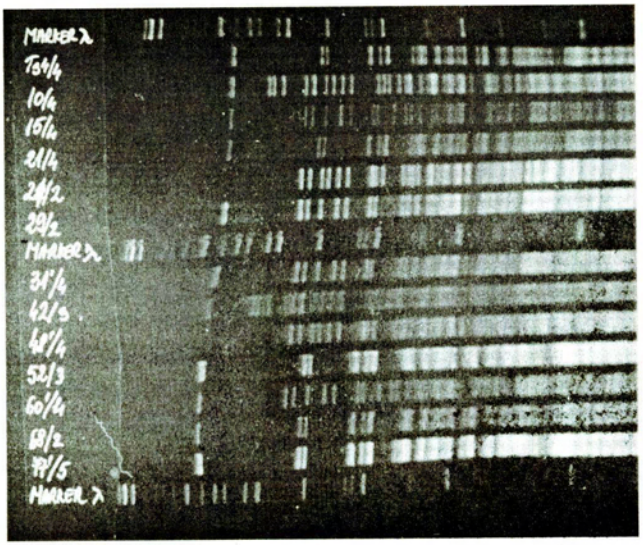
In figures 1 and 2 there are presented the electrophoretic profiles of genomic DNA belonging to 14 strains, digested with *Hha* I (two tests were performed in a succession of 14 days, for each test the bacterial genome being individually purified). In this case, the reproducibility of the restriction patterns could be observed.

The RFLP analysis of the restricted genome of the 14 strains shows the existence of 6 different genotypes which are grouped: **1.** Ts4, Ts21, Ts52, Ts77; **2.** Ts10, Ts42; **3.** Ts13, Ts15, Ts60; **4.** Ts24, Ts48; **5.** Ts29, Ts31; **6.** Ts68. Some of the isolates are differing by only one band (for example the strains Ts68 and Ts77; Ts15 and Ts24), by two bands (Ts24 and Ts29) and by many bands (Ts10 and Ts42 comparing to the others). As we expected, the serological groups (types) 1/2 and 3 are split in 4 different genotypes and the serotype 4 in other 4 different genotypes.

This observation is a proof for the real fact that there is a large genetic diversity of the strains belonging to a genera within the framework of a certain serogroup and also, this method can be used, after standardisation, for the studies of the source and transmission pathways of a pathogenic microorganism involved in an epidemic.

Figures 3 and 4 are comparatively representing the electrophoretic restriction patterns of the total (genomic) DNA of the 12 strains digested with the en-

Fig. 1. – The photograph of the restriction patterns of 13 strains of *Listeria monocytogenes* after digestion with endonuclease *Hha* I. The lines 1, 8, 16 represent the mixture of markers λ (digested with *Hin*d III and *Sty* I) and X. From the picture is missing the strain Ts13.



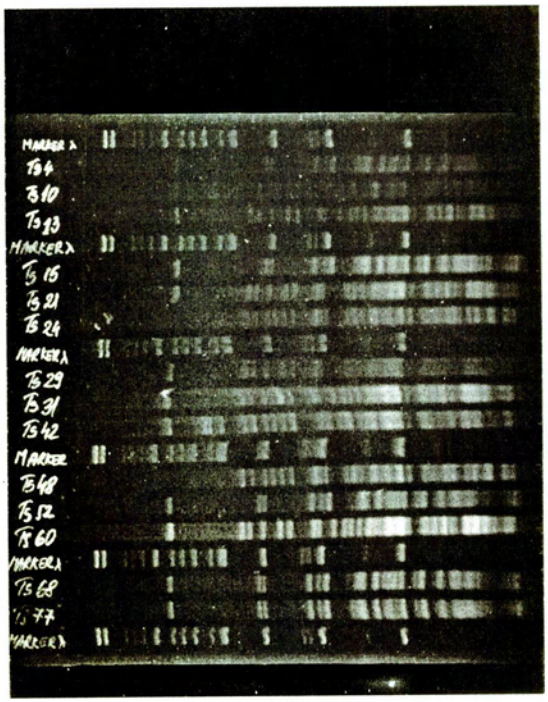


Fig. 2. – The photograph of restriction electrophoretic patterns of 14 strains of *Listeria monocytogenes* after digestion with the endonuclease *Hha* I. The lines 1, 5, 9, 13, 17, 20 are representing the mixture of markers λ (cut with *Hin*d III and *Sty* I) and X.

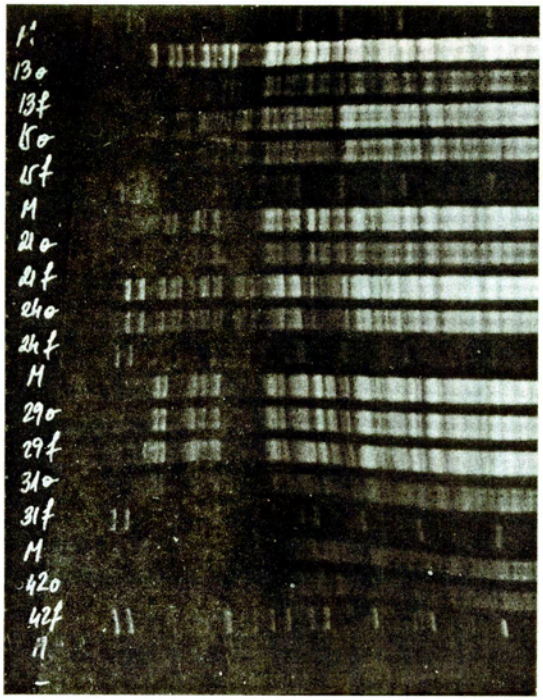


Fig. 3. – The photograph of the electrophoretic restriction profile of the genome of 7 strains of *Listeria monocytogenes* digested with *Hae* III. Lines 1, 6, 11, 16, 19 represent the mixed marker λ (cut with *Hin*d III and *Sty* I) and X.

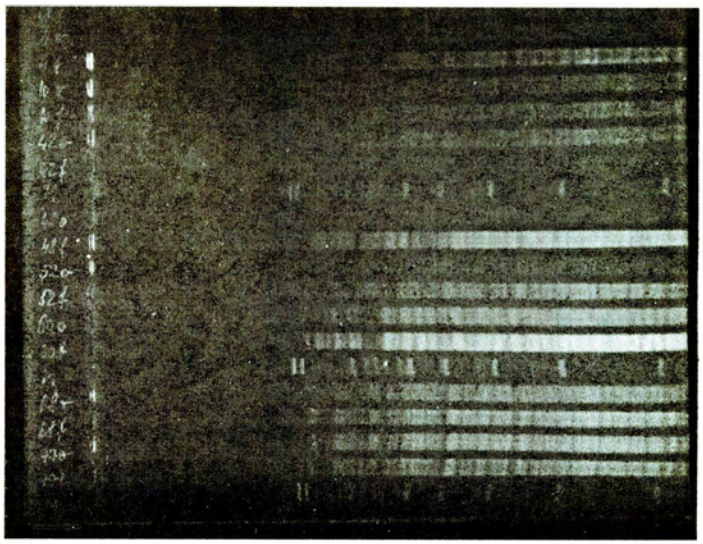


Fig. 4. – The photograph of the electrophoretic restriction profile of the genome of the other 7 of the strains of *Listeria monocytogenes* digested with *Hae* III. Lines 1, 6, 11, 16, 19 represent the mixed marker λ (cut with *Hin*d III and *Sty* I) and X.

zyme *Hae* III. The strains Ts4 and Ts10 are missing from the photograph, because we were not able to obtain an adequate quantity of DNA and sufficiently well purified, thus we could not examine them.

For this restriction enzyme, there are produced a bigger number of fragments with a less resolution of the gels as compared with the enzyme *Hha* I, that makes the interpretation of the patterns more difficult. A preliminary framing of the restriction patterns shows 6 different types: 1. Ts21, Ts29; 2. Ts31; 3. Ts68, Ts52; 4. Ts15, Ts77; 5. Ts13, Ts42; 6. Ts48, Ts60. Some of the profiles differ by only one supplementary band, for example Ts31 and Ts29) or by many bands (Ts15 and Ts24 from the others).

In the case of the utilisation of this endonuclease, it is evident the fact that 5 from 6 serotypes present a big homogeneity, being enclosed in the same serogroup.

It is obvious the fact that for the use of the enzyme *Hha* I in the limits of a genetic subtype there are enclosed strains belonging to both serotypes, in the case of the enzyme *Hae* III into a genotype are enclosed only strains belonging to the same serogroup.

CONCLUSIONS

– The technique used points out a high specificity. It is able to make subtle discriminations between the subtypes of a species, even for the analysis of a small number of strains (14 strains enframed in 3 serotypes, but really in 12 genetic subtypes – 6 obtained by digestion with *Hha* I and the other 6 with *Hae* III, in each genotype being enframed strains from all three serogroups);

– If the resolution of the gel is big, it is possible to observe the presence or absence of only one band or the position of bands as compared with the size marker;

– The duplicates were correctly identified, testifying the validity of the obtained results;

– The technique has a high reproducibility when the conditions are standardised;

– As a taxonomic significance for infraspecific level, our results acquired by the application of the RFLP technique for the analyses of the 14 strains of *Listeria monocytogenes* are confirming the possibility of an identification with a bigger accuracy (inside some taxa) than the serological methods.

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University of Bucharest, Faculty of Biology,
Department of Microbiology, Aleea Portocalilor 1–3,
Bucharest 6

CHROMOSOMAL BANDING IN PLANTS – AN OLD BUT NECESSARY TECHNIQUE

VERONICA STOIAN, ANDREEA CUNIȚĂ, NICOLETA JANTALĂU

The genetic material has basically the same structure in the whole eukaryotic world; nevertheless, there are some differences between animal and plant kingdoms. Banding techniques represent methods to explore them; besides, these methods have other applications, such as karyotyping and population screening (i.e. for additions, deletions, translocations, substitutions).
In the present study, we designed C- and NOR banding techniques "adapted" to our species of concern, in order to produce reliable devices for characterizing our subjects. Beside the methods, their results are also exposed.

INTRODUCTION

Genetic material organization is considered to be similar within the whole eukaryotic world. Chromosomal DNA structure is basically the same from yeast to man. Nevertheless, between plant and animal kingdoms (referring to angiosperms and mammals, respectively) some differences – which cannot be neglected – do exist:

- nuclear DNA content varies among different mammalian species with a factor of maximum 2.5, while in angiosperms it equals 1600 [3]. It is believed that the greatest variation could be attributed to repetitive DNA [8];
- plants chromosomes generally have a higher chromatin packaging ratio in various stages of cell division; they are 3.5 to 6.5–fold more condensed than mammalian chromosomes [11];
- in plants, consequences of the more compact packaging of the nucleoprotein fibers (and of greater chromatin density) are: 1) higher degree of order of chromatin fibers, and 2) absence of longitudinal differentiation encountered in animals (i.e. G bands), at least at the light microscopy resolution level [11, 21].

An approach of these structural differences by means of cytogenetic markers – constitutive heterochromatin, nucleolar organizing regions – is represented by banding techniques, C- and N / NOR, respectively. In the literature, one could find a huge number of variants (part of them adapted from those applied in animals), which permit to distinguish longitudinal banding patterns in plant chromosomes. However, as a consequence of the differences emphasized above, there is not always a direct correspondence between the results elicited in animals, respectively in plants. The most striking example is that of trypsine-banding, which in animals

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yields species – specific patterns, but in plants is ineffective, probably because of the higher degree of chromosomal condensation (the treatment of vegetal material with trypsine, followed by acetic orcein coloration produces C-bands) [16].

C-banding implies nucleic acids' denaturation, most frequently in basic medium (sodium or barium hydroxide), followed by renaturation; the differences in base composition and in proteins between eu- and heterochromatin are illustrated by the banding patterns. In plants, but not in animals, variants of this method were claimed to make evident kinetochores [22], centromeres [18] or constitutive heterochromatin in all [10, 17, 19, 20, 23, 25]. On the other hand, C-bands' polymorphism is much greater in plants; these markers vary not only within species, but also within subspecies, cultivars, among different populations and even within population.

Similarly, N / NOR banding, initially applied in animals in order to distinguish nucleoli / nucleolar organizing regions, elicits different results when used in the same species, sometime as a consequence of a different variant of technique: the patterns produced have not always been identical with those of the zones known as true NORs [9]. One could wonder if the nucleolin protein (known as associated with nucleoli and NORs and which couples with silver in $AgNO_3$-banding [1]) is indeed present in these "uncertain regions" and which is its relationship with the true NORs; on the other hand, interpreting these bands as NOR could be an error, because they have not always been correlated with the study of the nucleolus-associated spots.

Nowadays, as molecular techniques evolve, banding methods' role becomes auxiliary, i.e. to correlate molecular markers with cytogenetic markers (and not as a unique means to explore chromosomal DNA's structure and function) or to screen populations – in amelioration programs, as an example.

The present paper deals with banding analysis on *Vicia faba*, *Secale cereale* cv. Harkovskaja and on one wheat–rye hybrid created at R.I.C.I.C.–Fundulea. Primarily, our purpose is to develop reliable methods to perform population screening, karyotyping and visualizing genomic and chromosomal alterations (i.e. additions, substitutions, translocations, deletions); subsequently, it was to analyze, characterize and select hybridization products created in amelioration programs.

MATERIALS AND METHODS

Our study concerns *Vicia faba* (2n = 12) and *Secale cereale* cv.Harkovskaja (2n = 14), from which we used the meristematic part of root tips, and a first generation hybrid between a double haploid lineage of *Triticum aestivum* cv.Fundulea 132 and *Secale cereale* cv.Harkovskaja (2n = 28), from which we used anthers. This hybrid contains a *T.aestivum* haploid genome (21 chromosomes), and a rye

one (7 chromosomes); it was created at R.I.C.I.C.–Fundulea, county Giurgiu, Romania as a part of a more ample wheat amelioration program.

The meristematic tips were cut from 0.5–2 cm long roots from seeds germinated in semisterile conditions; they were pretreated in distilled water, on ice, for 26 hours, and fixed and conserved in 45% acetic acid till use. Before squashing, the tissue was softened by hydrolysis in 1N hydrochloric acid, at 60°C, for 15 minutes; the rye meristems needed enzymatic treatment (cellulase – 2%, pectinase – 20%, at 37°C, for 45 minutes), also. The spikes were fixed in modified Carnoy's mixture (ethanol 100%:chloroform:glacial acetic acid = 6:3:1) and conserved in 70% ethanol, at 4°C till use. The anthers were dissected and the content of the anthers' sacks was squeezed out. In both cases the squash was performed in a drop of acetic acid 45%; the best slides were chosen by phase contrast microscopy and those selected were kept in exsiccator, at room temperature, after removing the coverslip with dry ice.

The C-banding variant represented a method adapted from that described by Lukaszewski & Xu [17]; slides were treated with 45% acetic acid for 5 min., at 60°C, after dehydration in 100% ethanol; subsequently, they were immersed in saturated barium hydroxide solution, at the same temperature, washed in tap water for an hour, and incubated in 2XSSC solution, at 60°C, for 2 hours. 2% Giemsa in Sörensen buffer was used for coloration.

NOR banding was adapted after Bloom & Goodpasture's technique [4]; the dehydrated slides were treated with 50% $AgNO_3$ solution in distilled water, for 2 hours, in humid chamber. The deposits formed at nucleolar organizer regions/nucleoli were enhanced using a mixture of $AgNO_3$:NH_3:distilled water = 5g:5ml:5ml with 36% formaldehyde (neutralized with sodium acetate and pH – adjusted at 5–6 with formic acid).

RESULTS AND DISCUSSIONS

Our first goal was to design a reliable method to analyze plant chromosomes, mainly in plants from Triticeae tribe. On this purpose, we tried to adapt the parameters from the literature to our material. One of them was slides "maturation time", meaning the time passed between the moment of squashing and that of performing banding procedure. We find this one as being optimal as 2–3 weeks. Fiskejö [7] indicates different appearance times of bands: first of all, the telomeric ones appear, next the intercalary ones and finally the centromeric ones and those at secondary constrictions. Indeed, rye possesses telomeric and intercalary bands, but, as the majority of the researchers agree [12], no centromeric bands. So, we conclude that our technique is reliable enough to make the complete pattern of existing bands evident.

In *Vicia faba*, C-banding illustrates constitutive heterochromatin, in a pattern comparable with those from literature [5]. Nevertheless, the metacentric chromosome (Fig. 1, 2) presents a supplementary band comparatively with the standard idiogram (Fig. 3).

In interphase nuclei, chromocenters are easily observed, peripherally disposed (Fig. 4). This represents an illustration (and a confirmation, meantime) of the body–guard hypothesis [12]: heterochromatin protects euchromatin (more susceptible at physical and chemical mutagens), owing to its peripheral localization and its ability to buffer mutagens' action; its buffering capacity due to the plasticity conferred by its base composition and its repetitive structure.

In the rye metaphase (Fig. 5) and in the explanatory drawing, the terminal localization of the large bands, on one or both chromosomal arms can be seen;

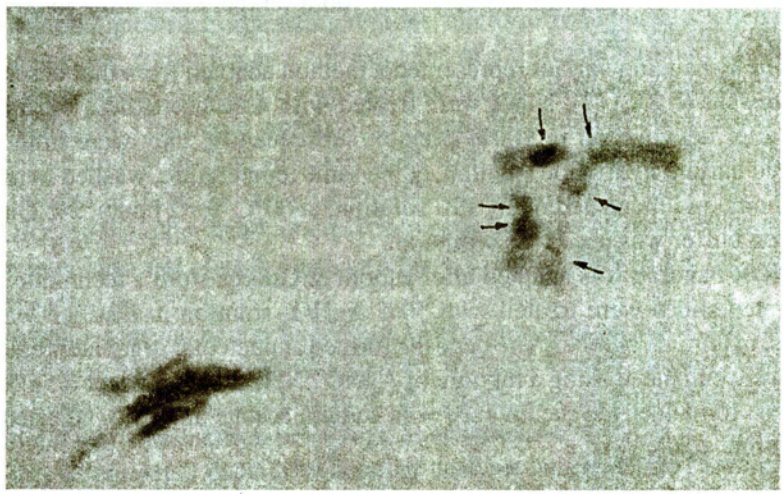

Fig. 1. – Partial mitotic metaphase in *Vicia faba*.

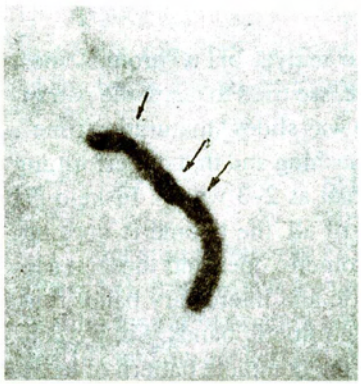

Fig. 2. – Metacentric chromosome of *Vicia faba* and its idiogram.

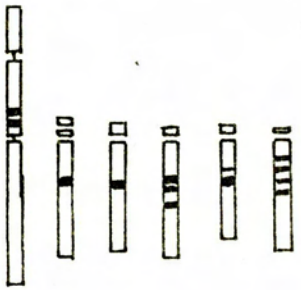

Fig. 3. – *Vicia faba* idiogram (after Döbel & collab. [5]).

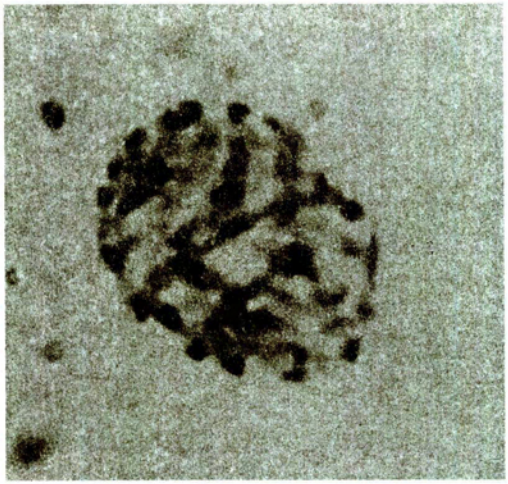

Fig. 4. – Interphase nucleus in *Vicia faba*.

pairs B, D, G of chromosomes carry narrow to very narrow intercalary bands. Centromeric bands have never been seen. Using this metaphase we produced Fig. 6 karyotype. As it can be noticed, there is not a perfect homology between the members of the chromosome pairs, as a consequence of the exogamous reproduction characteristic to rye [25].

Using this technique we observed chromosomal interconnections, also (Fig. 7); these were previously mentioned in literature [10]. As telomeres have large C-bands, it is supposed that the material existing in these interconnections is heterochromatic, too. Their presence confirms the suprachromosomal organization hypothesis, but, at least for the moment, we cannot appreciate the spatial relationship between homologous chromosomes and between one pair's members and the rest of chromosomes. Generally, it is considered that in the stationary stages of the cell cycle (telophase–interphase–prophase), homologous chromosomes are closer to each other, comparatively with the other members of the complement; the intimate association is disrupted at metaphase and subsequently is established again during anaphase. By measuring the distance between chromosomes, their arrangement in interphase nucleus could be deduced [2].

An interesting aspect is represented by the perinucleolar localization of the chromocenters in the interphase nucleus in Fig. 7, contrary to their normally peripheral one. We cannot find a logically reasonable explanation of this finding.

Nevertheless, contrary to our expectations, our tentative to band meiotic chromosomes in F_1 hybrids wheat-rye had a limited success, allowing only the identification – at low resolution (considering also the morphology of chromosomes) – of the rye genome; a possible explanation would be the condensation state modification of the chromosomes in the new genomic environment of the hybrid. Our

Fig. 5. – Mitotic metaphase and explanatory drawing in *Secale cereale* cv.Harkovskaja.

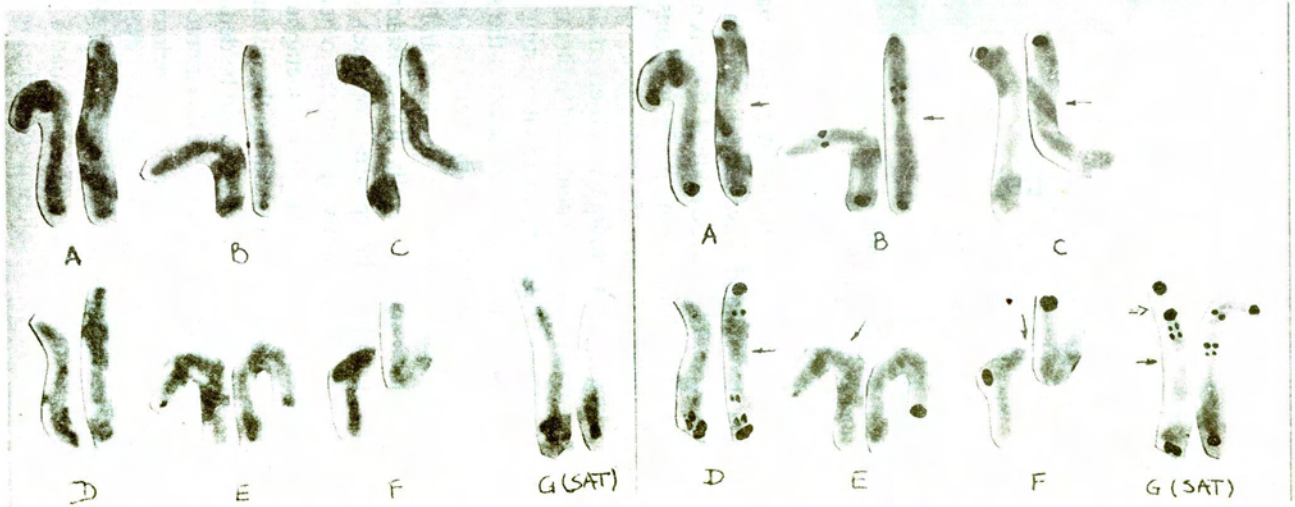

Fig. 6. – Karyotype and explanatory drawing in *Secale cereale* cv.Harkovskaja.

Fig. 7. – Chromosomal interconnections (→) and perinucleolar chromocenters in *Secale cereale* cv.Harkovskaja.

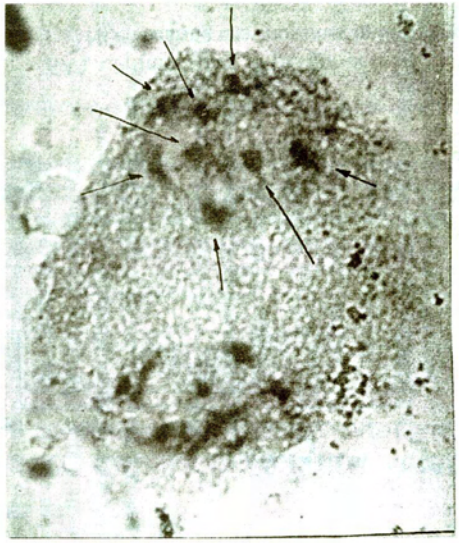

Fig. 8. – Interphase tappetum binucleated cell (→ indicates the chromocenters of one of nuclei).

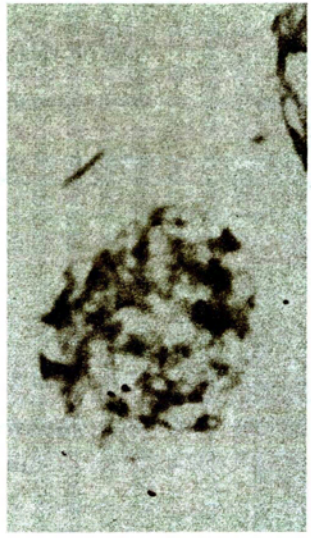

Fig. 9. – Pollen mother cell at leptotene in F_1 hybrids.

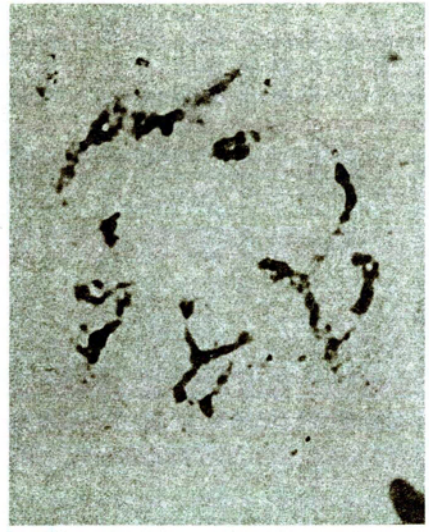

Fig. 10. – Pollen mother cell at pachytene in F_1 hybrids.

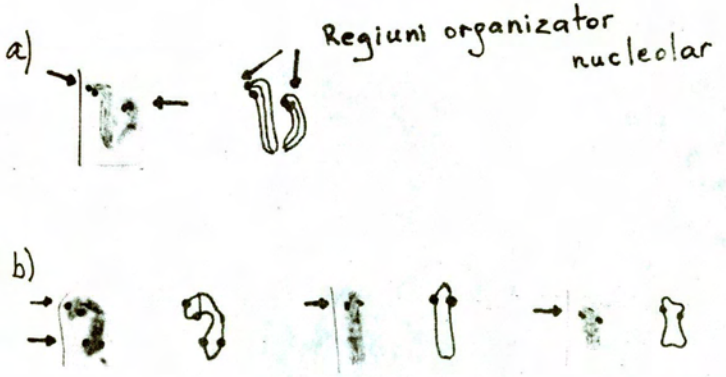

Fig. 11. – SAT chromosomes in *Secale cereale* cv.Harkovskaja (a) and NOR-bearing chromosomes in F_1 hybrids (b).

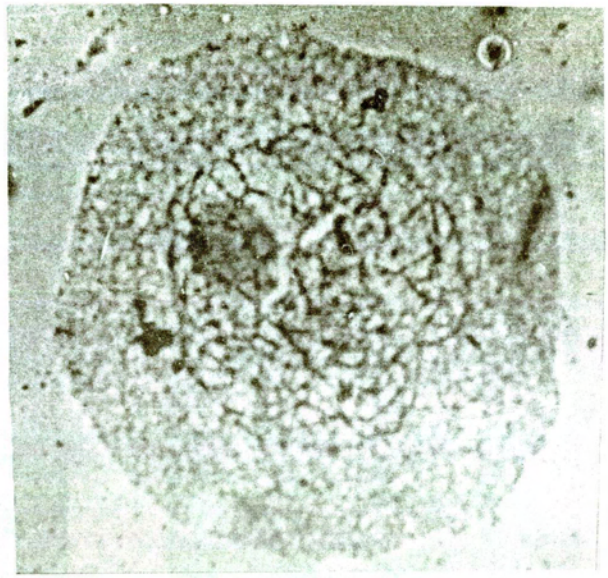

Fig. 12. – Pollen mother cell at leptotene, with one nucleolus, in F_1 hybrids.

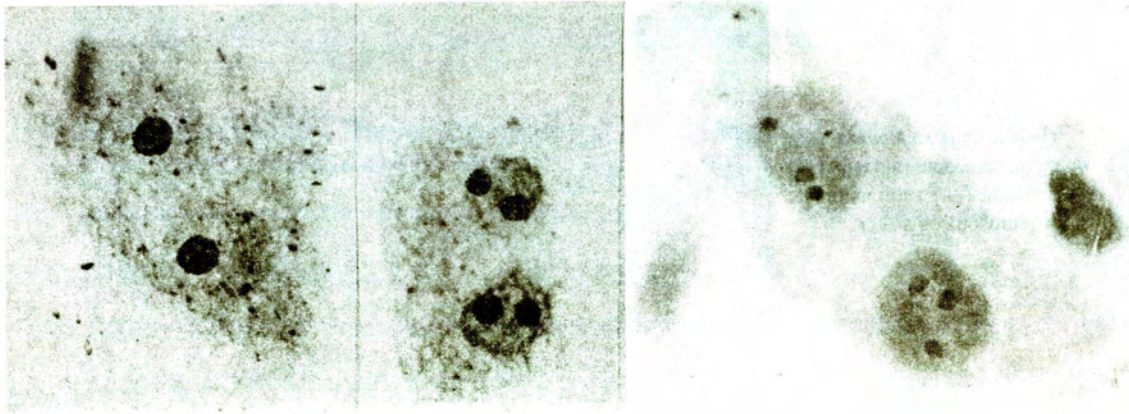

Fig. 13. – Interphase tappetum binucleated cell in F_1 hybrids.

technique made evident chromocenters in the tapetum binucleated cells and in pollen mother cells (PMC) (Fig. 8, 9, 10). The small number of chromocenters correlates with the relative low number of bands in rye (and wheat) complement.

Lacadena & Vasquez [15], Klasterska & Ramel [12], Viinikka & Nokkala [24], established 8 stages in meiotic prophase of *S. cereale* and *Najas marina*. Klasterska & Ramel proposed the following: leptotene, synizesis, pachytene, early diplotene, diffuse stage, second contraction, late diplotene, diakinesis. We appreciate that the cell in fig. 10 is in leptotene, the chromosomes beginning to form the synizetic knot, and that in fig. 11 – in pachytene*.

Banding method for nucleolar organizing region permitted identification of satellite chromosomes in rye (Fig. 11), paternal parent of F_1 hybrid. In this last one, NOR banding:

– established the existence in prophase (leptotene–zygotene) PMC of a unique large nucleolus; it is crossed by chromatic fibrils, probably of many chromosomes (Fig. 12);

– made evident, in interphase tappetum binucleated cell, one bigger to three smaller nucleoli per nucleus; this suggests the existence of three NOR-bearing chromosomes, at least (Fig. 13).

These observations concord well with the fact that in metaphase I meiotic PMCs have been certainly identified three NOR-bearing chromosomes (Fig. 12): in rye, there is one SAT chromosome per haploid complement and in wheat, at least at "Chinese spring" cultivar – only two per haploid complement (1A and 5D) [9].

To conclude we appreciate that our methods offer promising results, reliable enough to karyotype, characterize plant genomes and screen population, with good expectations to be an intermediary device between microscopy and molecular levels.

Acknowledgments. We wish to express our warm thanks to Mr. Aurel Giura, Ph.D. and to Mrs. Alexandrina Mihailescu, Ph.D. (from I.C.C.P.T.–Fundulea) for the biologic material they kindly offered us and for valuable discussions; we also are very grateful to Mrs. Agripina Lungeanu, Ph.D., for helpful suggestions and constant encouragement.
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* In synizesis stage, the chromatin fibers with a very low degree of condensation form a relatively tight mass around the nucleolus, with paired regions and heterochromatic blocks fusioned to form a synizetic knot; the diffuse stage follows early diploten: heterochromatic regions are distinct, not paired, and the euchromatic ones are diffuse; at second contraction stage – bivalents occupy a relatively small area and heterochromatic regions tend to form paired rings. The other stages have the same known meanings.

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University of Bucharest
Faculty of Biology
Department of Genetics

THE EFFECTS OF *ARMILLARIA* AND *BOLETUS* (FUNGI, BASIDIOMYCETES) WHOLE WATER EXTRACTS, INTERFERING WITH PAVSTIM – A STEROIDIC BIOREGULATOR – ON GENETIC MATERIAL OF *ALLIUM CEPA*

L. GAVRILĂ*, A. JACOTĂ**, P. CHINTEA**,
GR. MIHĂESCU*, LORELAI-BIANCA GAVRILĂ***, VALERIA-ROSALINDA GAVRILĂ***

The testing of effects of *Armillaria melea* – an edible mushroom –, *Armillaria* sp. – an unedible mushroom and of *Boletus satanas* – a poisoning mushroom – whole water extracts on genetic material of *Allium cepa*, have been performed. The edible mushroom *Armillaria melea* whole water extract has neither clastogenic nor mutagenic effects, while unedible mushroom *Armillaria* sp. whole water extract has obvious clastogenic effect. *Boletus satanas* whole water extract has both clastogenic and mutagenic effects, while in combination with PAVSTIM – a steroidic bioreglator of vegetal origin –, exerts an abnormal nucleolar (i.e. ribosomal cistrons) amplification, followed by nuclear disorganization.

The investigations of different microorganisms or high plant extracts effects on eukaryotic cell have both practical and theoretical applications in a time when naturistic medicine is reconsidered.

Long time ago there were discovered the toxic or lethal effects of some bacteria strains. Recent papers described the neurotoxic effect of some toxins produced by cyanobacteria (e.g. *Anabaena flos-aquae* and *Nodularia spumigena*). The cyanobacterium *Microcystis aeruginosa* produces toxins with disastrous effects on the liver as well as with carcinogen effects. These cyanobacteria toxins are called microcistin and nodularin, respectively, and are peptides consisting of 7 and 5 aminoacids, respectively (Carmichel, 1994). According to our knowledge, the effects of these cyanobacteria toxins on the genetic material have not been tested yet. Our recent investigations (Gavrilă et al., 1994) using as bacterial extract the streptolysin O from *Streptomyces pyogenes* and eight whole alcoholic vegetal extracts from different species of gymnosperm and angyosperm plants evidenced in some of them clear clastogenic effects.

The streptolysin O inhibits the cell division in meristem root cells of *Allium cepa*, producing nuclear pulverisation with extensive damages on interphase nucleus chromatin, leading to the death of the cells. In variants in which the streptolysin O acted after root emergence, it produced modifications in chromatin organization, affecting primarily the euchromatic regions of chromosomes. The nuclear chro-

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matin appears with a peculiar appearance in which the "beads on string" formations at the level of light microscope are the most specific aspects. These do not represent nucleosomes (which can be visualized only at the ultrastructure level), but merely heterochromatic regions of the chromatin fibers, exhibiting an ordered pattern of their distribution. The transition from mitosis to amitosis is often encountered in the cells treated with 1% streptolysin O, with an unusual extension of nucleolar areas.

The *Artemisia absinthium*, whole alcoholic extract proved to be a powerful clastogenic agent, determining the appearance of micronuclei in interphase cells, chromatic bridges in Anaphase, Telophase and Interphase, chromosomes out of the metaphase plate and ring chromosomes (Gavrilă et al., 1994).

The *Euphorbia* sp. whole alcoholic extract proved to have also strong clastogenic effects with the breakage of chromatin fibers followed by translocations, with the appearance of acentric fragments, ring chromosomes, chromatic bridges and chromosomal stickness.

The present paper is dealing with the effects of whole water extract from unedible and edible mushrooms of *Boletus* and *Armilaria* genera.

It is known that necatorin, produced by edible mushroom *Lactarius necator*, has a strong mutagenic effect in bacteria. The gene transcription is blocked by alpha-amanitin which is produced by extremely poisonous mushrooms like *Amanita muscaria* and *A. phaloides*.

MATERIALS AND METHODS

The mushrooms were collected from forests in village Bertea-Prahova district (*Boletus satanas*), from the Botanical Gardens in Bucharest or from the Băneasa forest near Bucharest (*Armillaria mellea* – edible, and *Armillaria* sp. – unedible).

The modified *Allium cepa* test (Rank et al 1993) was applied in order to test for the effects of whole water extracts of these mushrooms on cell division mechanics, as well as on chromosome structure. In doing so, two variants in each case were used: with prefixation in colchicine 0.3% solution and without prefixation, i.e. direct fixation of onion roots in 45% acetic acid solution, at room temperature for 12 hours. The onion bulbs were put in contact with different concentrations of whole water extracts of these mushrooms or the roots were put in contact with these extracts after the emergence in common tap water. For the study of interference of mushrooms extracts and PAVSTIM (a steroidic bioregulator of vegetal origin, produced in the Genetics Institute from Kishinew, Moldova Republic), 0.5 mg PAVSTIM in 100 ml tap water was dissolved and equal quantities of these solutions and mushroom extracts were mixed and the onion bulbs having emerging roots were put in contact with this mixture for 48 hours.

The microscope slides were realized using the squash method after Feulgen reaction for onion roots and the microscope analysis was performed using an Amplival light microscope.

RESULTS AND DISCUSSIONS

The toxic principle from *Boletus satanas* is called boletoxin-boletol and is mainly composed of resinoid residues of cetone or kinone type. Having three cyclic nuclei of benzene derivative type boletoxin is considered to be similar to muscarin. The boletoxin is resistant to boiling, being destroyed only at 275–280°C.

In a separate paper (in preparation) we will present the effect of whole water extract of *Boletus* on the electrophoresis profile of nucleic acids from *Escherichia coli*. This extract, at its highest concentration, inhibits the cell division process and affects, at the same time, the process of DNA synthesis. If at the molecular level these preliminary investigations are not yet relevant, concerning the effects of whole water extract from *Boletus satanas*, at the chromosomal level these are really relevant.

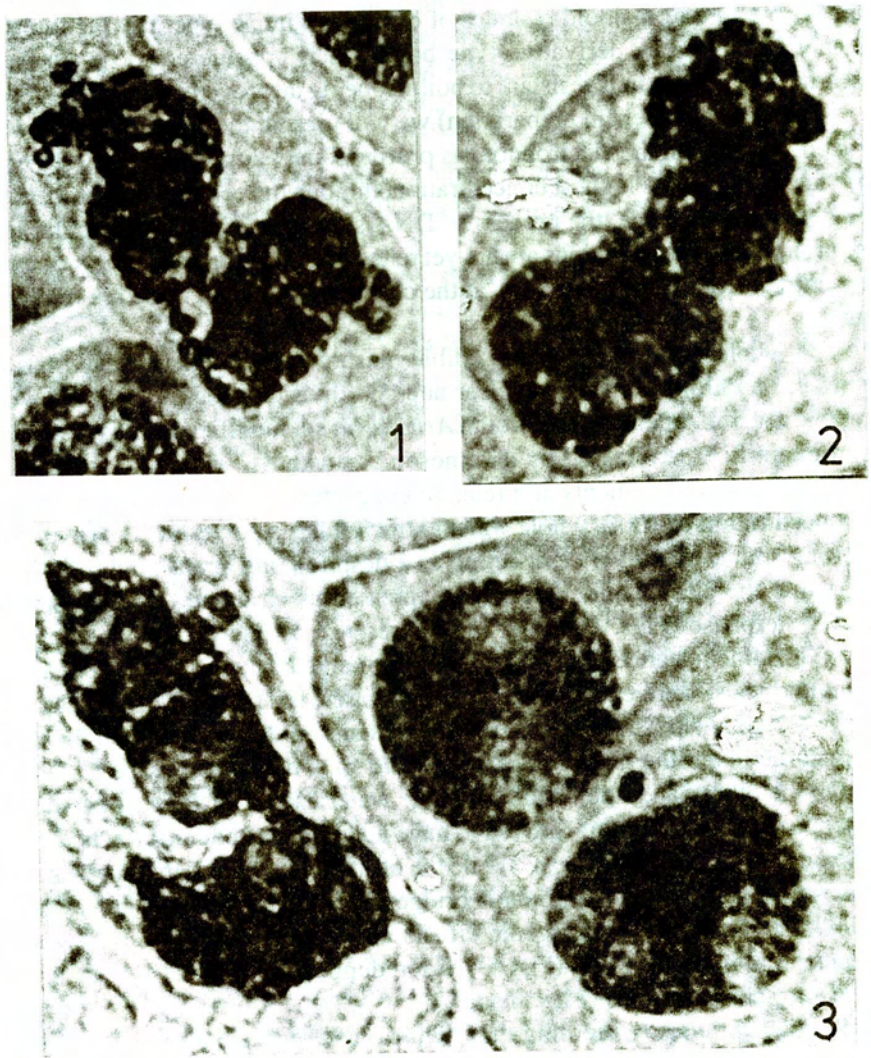
The whole water extract from edible mushroom *Armillaria mellea* has not any effect on chromosomes or nuclear and cell division process. But, the whole water extract from unedible mushroom *Armillaria* sp. modifies the physical status of chromatin fiber, expressed by chromosomal stickness, without any detectable chromosomal rearrangements at a relative concentration of 50%, while at a relative concentration of 100% whole water extract, it has a powerful corrosive effect on chromosomes of *Allium cepa* acting as a despiralizing agent of chromosomal fiber, while the clastogenic effects are expressed as chromatic bridges in Telophase – Interphase (Figs. 1–3) and as the presence of micronuclei in Interphase (Fig. 3). In some instances, the blocking in the daughter-nuclei separation, as well as of cytokinesis, were encountered. In other cases, the picnosis of nuclear chromatin is obvious. No chromosomal rearrangements could by noticed in the examined material under the influence of *Armillaria* sp. whole water extract.

On the other hand, the action of whole water extract of *Boletus satanas* – a strong poisoning mushroom – proved to have not only clastogenic effects, but also well expressed mutagenic effects. While the clastogenic effects are represented by chromosomal stickness and chromatic bridges in Telophase (Fig. 4) and cytokinesis blocking (Fig. 5), the mutagenic effects are expressed by the appearance of micronuclei in interphase cell (Fig. 5), endoreduplication (Fig. 6) and the appearance of ring-chromosomes (Figs. 6 and 7).

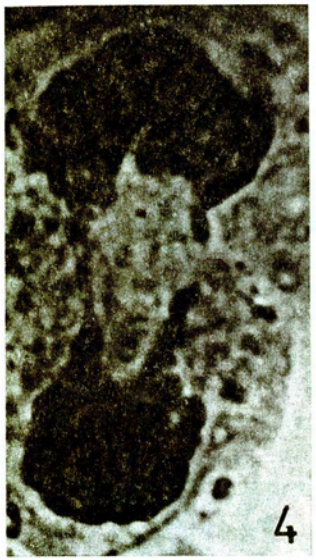
We have no idea concerning the chemical nature of the toxin from unedible *Armillaria* sp. and, as a consequence, no possibility to speculate on the mechanism of its action on genetic material of *Allium cepa*. This is not the case for *Boletus satanas* where the chemical nature of boletoxin or boletol is well established. The

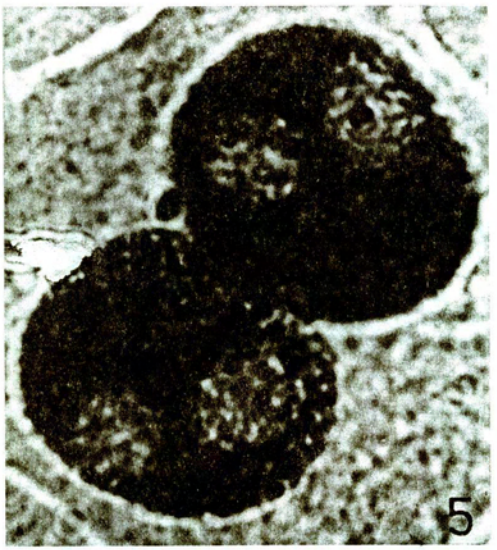
boletoxin (i.e. boletol) contains an anthrakinonic cycle, having mutagenic properties similar to anthrakinons produced by plants from Rubiaceae family (for a review, see Gavrilă, 1986) whose mutagenicity is considered to be due to the groups 1,3–OH.

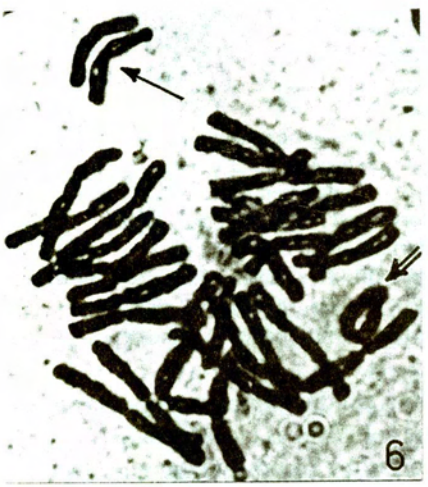
In another fungus toxin, called faloidin, produced by *Amanita phaloides*, there are several CH_3 groups which could confer some alkylating properties to this toxin. The radiomimetic effects of these fungi toxins can be put on behalf of such active groups which can introduce chemical modifications in the bases or other compo-

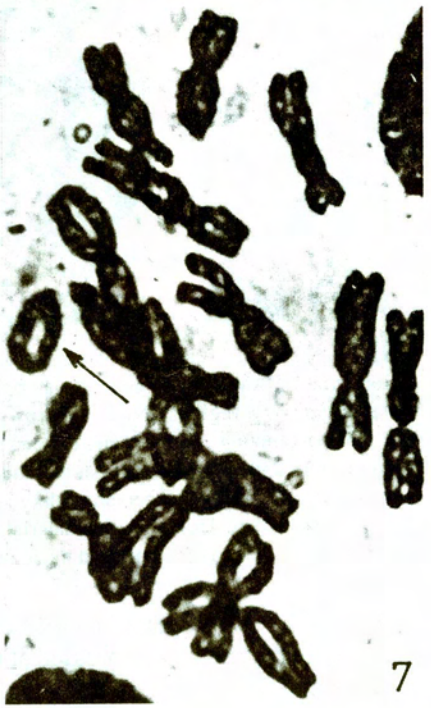


Figs. 1–3. – The effects of whole water extract of *Armillaria* sp. – unedible mushroom – on genetic material of *Allium cepa*: 1, 2 – chromatic bridges in Telophase – Interphase; 3 – Chromatic bridges in Telophase – Interphase and micronucleus in Interphase.

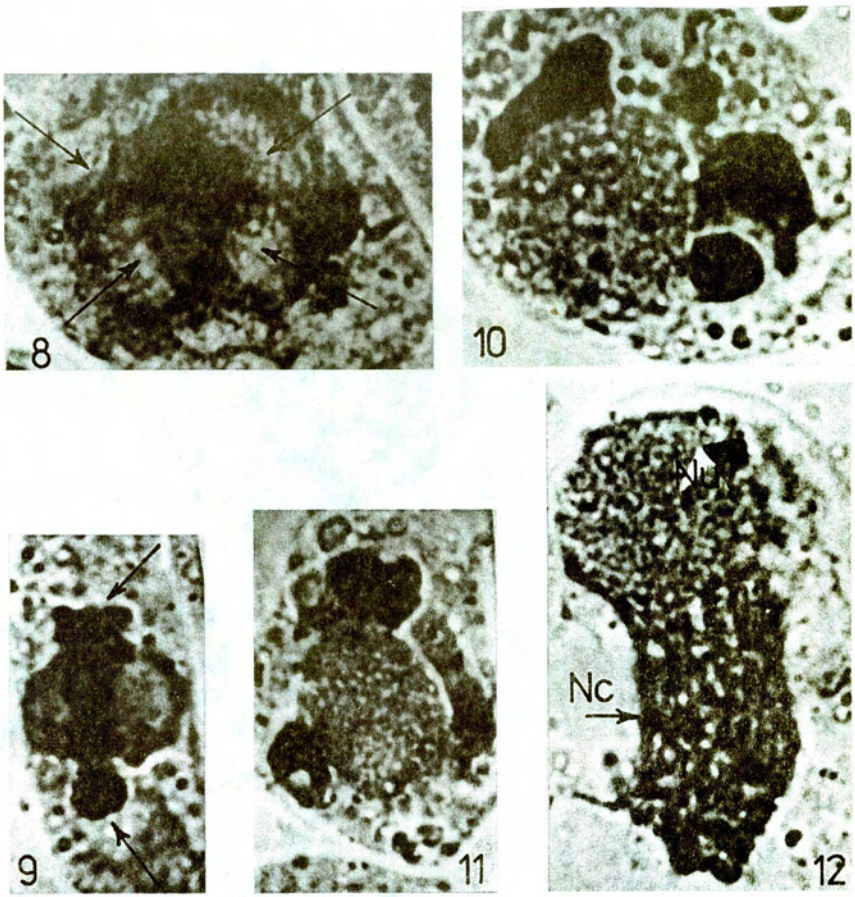








Figs. 4–7. – The effects of *Boletus satanas* whole water extract on genetic material of *Allium cepa*. 4 – chromatic bridges in a late Telophase; 5 – daughter-nuclei separation and cytokinesis blocking; note also the presence of a micronucleus; 6 – endoreduplication (simple arrow) and a ring chromosome (double arrow); 7 – ring chromosome (arrow).



Figs. 8–12. – The effects of *Boletus satanas* whole water extract combined with steroidic bioregulator PAVSTIM on genetic material of *Allium cepa*: 8 – the multiplication of nucleolar organizing regions (NORs) (arrows); 9 – impairments in nucleus division with genome-organizing chromosomes (arrows) forwarded to the poles, well separated from the bulk of remained chromosomes at the equatorium of the cell. Note the persistence of the nucleolar structures in a metaphase-like stage. 10–11 – Nuclear disaggregation with the amplification of nucleolar fibrillar material; 12 – "Nuclear segregation" with a nuclear chromatin mass (Nc) at one pole and nucleolus fibrillar material (Nu) at the opposite pole.

nent of DNA molecule. Such modifications produce impairments in the function of the hereditary material, expressed at the cell level as clastogenic effects or chromosomal damages of breakage type, followed by chromosomal rearrangements of ring-chromosome type.

The combined effects of *Boletus satanas* whole water extract and PAVSTIM proved to be very strong, affecting the nuclear structure with specific action on nucleolar organizing region (NOR) as well as on genome organizing chromosomes

(GOC) (Gavrilă et al. 1995). Previous investigations with PAVSTIM allowed us to detect its stimulatory effects with an obvious increase in mitotic index value.

The combined effects of *Boletus satanas* total water extract and PAVSTIM put the cell of onion in an unusual situation: while PAVSTIM stimulated the synthetic processes (i.e. DNA, RNA and protein syntheses), the fungus extract acted as clastogenic agent, blocking the cell division, mainly cytokinesis. As a consequence, the cell reached big sizes, while the nucleus became progressively larger and larger, sometimes occupying the entire space inside the cell (Fig. 8), exibiting several nucleolar-organizing regions (NORs).

In the cells being in division process, with genome-organizing-chromosomes reaching the poles (Gavrilă et al. 1995) some impairments in the nucleus division are encountered (Fig. 9). The most intriguing aspect of this combined effect of *Boletus satanas* whole water extract and PAVSTIM is the crack of the nucleus in heterochromatic blocks, while its fibrillar euchromatic part, representing highly amplified ribosomal cistrons (rDNA), remained intact and very well delimited (Figs. 10–12).

This experiment in which the *Boletus satanas* whole water extract was mixed with a steroidic bioregulator of vegetal origin allowed us to speculate on the nature of stimulating effect of PAVSTIM: the amplification of ribosomal cistrons and intensification of ribosomal gene transcription. As a consequence, having a rich ribosomal population, the cell performs the other syntheses at a high rate, leading to the enlargement of the whole cell. This scenary remains to be proved concretely, at molecular level.

CONCLUSIONS

1. The whole water extract of *Armillaria mellea* – an edible mushroom has no clastogenic or mutagenic effect.
2. The whole water extract of *Armillaria* sp. – an unedible mushroom – has clastogenic effects. In our experiments we were not able to detect any mutagenic effects, at the chromosomal level, except for chromosomal corrosion, chromosomal stickness, chromatic bridges, chromatin fiber despiralization, cytokinesis blockage and nuclear pycnosis.
3. The whole water extract of *Boletus satanas* – a poisoning mushroom – exibits both clastogenic and mutagenic effects, the latter being of radiomimetic type, expressed by the appearance of ring-chromosomes.
4. The chemical composition of boletol (boletoxin), which is a component of whole water extract of *Boletus satanas* and contains anthrakinone cycle, is perhaps responsible for the mutagenic effect of this extract.
5. The combined effect of whole water extract of *Boletus satanas* and of the steroidic bioregulator PAVSTIM is mainly exerted on ribosomal cistrons, inducing their amplification, but finally, the nuclear disaggregation appears.

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* *Bucharest University, Genetics Department*
** *Genetics Institute, Kishinew*
*** *Biotehnos, Romania,*
Genetics, Mutagenesis and
Genetic Engineering Department

THE MICROMORPHOLOGY OF FRUIT EPICUTICULAR WAX OF SOME APPLE ABORIGINAL VARIETIES

NAELA COSTICĂ, C. TOMA

We analysed and described the micromorphological details of the epicuticular wax from the fruits of some aboriginal apple varieties.

HISTORICAL REFERENCES

The functions, chemical compositions, mechanisms of synthesis and crystallisation of the epicuticular wax are well illustrated in the speciality literature, representing the theme of an anterior contribution [2].

The morphology of the epicuticular wax is little described for the apple fruit, and especially for the aboriginals apple varieties.

Recent studies treat this aspect only indirectly, within the framework of the elimination ways of the wax peculiarities (as a whole) of the protector peripherical structures at different groups of varieties or of the influence of ecological conditions [5, 6]. The epicuticular micromorphology varies, depending on the peculiarities of the epidermal cells and to a certain extent on the apple variety. The differences noticed among the fruits of the same apple variety depend on the ripening degree, cultivation conditions and altitude. The fruits of the early varieties present a wax layer thinner than those of late varieties, at which the depositions are massive. The altitude growth determines modifications of the epicuticular surfaces, in the sense of reducing the thickness of the wax layer and the density growth of the plates with uniform distribution [5].

The necessity of these researches results from the role of wax layer for the apple fruit; the wax extraction is followed by a significant increase of the permeability and cuticular transpiration, and by micromorphology modification in the wax, which is responsible for the humidity degree of the surface [4]; at these there is added the fact that the fruits of the aboriginal apple varieties have not been researched, from this point of view, until now.

MATERIAL AND METHOD

From *Moți, Procovene, Șiculane, Țigănci* and *Viești* varieties, pericarp fragments with untouched epicuticular surfaces have been fixed through lyophilization, then metallized with Ag, and examined at the scanning electron microscope Tesla BS-301.

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The significant images of the studied surfaces are rendered on 8 microphotographs, grouped in 2 plates.

The fruits from the mentioned varieties were harvested at the ripening, from the pomological collection of the Agronomic University in Iași.

RESULTS AND DISCUSSIONS

The micromorphology description of the epicuticular wax has been realised in accordance with the existing terminology from the literature. The wax crystalloids of the fruits from *Moți* variety are of granular type with high density and a light tendency of elongation, juxtaposed in broad stripes, which are parallelly oriented, and which prefigures the characteristic disposition of typical lamellar crystalloids (Fig. 1). Between the stripes they appear rarely and with aleatory disposition. Towards the equator of the fruits, the crystalloids are little tall and massive with tendency of torsion and little density, often irregularly disposed and accompanied by very little granular elements. The epicuticular morphology of the fruits of this variety frame in an intermediary passing category between granulates and plates and would constitute a proof of transformation of crystalloid forms.

In the *Procovene* variety the dominant morphological elements of the surfaces are granulated sometimes disposed in groups. It has been established, in this case, the same tendency of elongation of granules and the formation of lamellar type crystalloids.

It has also been observed a recrystallisation phenomenon of the wax. A "situs" of secretion, alike with a volcano cone would explain the radiar-concentric distribution of crystalloid form (Fig. 2).

The basal coarse relief of the fruit surfaces from *Șiculane* variety is covered with granular elements, disposed in balled or lineal groups. The latter can develop towards massive lamellar crystalloids with little waved border, which appear in the neighbourhood of wax zone in palisade form (Fig. 3). The fruit extremities are covered with granular, spherical wax with great density, sometimes with grouping tendency, rarely existing isolated and elongated crystalloids.

The surfaces from fruits extremity of *Țigănci* variety are dominated by orderly distributions of lamellar crystalloids or transitory phase towards this disposition (Fig. 4). As they fade away from the extremities, the regulator distribution of stripes demises and their density is evidently reduced, on the other hand it grows the number of morphological elements with transitory character towards elongated wax until this dominance. On limited surfaces appear stripes which seem to proceed by breaking off the basal wax layer and from little granular elements. There have been emphasized possible "situses" of specific secretions of elongate type or of their transitory forms (Fig. 5).

The epicuticular surfaces of the fruits from *Viești* variety have large fields of granular wax (Fig. 6) among which there have been observed rare elements of

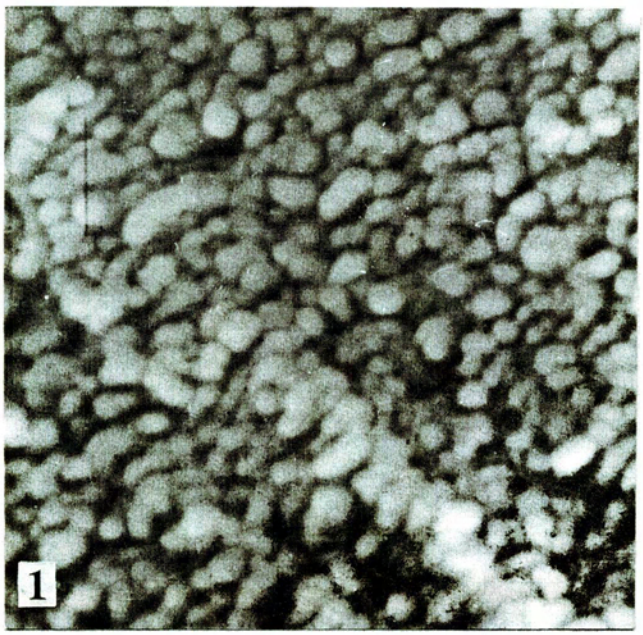
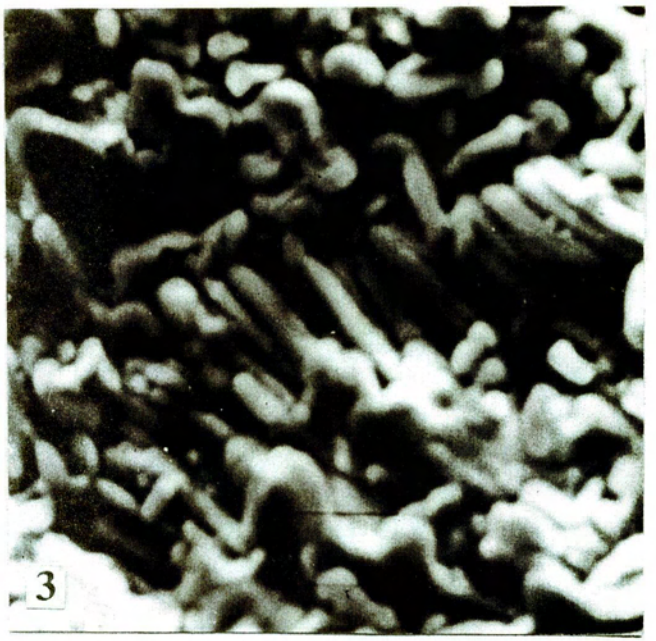


Plate 1. – The micromorphology of epicuticular wax at the analysed apple fruit varieties: Fig. 1 – *Moți* (× 5000); Fig. 2 – *Procovene* (× 2500).



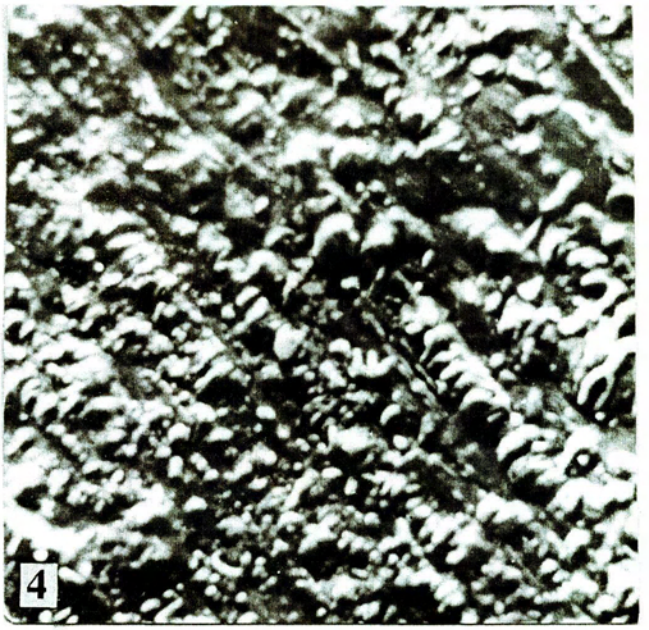
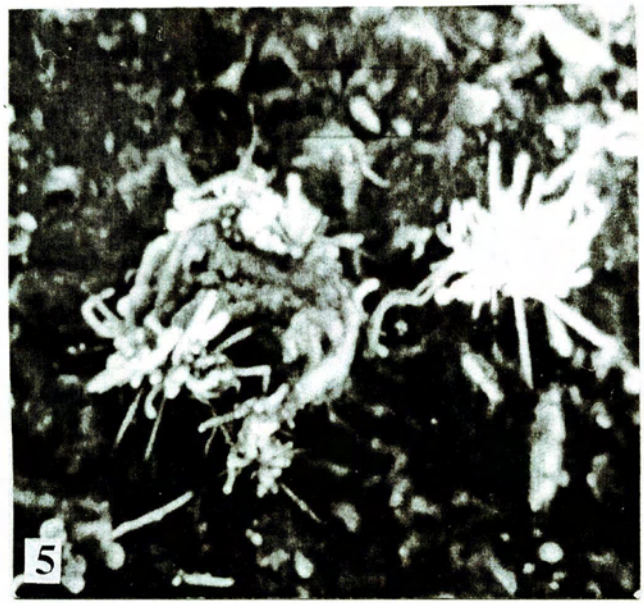


Plate 1. – The micromorphology of epicuticular wax at the analysed apple fruit varieties: Fig. 3 – *Șiculane* (× 5000); Fig. 4 – *Țigănci* (× 2000).



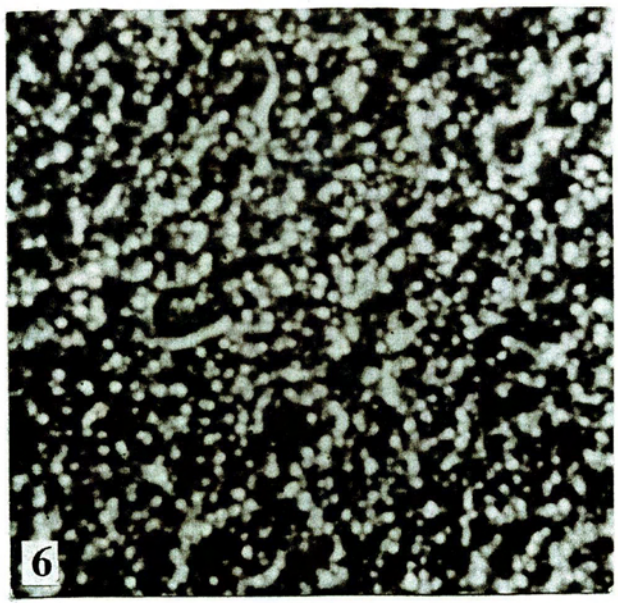
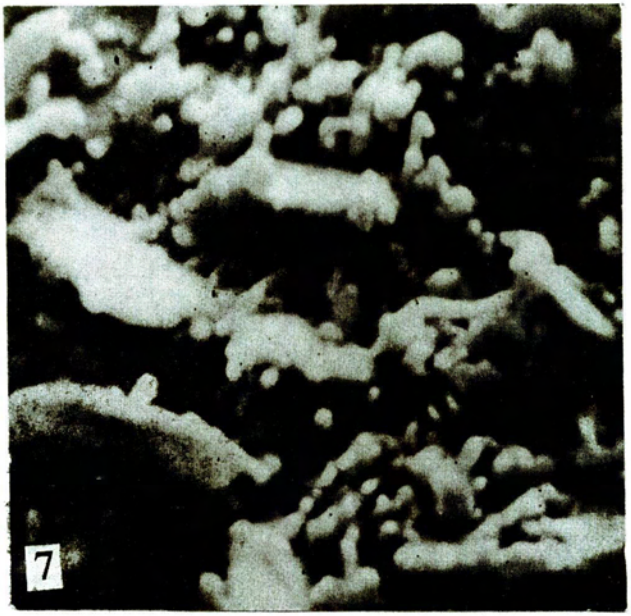


Plate 2. – The micromorphology of epicuticular wax at fruit which belong to the researched varieties: Fig. 5 – *Țigănci* (× 2500); Fig. 6 – *Viești* (× 2000).



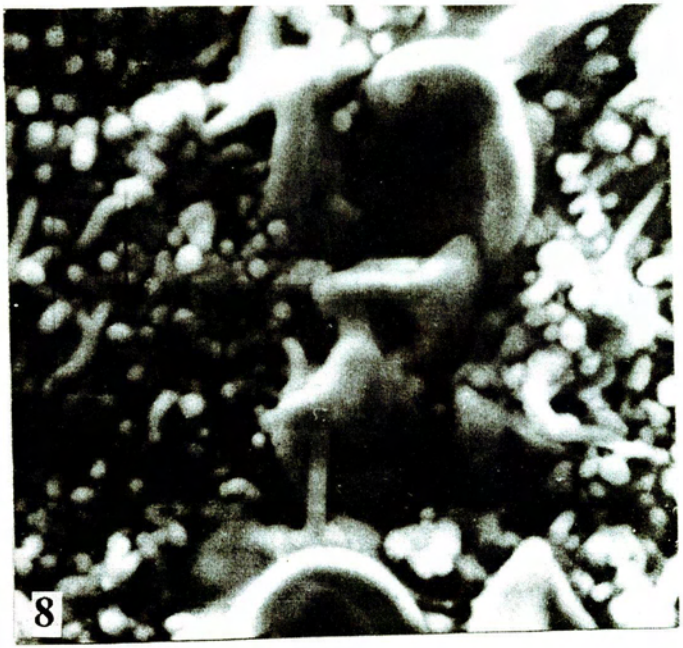


Plate 2. – The micromorphology of epicuticular wax at fruit which belong to the researched varieties: Fig. 7 – *Vieşti* (× 4800); Fig. 8 – *Vieşti* (× 5500).

lamellar wax, besides sticks wax (Fig. 7). It has also been presented lamellar wax, extremely massive with an auditory pavilion aspect, sometimes in groups of bi- or three components, which delimit an internal space (Fig. 8).

The epicuticular wax morphology is determined, to a large extent, by its chemical composition, but also by the physical and chemical properties of the cell wall and of the cuticle. The form, dimensions and the distribution of the secretion "situs" are determined by the types of macromolecules which compound the cell wall and the cuticle by the intermolecular bounds and by the action direction of the foke resultant of extent which accompany the growth. The crystallised wax, which appears at the level of some zone of minimal resistance of the basal texture, has first of all the role to cover the formed slits. In the median zone of the fruits, where the biosynthesis and growth processes are more intense, the crystalloids usually have larger dimensions and less density than in their polar zones, where the epidermal cell has a linear orientation and is approximately parallel to the long axle of the fruit and the elongation stress is weak, determining a regular distribution, with a great density of the crystallised wax. A proof for the production of elongation phenomenon is the presence of the "palisade" wax which always appears between two parallel zones of massive wax and which represent "pillars", the elongation happening between them.

CONCLUSIONS

The investigations concerning the micromorphology of the epicuticular wax from the fruits of five apple varieties, spotlight 3 types of crystalloids: lamellar, granular and elongated.

It is established the existence of some dimensional, numerical and distribution differences of crystalloids between the extremities and the median sections of the fruits. There are established some correlations between the types and the distribution of crystalloids and their protective efficiency. Thus, the lamellar wax in an orderly arrangement is the characteristic of the fruits with a long keeping period, substituting, to a certain extent, the function of a thin cuticle.

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"Al. I. Cuza" University
Iassy, Copou 20A

THE ASSESSMENT OF CELL RADIOSENSITIVITY FOLLOWING THE TREATMENT OF GAMMA RADIATIONS COMBINED WITH PROCAINE AND TYASTIME IN *PHASEOLUS VULGARIS* L.

RODICA DUMITRESCU*, M. ANDREI**, D. POPESCU*

The present study shows the Procaine 1‰ and the Tyastime 1‰ interaction, on the one hand, in the relationship between nuclear volume, interphase chromosome volume and nuclear radiosensitivity, on the other hand, in the correlation between nucleus/cytoplasm ratio and cell radiosensitivity. The Procaine 1‰ and Tyastime 1‰ in combination with gamma radiation at 10 Gy and 30 Gy doses at 30 Gy/min rate had a synergetic action decreasing the average values of nuclear volume below the control value. The Student test showed that the decrease was very significant ($p < 0.001$) in variants treated with Procaine 1‰ and Tyastime 1‰ in association with 10 Gy dose in cells of root elongating zone. The changes of cytoplasm volumes are very low significant in variants treated with Procaine 1‰, in cells of root meristematic zone and significant in cells of elongating zone. The nucleus/cytoplasm ratio varies close to control, that proves a low cell radiosensitivity, which in turn shows that the proliferative ability is not affected.

The gamma radiation differently influences the plants, depends on the dose and rate used, causing a radiosensitivity on the cell level. ANGHEL and TOMA (2) showed that a constant nucleus/ cytoplasm ratio, which characterises each species, always exists in the untreated plants. The studies of MILLER and SPARROW (11), DUMITRESCU et al. (5) evidenced that gamma radiations cause severe damages to interphase nucleus. RAICU (16) specified that gamma radiations block the DNA synthesis in interphase and consequently the nucleus volume modifies. DUBININ (4), PATIL et al. (15) related that different nucleus volumes in cells of various tissues in the same species also may appear. AL-RUBEAI (1) concluded that nucleus volume modifications could be responsible for the species radiosensitivity.

SPARROW (18) considered that nuclear volume variations are correlated with those of interphase chromosome volume (ICV).

The studies on the direct effect of gamma radiations on the cell radiosensitivity in *Phaseolus vulgaris* L., performed by DUMITRESCU, ANDREI, POPESCU (5), demonstrated that gamma radiations differently affected the two root areas (meristematic and elongating zones). The increase of nuclear volumes and of ICV denotes a rise of nuclear radiosensitivity. Also, the fluctuations of nucleus/cytoplasm ratio suggest a different cell radiosensitivity as a function of root zone and treatment.

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In the present paper we attempt to demonstrate the Tyastime 1‰ and Procaine 1‰ interaction, on the one hand, in the relationship between nuclear volume, interphase chromosome volume and nuclear radiosensitivity, on the other hand, in the correlation between nucleus/cytoplasm ratio and cell radiosensitivity.

MATERIALS AND METHODS

The *Phaseolus vulgaris* L. seeds moistened 24 h. in running water, or Procaine 1‰, or Tyastime 1‰, were subjected to the following doses of gamma radiations at 10 Gy/min. rate: 10 Gy and 30 Gy. The roots resulted 6 days after irradiation were fixed in Carnoy solution (3:1), 24 h, at 4°C. They were subjected to hydrolysis and staining with acetic carmine solution (45%) and then placed on the slides by the squash method. The major and minor axes of nucleus and cell were measured using an eye micrometer at 100 cells for each treatment series. The nuclear and cytoplasm volumes were calculated using the formula quoted by DAVIDSON (3), ICV according to MORTON and MILLER (12) and the nucleus/cytoplasm ratio according to ANGHEL and TOMA (2). The significance of differences between mean values was assessed by Student test according to MUREȘAN (13).

RESULTS

1. THE NUCLEAR VOLUME AVERAGES

a) In the *root apical meristematic zone* (Table 1) the chemical substances utilised in combination with gamma radiation had a synergetic action, decreasing the average values of nuclear volume (Fig. 1, a) below the control value. The

Table 1

Apical meristematic zone

Treatment	Nuclear Volume (μm^3)	Significance	Cytoplasm Volume (μm^3)	Significance	I.C.V (μm^3)	N/C Ratio
CONTROL	128.61±9.71	–	3211.6±271.6	–	5.84	0.040
Procaine 1‰+10 Gy	111.10±8.32	NS	2312.46±204.45	$p < 0.01$	5.05	0.048
Procaine 1‰+30 Gy	92.61±10.54	$p < 0.02$	2243.86±398.95	$p < 0.05$	4.20	0.041
Tyastime 1‰+10 Gy	113.25±8.36	NS	2658.57±311.8	NS	5.14	0.042
Tyastime 1‰+30 Gy	115.91±8.84	NS	3198.08±526.02	NS	5.26	0.036

$p < 0.05$ very low significant, $p < 0.02$ low significant, $p < 0.01$ significant, $p < 0.001$ marked significant

Student test showed that a low significant difference was registered only in the treatment of Procaine 1‰ + 30 Gy (91.61 μm³) ($p < 0.02$).

b) In *root elongating zone* (Table 2, Fig. 1, b) both Procaine 1‰ (265.66 μm³) and Tyastime 1‰ (270.88 μm³) determined a decrease of nuclear volumes below the control value (390.6 μm³), at 10 Gy dose, but this represents an increasing ten-

Table 2

Elongating zone

Treatment	Nuclear Volume (μm³)	Signi-ficance	Cytoplasm Volume (μm³)	Signi-ficance	I.C.V. (μm³)	N/C Ratio
CONTROL	390.60±9.22	–	10460±945.2	–	17.75	0.037
Procaine 1‰+10 Gy	265.66±17.34	$p < 0.001$	8023±747.62	NS	12.07	0.033
Procaine 1‰+30 Gy	433.52±66.60	NS	11610.0±1414.3	NS	19.70	0.037
Tyastime 1‰+10 Gy	270.88±16.01	$p < 0.001$	8537.88±703.18	NS	12.03	0.031
Tyastime 1‰+30 Gy	390.91±40.62	NS	13801.5±1693.2	NS	17.76	0.028

$p < 0.05$ very low significant, $p < 0.02$ low significant, $p < 0.01$ significant, $p < 0.001$ marked significant

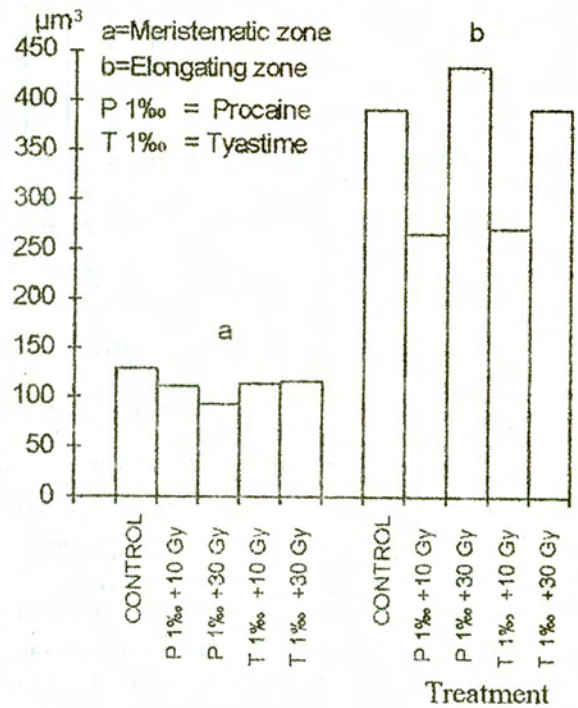

Fig. 1. – Nuclear volume (combined effect) – *Phaseolus vulgaris* L.

dency in comparison with the direct treatment of 10 Gy (240.72 μm³) (5). The Student test revealed that the differences are very significant against control ($p < 0.001$).

2. INTERPHASE CHROMOSOME VOLUME (ICV)

After SPARROW et al. (19) the ICV varies as a function of treatment in correlation with nuclear volumes. ICV represents the chromosome volume and other nuclear substances named "nuclear shap" (8, 10). The nuclear volume modifications and of ICV lead to the changes of the species radiosensitivity (1, 11).

The data in Tables 1, 2 and Fig. 2 a, b evidence that in both root areas, a good correlation between ICV values and that of nuclear volumes exists at cellular level.

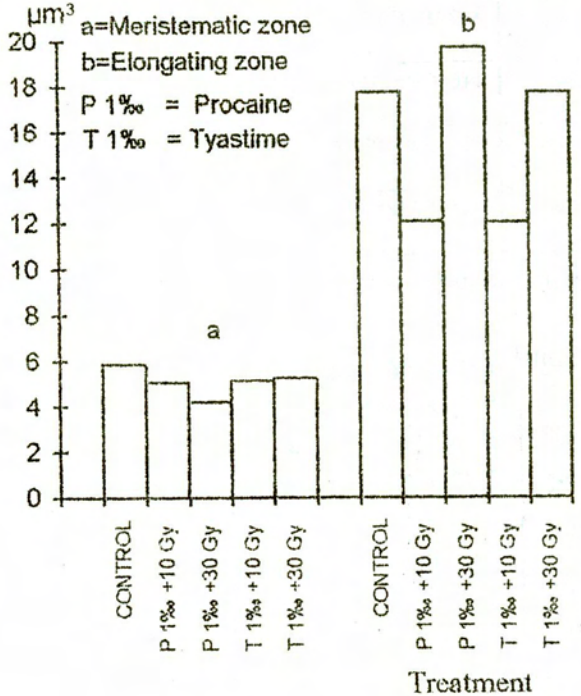

Fig. 2. – ICV (combined effect) – *Phaseolus vulgaris* L.

3. THE CYTOPLASM VOLUME AVERAGES

a) Root apical meristematic zone (Table 1, Fig. 3 a)

The direct treatment with gamma radiation at 10 Gy and 30 Gy doses (RODICA DUMITRESCU et al. 1996) (5) caused the increase of cytoplasm volumes against control (3211.6 μm³) in meristematic cells.

The combination of Procaine 1‰ and Tyastime 1‰ with gamma radiations, at the above mentioned doses, diminished the averages of cytoplasm volumes as compared to control, but significant differences were only in the Procaine 1‰ treatment.

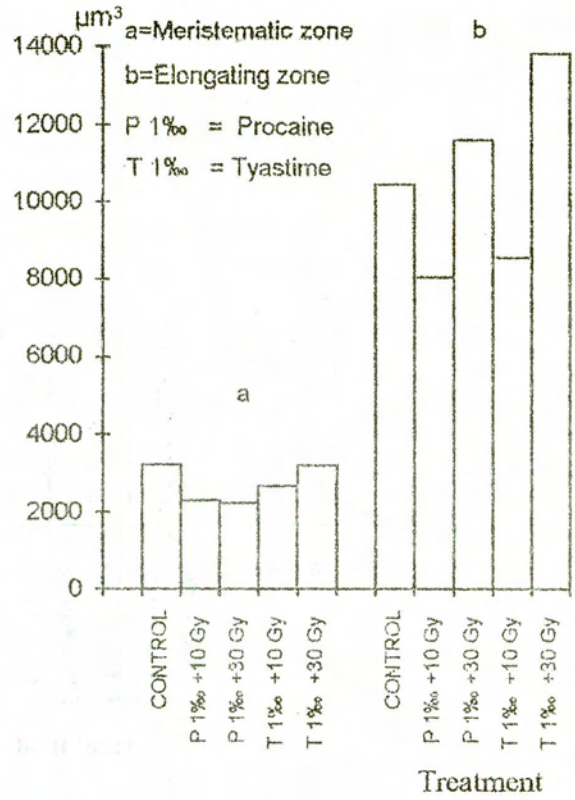

Fig. 3. – Cytoplasm volume (combined effect) – *Phaseolus vulgaris* L.

b) Root elongating zone (Table 2, Fig. 3 b)

The cytoplasm volumes of cells in this zone are greater than those of cells in the root meristematic zone.

The two chemical substances in combination with 10 Gy dose determined the decrease of cytoplasm volumes below the control value, while in combination with 30 Gy dose, caused increases of these above control values. The statistical evaluation indicated that these variations of cytoplasm volumes are not significant, and the cell radiosensitivity is not modified.

4. THE NUCLEUS / CYTOPLASM RATIO (N/C) (TABLES 1, 2, Figs. 4 a, b)

Fluctuates close to control depending on treatment, in cells of both root zones studied. The values of nucleus/cytoplasm ratio range between 0.036–0.048 in the meristematic zone of root, and between 0.028–0.037 in the elongating zone.

In meristematic zone, both Procaine 1‰ and Tyastime 1‰ in combination with 10 Gy dose caused increases of nucleus/cytoplasm ratio against control. In cell elongating area, the Procaine 1‰ in combination with 30 Gy dose gave nucleus/cytoplasm ratio identical to control.

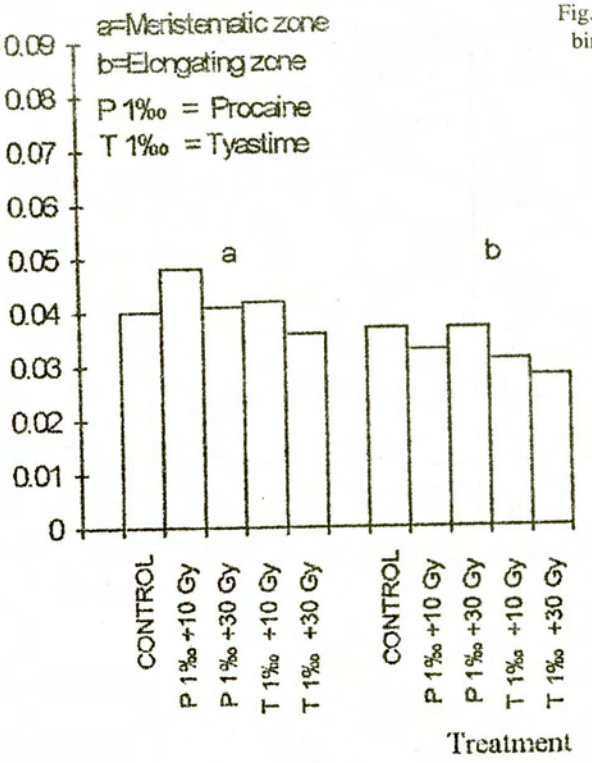

Fig. 4. – Nucleus/cytoplasm ratio (combined effect) – *Phaseolus vulgaris* L.

DISCUSSIONS

The appeared differences between nuclear volumes in cells of two root zones might depend on the distinct response of cells to the gamma radiation and chemical substances impact, as well as on the different quantities of "nuclear shap" material present in the nucleus.

DUBININ (4) specified that the nucleus activity depends on its structure characteristics, during mitosis and interphase. WATSON (20) in turn confirmed that the cell metabolism is realised on the principles of a controlled system, in which the information carried by nucleus depends on its structure.

The increase of DNA quantity in interphase is connected to the increase of nucleolus matter, non-nucleolus matter, including the chromosomes. Thus, SPARROW (18) evidenced a correlation between nuclear volume and DNA quantity/cell, and subsequently (19) demonstrated a correlation between ICV and DNA quantity/chromosome.

The studies done at molecular level by FRAIFELDER (7), WATSON and HOPKINS (20), RAICU (16) demonstrated that gamma radiation at specific doses generates monocatenary and bicatenary breaks of DNA catene coupled to changes

of adjacent bases. These are responsible for the block of replication and transcription which in turn causes a mitosis delay.

NASAZZI et al. (14) showed also that DNA catene break and base recombinations cause chromosomal aberrations in mitosis. These induce the dispersion of chromosomes in the nucleus which in turn could determine the variation of nuclear volume. AL-RUBEAI (1) suggested that nuclear volume modifications lead to changes of the nucleus radiosensitivity.

Procaine 1‰ and Tyastime 1‰ contain sulphur in their molecule determining a diminishing of the gamma radiation effect by a mechanism similar to anoxia, the removal of intracellular oxygen [FORSBERG, 1953, cited by GHIORGHIȚĂ (9), SHAW and CADET (17)] According to the proposed model, it is thought that two SH- groups and oxygen combine to make one water molecule and a disulphide bond.

The effects produced by gamma radiation are distinct depending on the species. In *Phaseolus vulgaris* L. the number of chromosomes is relatively high ($2n = 22$), while the nuclear volume is small, thereby according to resistance to radiation.

FEHRENBACH et al. (6) consider that cytoplasm/nucleus ratio changes are determined by individual fluctuations of the nucleus and cytoplasm volumes, and prove an alteration of subcellular structures. The modifications of nucleus/cytoplasm ratio cause at last changes of cell radiosensitivity which influence the proliferative ability of cells.

In the case of *Phaseolus vulgaris* L., the combined treatment of gamma radiation and chemical substances resulted in nucleus/cytoplasm ratio values close to control which denotes a low cell radiosensitivity.

The Student test showed that modifications of all cell parameters studied were not always significant.

CONCLUSIONS

1. Procaine 1‰ and Tyastime 1‰ used in combination with gamma radiations at 10 Gy and 30 Gy doses, at 10 Gy/min. had a synergetic action diminishing the values of nuclear volumes below the control value.

2. The significant changes ($p < 0.02$) of nuclear volumes were registered in the root meristematic zone for the treatment with Procaine 1‰ +30 Gy and very significant ones ($p < 0.001$) in root zone of cell elongating at variants: Procaine 1‰ +10 Gy and Tyastime 1‰ +10 Gy.

3. A good correlation exists between nuclear volumes evolution and ICV, respectively the nuclear radiosensitivity.

4. Cytoplasm volumes decrease against control in meristematic zone of root, being significant ($p < 0.01$) in variant: Procaine 1‰ +10 Gy and very low significant ($p < 0.05$) in variant Procaine 1‰ +30 Gy.

5. In root zone of cell elongating the fluctuations of cytoplasm volume are not significant.

6. The nucleus/cytoplasm ratio variation is close to control, evidencing that cell radiosensitivity is low in this treatment and at this species, and that the proliferation potentiality of cells is not affected.

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* *Institute of Biology*
Bucharest, Spl. Independenței 296
** *Faculty of Biology*
Bucharest

INFLUENCE OF SELENIUM ON PHOTOSYNTHESIS AND ULTRASTRUCTURE OF PHOTOSYNTHETIC APPARATUS IN MAIZE LEAVES

H. TIȚU, GABRIELA VLĂDEANU, NICULINA MITREA

The paper is dealing with the effect of 1, 10, 100, 1,000 and 10,000 ppm of Na_2SeO_4 on the chlorophyll content, photosynthesis, carbohydrate compounds and ultrastructure of the photosynthetic apparatus in maize leaves (*Zea mays* L.). Selenate decreased the biosynthesis of chlorophyll *a*; chlorophyll *b* has been stimulated by the concentrations of 1–100 ppm during the first 7 days of treatment, but decreased after 14 days. The photosynthetic rate decreased along with increase of selenate dose; the carbohydrate compounds were stimulated by all selenate doses. High dose of selenate induced disintegration of stroma lamellae in mesophyll chloroplasts; sheet bundle chloroplasts contain more starch inclusions than in control plants.

The research concerning selenium toxicity began to intensify starting with the finding out of the so-called alkaline disease at the farm animals from the USA that consumed forage made the selenium-accumulators plants. Then it was found that selenium, in high concentrations, is toxic for most of the organisms: microorganisms, plants and animals. Plants which absorb high quantities of selenium without their growth diminution are some herbs from the *Astragalus*, *Neptunia* a.o. species (9–10). Some higher mushrooms from the *Amanitaceae* group also contain high quantities of selenium (6). On the other side, literature data show that selenium, in small quantities, stimulates the growth of some cultivated plants (17). The acute deficiency of selenium provokes severe diseases such as muscle degeneration, hepatic necrosis, malignant tumors a.o. at animals and humans. These diseases can be prevented or cured by administrating of appropriate selenium doses as well as with different drugs: E vitamin, carotenoids, a.o. (8), (11). The explosion of informations concerning the toxicity and the tolerance of selenium is comprised in many reviews published in the last decades (3), (10), (12), (15).

In the following paper we present preliminary data concerning some physiological and ultrastructural peculiarities at maize plants treated with different doses of selenium.

MATERIAL AND METHODS

Maize plants (F 365/93), with three leaves, resulted from seeds which were kindly obtained from the Research Institute for Cereal and Industrial Crops Fundulea

and were grown 7 and 14 days (7d and 14d) on Knop medium with Arnon micro-elements. The selenium was supplied as a Na_2SeO_4 in concentrations which are ranged from 1 to 10,000 ppm Se. Photosynthesis was measured by Ivanov's method (cited in Boldor et al. 1973) and chlorophylls and carotenoids according to Holm (5). Carbohydrate compounds were determined by the Hagedorn-Jensen method (cited in Skazkin et al. 1958).

Electron microscopy. Samples of the first leaf tissue with approximative dimension 1/10 mm from the above control and treated with selenate plants, were fixed in 4% glutaraldehyde (0.1 M phosphate buffer, pH 7.4) for 4 hours and postfixed in 2% osmium tetroxide (0.1 M phosphate buffer, pH 7.2) for 2 hours. Fixed material was dehydrated in ethanol and embedded in *Epon* 812 (*Serva Feinbiochemica*). Ultrathin sections were cut on an ultramicrotome Tesla BS 490A and stained with 2% aqueous uranyl acetate and lead citrate. The electron microscopic examinations were performed with *Tesla* 500.

RESULTS

Metabolism peculiarities. The treatment of maize plants during 7 days with 1–100 ppm Se resulted in an increase in the content of assimilatory pigments, which is more pronounced in the case of chlorophyll *b*; at the 1,000 and 10,000 ppm Se samples, the chlorophylls and carotenoids had less values than with the control plants. The diminution of the total chlorophyll is accompanied by the decrease of the photosynthetic rate. In these conditions the leaves have a chlorotic aspect. But the selenium toxicity is more evident after 14 days of treatment with natrium selenate, probably due to the translocation and the accumulation of this compound in all the plant's organs; the pigment composition and the rate of photosynthesis are less at all the selenium doses comparatively with the control plants and with the shorter duration of treatment (Table 1, Figs. 1, 2). On the contrary, at the plants

Table 1

Effect of selenium upon assimilatory pigments from maize leaves after different duration of treatment

Samples ppm Se	Pigments mg/ g dry wt							
	7 days				14 days			
	chlorophyll *a*	chlorophyll *b*	chlorophylls *a+b*	carotenoids	chlorophyll *a*	chlorophyll *b*	*a+b*	carotenoids
Control	7.58	1.79	9.37	2.51	7.54	2.79	10.83	2.25
1	7.82	4.14	11.94	1.52	3.44	2.24	5.68	1.24
10	7.69	4.24	11.93	2.55	5.70	2.37	8.07	2.94
100	7.55	4.31	11.85	2.47	4.94	1.99	6.83	1.66
1,000	7.43	2.79	10.22	2.55	4.61	2.03	6.64	1.75
10,000	7.20	1.58	5.78	1.87	3.44	2.24	5.68	1.24

leaves treated for 14 days with 1–10,000 ppm Se it is observed a positive relation between this element concentration and the accumulation of carbohydrate compounds (Table 2). The reports presented above show that the treatment of plants

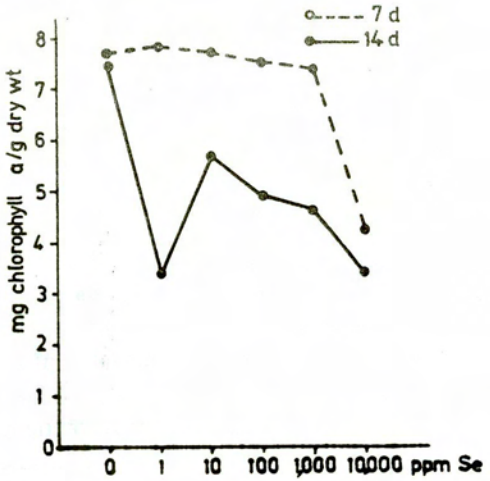

Fig. 1. – Effect of selenium on biosynthesis of chlorophyll *a* from maize leaves.

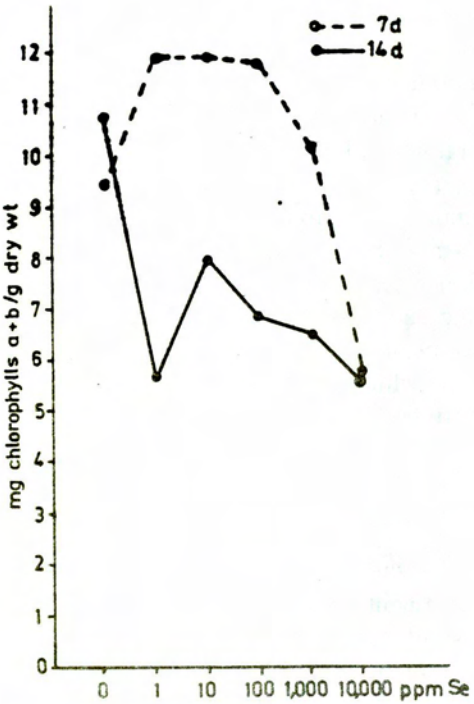

Fig. 2. – Effect of selenium on chlorophylls *a* + *b* from maize leaves.

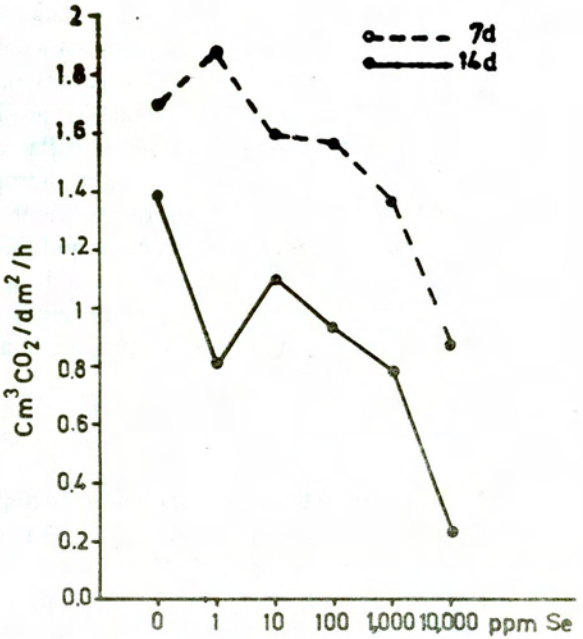

Fig. 3. – Effect of selenium on photosynthetic rate from maize leaves.

Table 2

Effect of selenium upon carbohydrate compounds from the maize leaves

Samples ppm Se	mg/ g dry wt			
	Monosaccharides	Disaccharides	Polysaccharides	Total carbohydrates
Control	2.16	0.20	8.84	12.21
1	2.99	1.71	10.79	16.79
10	2.60	1.77	10.52	16.14
100	2.04	2.62	9.91	15.82
1,000	2.00	2.06	8.64	13.73
10,000	3.42	4.53	10.35	19.69

for a short period of time, with low concentrations of selenium, stimulated the biosynthesis of chlorophylls and carotenoids and the rate of photosynthesis.

Photosynthetic apparatus. Electronmiscroscopic examination of chloroplasts from control samples showed they have ultrastructural peculiarities similar with the ones of the organelles from the C_4 plants. The mesophyll chloroplasts have a well developed system of photosynthetic lamellae assembled into grana stacks with numerous thylakoids (Fig. 4); the bundle cell chloroplasts contain lamellae differentiated only in very few rudimentary grana, but are filled with voluminous starch inclusions (Fig. 5). The unique feature of mesophyll and bundle cell chloroplasts is the pheripheral reticulum that is made of numerous vesicles. The ultrastructure of the chloroplasts from plants grown 7 days in the medium with 1–1,000 ppm Se does not present degradative changes of the photosynthetic apparatus; at the 10,000 ppm Se, the grana thylakoids are less numerous than in the chloroplasts from the control plants, stroma lamellae are ruptured, but abundance of osmiophilic globules is present (electronmicrographs not shown). Selenium toxicity amplifies itself after 14 days of treatment. At higher doses, changes in mesophyll chloroplasts consist in the rupture and the disintegration of stroma lamellae and ribosomes, the diminution of thylakoids per granum and the accumulation of large osmiophilic globules (Fig. 6); in the bundle sheet cell chloroplasts numerous starch inclusions and compact rudimentary grana are visible (Fig. 7).

DISCUSSIONS

Selenium toxicity is shown in the diminution of the chlorophyll *a* biosynthesis starting with 100 ppm dose after 7 days of treatment; after 14 days, the same pigment decreases significantly at 10 ppm Se. A similar rule is noted regarding the intensity of photosynthesis. In opposition, the content in carbohydrate compounds increases at selenium doses, fact that is confirmed by the presence of numerous starch inclusions in the ultrastructure of chloroplasts from sheet bundle cells. Probably, the phenomenon of carbohydrate compounds accumulation also represents a

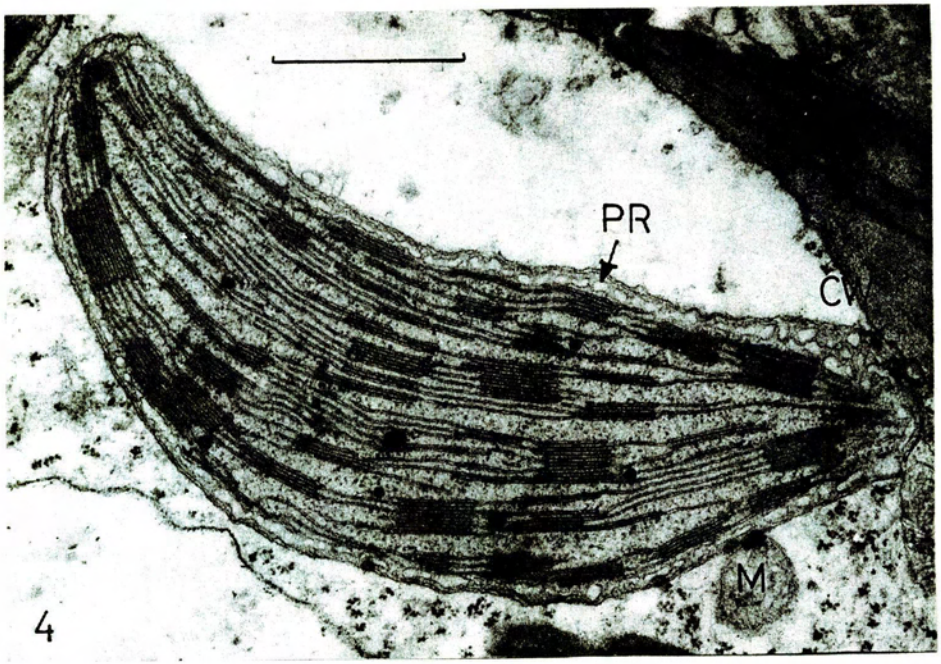

Fig. 4. – Ultrastructure of chloroplast from mesophyll cell; control plants. PR, peripheral reticulum; M, mitochondrion. CW, cell wall. Bar = 1 μm.

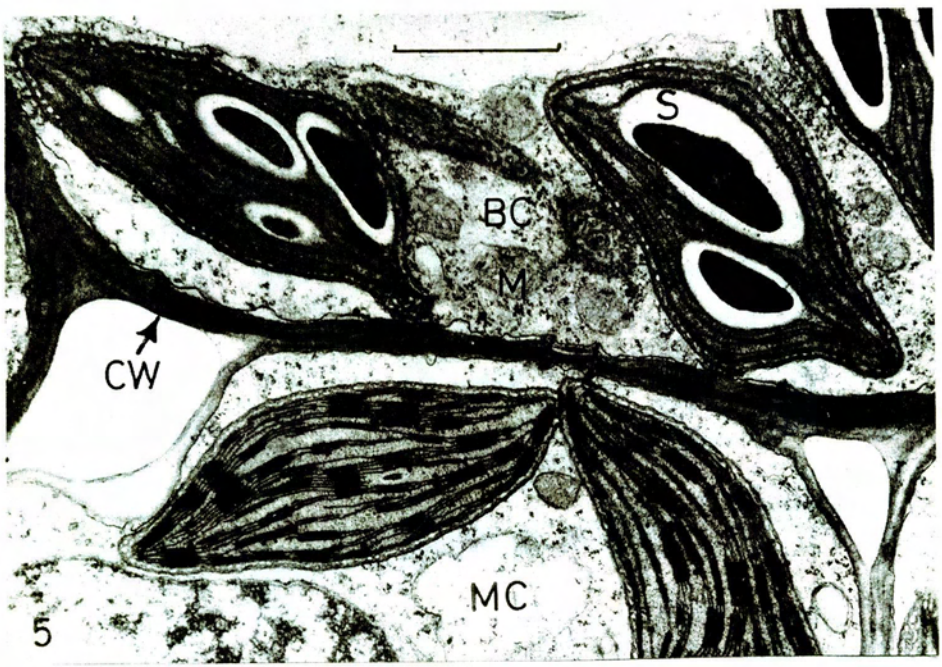

Fig. 5. – Chloroplast dimorphism in maize leaf cells. MC, mesophyll cell; BC, bundle sheet cell; S, starch inclusions. Control plants. Bar = 2 μm.

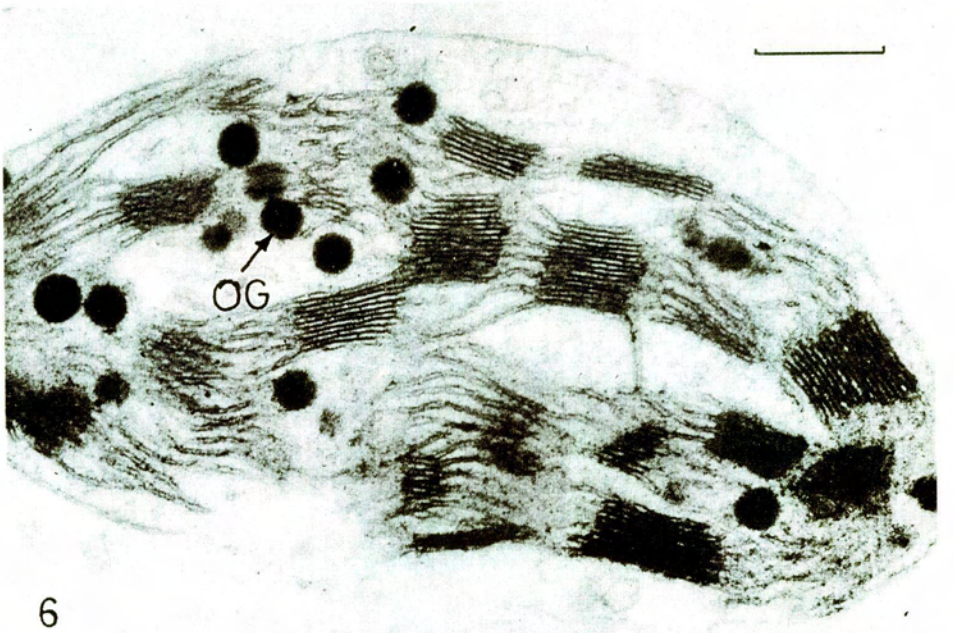

Fig. 6. – Ultrastructure of mesophyll chloroplast from plants treated 14 days with 10,000 ppm Se. Stroma lamellae and chloroplast envelope are ruptured. OG, osmiophilic globules. Bar = 0.5 μm.

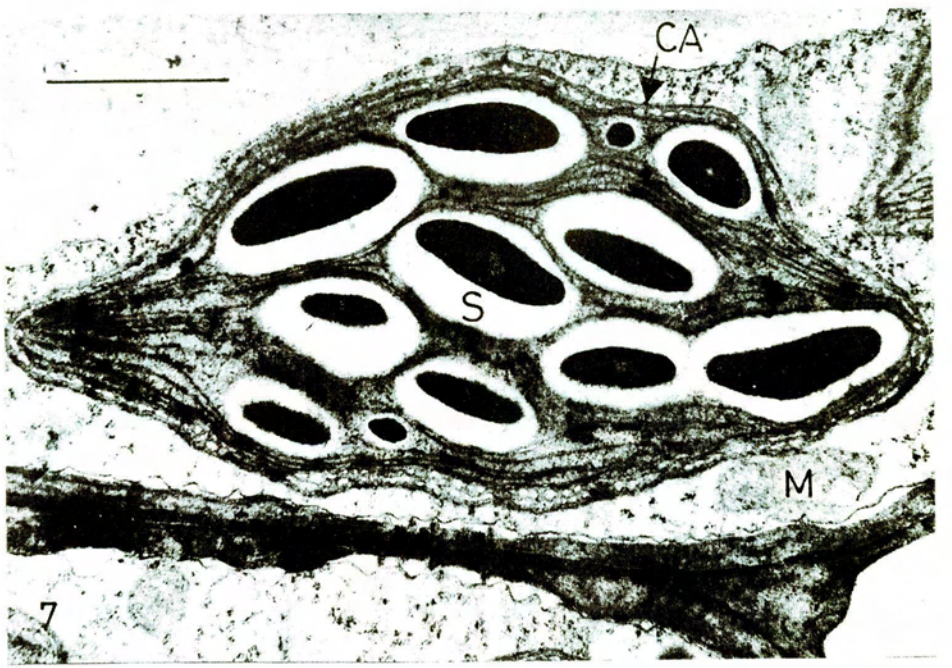

Fig. 7. – Ultrastructure of chloroplast from bundle sheet cell from plants treated 14 days with 10,000 ppm Se. CA, chloroplast envelope. Bar = 1 μm. Explanations in the text.

result of the selenium toxicity consisting in the blocking of the translocation of these substances. Our observations confirm, in this way, the data obtained by Wiliams (cited in Shkolnik, 1984) which include maize in the group of non-selenium accumulators. Broyer et al. (4) established that, in the case of alfalfa and clover plants, the growth is suppressed when the selenium concentration in the leaf tissue reaches 0.2–0.8 μg atoms/g of dry weight. The establishing of the biochemical differences between the accumulators and non-selenium-accumulators plants started from the finding due to which the selenium can replace the sulphur from the amino acids that contain this element, resulting selenocysteine, selenomethionine, a.o. These seleno aminoacids can be incorporated in selenoproteins that are generally very toxic for non-selenium-accumulator plants. At the selenium-accumulators Se-methylselenocysteine is the main free amino acid that is not incorporated itself in proteins at the four accumulator *Astragalus* species. Therefore, it is considered that the presence of selenium in the fraction of free amino acids might be a feature of the selenium-accumulator species (7, 13, 18). The competition between selenium and sulfur was also pointed out by Bosma et al. (2) who found that selenate (Na_2SeO_4) provokes chlorosis at spruce needles; it is proposed that the selenate-induced decrease in glutathione content of spruce needles is caused by a strongly reduced sulfur reduction, due to competition between selenate and sulfate, in combination with rapid turnover of glutathione. Our research will be continued for pursuing the influence of selenium at biochemical level and during a longer period of vegetation at maize and for determining the quantity of this element in different organs of the plants.

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Institute of Biology
Spl. Independenței 296, C.P. 56–53
79651 Bucharest, Romania

CYTOGENETICAL OBSERVATIONS IN THE CALLUS OF GYNOGENETICAL ORIGIN AND AT THE REGENERATED WHEAT (*TRITICUM AESTIVUM* L.) AND SUNFLOWER (*HELIANTHUS ANNUUS* L.)

MARIA PRISECARU*, P. RAICU**, G.I. GHIORGHIȚĂ*

In the callus having gynogenetical origin obtained with *in vitro* ovary culture at wheat (*Triticum aestivum* L.), as at the regenerated plants from the callus and obtained directly from ovules of sunflower (*Helianthus annuus* L.), a cytogenetical study was accomplished. Both the callus and the root meristems of the gynogenetical regenerated plants were coloured according to the classic Feulgen method. In the callus obtained from wheat ovaries there were found out haploid cells (61.5%) and diploid (38.5%). Some of the cells had endoreduplicated nucleus being in the process of dividing. The plants regenerated from the gynogenetical callus at wheat or in the case of sunflower directly from ovules, proved to be haploids (60% at wheat, 50% at sunflower), diploids (32.5% at wheat, 38.4% at sunflower) and mixoploids (11.5% at sunflower).

During the growth process of the plants we notice a fast decrease of the haploid metaphases and a rising of the diploid ones. The presence of the endoreduplicated nucleus being in the process of dividing as the presence of the mixoploids prove the possibility of the spontaneous doubling of the chromosomes.

INTRODUCTION

By means of the important theoretical value and having a large number of potentialities in application, the haploid method comes with many prospects to the creation of some new genotypes.

The experimental gynogenesis is the process of regeneration of a whole plant starting from a single ovule. So, we obtain a plant which contains only the chromosome stock from its mother's, the mother being haploid. The globular structures, the proembryos-formed of one or more cells belonging to the embryo sack can give birth to embryos or to a callus. Later on, being transferred to a medium for the differentation phase, the callus develops embryos which can generate plants.

The ploidy level of the plants regenerated through direct or indirect gynogenesis, via callus, is highly differentiated because out of haploids we can obtain diploids, polyploids, aneuploids and cytochimerics. The non-haploid plants obtained are favourable in many cases. They can be important for the plants' improvement process both from a theoretical and a practical point of view.

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MATERIAL AND RESEARCH METHODS

The number and structure of chromosomes were established paying attention to the root meristem metaphases of the regenerated plants (minimum 50 metaphases/ plant) in the case of wheat (the varieties Podu-Ilioaiei, Turda$_{5190}$, Fundulea 4 and the hybrid F_1 40251), in the case of sunflower (the hibrids F_1 Select, Fundulea 206 and HS-2239) and in the gynogenetic callus obtained from the ovary only at the wheat (the varieties Podu-Ilioaiei, Turda$_{5190}$, Fundulea 4 and the hybrid F_1 40389).

Both the callus and the root meristems were remade according to the classic Feulgen method (prefixed in colchicine solution 0.1%, then fixed in acetic alcohol 3:1, hydrolysis in HCl 1 N at 60°C and the process of colouring with Schiff reagent). The observations were done on squash preparation.

THE RESULTS OF RESEARCH

From the gynogenetical callus obtained at some genotypes of *Triticum aestivum* ($2n = 4x = 42$), samples were taken during the differentation process, approximately 7 days from the complete changing of the ovary.

In the wheat's callus analysed, at Podu-Ilioaiei, Turda$_{5190}$, Fundulea 4, and 40389 genotypes (the 1st table), both the haploid and diploid cells were discovered, the former group being in a larger number (the 1st image). At Podu-Ilioaiei, Fundulea 4 genotypes and at the hybrid 40389 there were also discovered cells starting the dividing process, cells having endoreduplicated nucleus (the 2nd image).

The cytogenetical study in the root meristem of the plants obtained in the first subculture (approximately 2 weeks from the appearance of the plant) pointed out a high frequency of induction at the female in haploid plants at wheat and also at sunflower. The results of the study are presented in the 2nd table.

We find that with *Triticum aestivum*'s genotypes the proportion of analysed plants having a haploid status (60%) is higher than with the diploid (the 3rd image) excepting the single mixoploid plant belonging to Podu-Ilioaiei genotype. There were no other ploidy forms.

The cytogenetical examination of *Helianthus annuus* ($2n = 2x = 34$) proves that among analysed plants (26), 13 of them were haploids, 10 diploids and only

Table 1

The number of chromosomes of the analysed cells in the gynogenetical callus at *Triticum aestivum* L.

Genotype	The callus origin	The number of the analysed metaphases	The ploidy level		
			n	2n	other ploidy levels
Podu-Ilioaiei	Ovary	50	34	16	–
Turda 5190	Ovary	50	28	22	–
Fundulea 4	Ovary	50	35	15	–
40389	Ovary	50	26	24	–

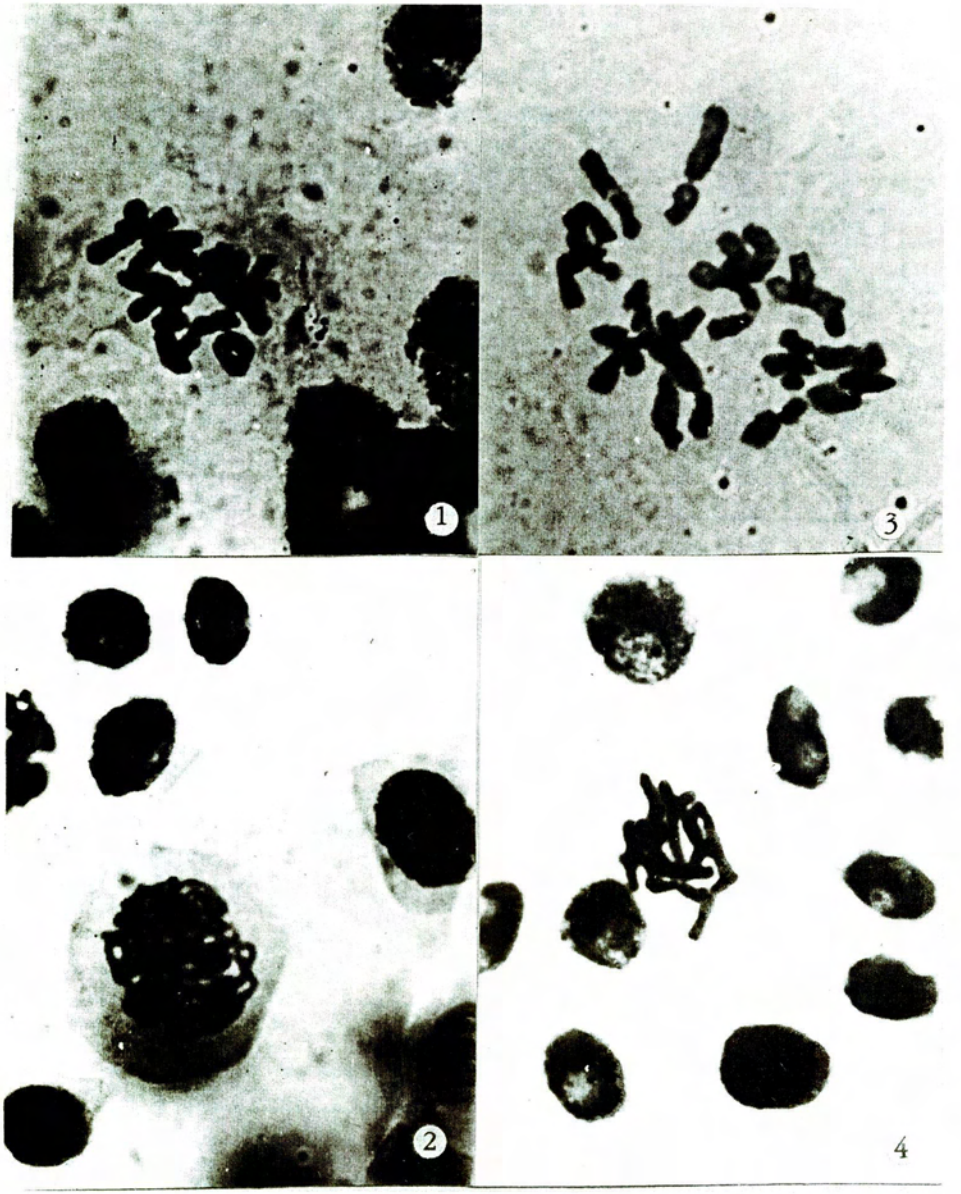

Fig. 1 – Cell having haploid number of chromosomes identified in the gynogenetical callus of the wheat.

Fig. 2 – Cell having endoreduplicated nucleus being in the process of dividing, identified in the gynogenetical callus of the wheat.

Fig. 3 – Cell having haploid number of chromosomes identified in the root meristem of the wheat gynogenetical plant.

Fig. 4 – Cell having haploid number of chromosomes identified in the root meristem of the sunflower gynogenetical plant.

Table 2

The ploidy level of the plants obtained from unpollinated ovaries and ovules in the first subculture

The species	Genotype	The plant's origin	The number of analysed plants	The ploidy level			
				n	2n	Aneuploids	Mixoploids
Triticum aestivum 2n = 6x = 42	Podu-Ilioaiei	ovarian callus	10	5	4	–	1
	Turda-5190	ovarian callus	10	8	2	–	–
	Fundulea 4	ovarian callus	15	8	7	–	–
	40251	ovarian callus	5	3	2	–	–
Helianthus annuus 2n = 2x = 34	Select	ovule	8	4	2	–	2
	Fundulea 206	ovule	13	7	6	–	–
	HS-2239	ovule	5	2	2	–	1

3 of them presented both haploid and diploid metaphases in the same root meristem (mixoploid plants). The percentage of the haploid metaphases at these plants was low (1–10/100 perceptible metaphases – the 4th image).

The haploid plants which did not degenerate and succeeded in their development during 2 or 3 passages in fresh environments of growth and development of the radicular system, at the transfer in the pots for their accommodation to natural conditions, were analysed further cytogenetically for having the right esteem on the development in time of the genotype of the gynogenetical plants. We found out a fast decrease of the haploid metaphases and a rising of the number of the diploid ones after approximately 8–12 weeks from the plants' appearance, during the process of development (the 3rd table).

Table 3

The ploidy level of the gynogenetical haploid plants after 8–12 weeks of development

The species	Genotype	The plant's origin	The number of analysed plants	The ploidy level	
				n	2n
Triticum aestivum 2n = 6x = 42	Podu-Ilioaiei	callus ovary	3	–	3
	Turda-5190	callus ovary	5	2	3
	Fundulea 4	callus ovary	5	1	4
	40251	callus ovary	1	–	1
Helianthus annuus 2n = 2x = 34	Fundulea 206	ovules	3	1	2
	Select	ovules	2	2	–

CONCLUSIONS

The results of the cytogenetical study in the gynogenetical callus, obtained with *in vitro* culture of the wheat ovaries, showed the presence of the haploid cells

in proportion 61.5% and the diploid 38.5%. There were also found out cells being in the process of dividing, cells having endoreduplicated nucleus.

The plants regenerated from the callus as the ones obtained directly from sunflower ovules were in majority haploid (60% at wheat and 50% at sunflower) but there were some diploids (32.5% at wheat and 38.4% at the sunflower). We found out during plants' development a decrease of the haploid metaphases and a rising of the diploid ones, after 8–12 weeks of growth.

The identification of the mixoploids and the one of the endoreduplication nucleus being in the process of dividing, proves the instability of the haploid genome firstly in the gynogenetical callus having the possibility of spontaneously doubling chromosome during *in vitro* stage. So, to obtain dihaploids is no more compulsory to use inductions with colchicine.

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* *University of Bacău, Faculty of Biology, Bacău, 157 Mărășești Street*
** *University of Bucharest, Faculty of Biology, Bucharest, Aleea Portocalilor 1*

MICROBIOLOGICAL STUDIES IN TECHNOGENIC SOILS FROM OLTENIA AREA

IOANA GOMOIU

This paper contains mycological studies of technogenic soils from lignite mines in Rovinari and Berbești branches. Fungi growing up in technogenic soils cultivated with maize are specific to this culture but are less than in control soil. In technogenic soils cultivated with poplar and acacia we found a small number and diversity of fungi. Technogenic soils from Berbești have a better evolution to an agricultural soil than the ones from Olteţu.

The process of making up the soil is complex and for a long time when the rocks under physical, chemical and biological factors get character of fertility. Microorganisms get an important role in making up the soil, and its stability by their number and their ability to produce exopolysaccharides or enzymes.

During the evolution of soils it was noticed an evolution of microbiota corresponding with age (8) and fertility.

There are soils in CIS where fungal biomass was high after three years (9, 10), but rhizosphere effect was higher in young technogenic soils than in zones ones. According to Machulla (8), after 18–27 years of soils revegetation, microbiota was comparable with the control.

Fresques and Lindemann (1) found a high number of microorganisms and dehydrogenase activity in native soils and recultivation soils. So, Fresquez et al. (2, 3) consider that by recultivation of soils takes place an increase of enzymic and microbial potential.

The soils nonoverburdened, after 6–8 years of recultivation, have a lower number of microorganisms and enzymic activity than overburdened ones. In the last case there is also a high diversity of microorganisms.

According to Persson and Funke (11), the amount of microorganisms and their diversity depend on the depth of soil. The number of eubacteria and filamentous bacteria is similar in a sample of 0–122 cm deep following an abrupt decrease. For fungi from 30 cm to 122 cm takes place a decrease of their number but for the same depth their number is increased depending on the time of storing.

Lindemann (7) obtained good results for microorganisms and penetrating of water spreading of ash up to 10 cm on the soil surface.

This paper contains mycological studies in technogenic soils from Rovinari and Berbești branches.

REV. ROUM. BIOL.–BIOL. VÉGÉT., TOME 42, Nos 1–2, P. 89–95, BUCAREST, 1997

MATERIALS AND METHODS

Studies had been done on technogenic soils from North Peșteana, South Peșteana, Gârla (Rovinari branch), Berbești and Olteț (Berbești branch).

Units forming colonies (ufc) were obtained using the serial dilution method and Czapek medium. For taxonomy it was used "A manual of soil fungi" (4).

RESULTS AND DISCUSSIONS

Mycological studies had been done on 18 soil samples (control and technogenic soils) from Rovinari branch: North Peșteana, South Peșteana and Gârla.

From North Peșteana there were taken samples from wooden soil as control: MP_1, MP_2 and from 10–15 years old soils covered with spontaneous vegetation: PH_1, PH_2 or cultivated with maize: MP_1C, MP_2C, poplar and acacia: 5,8 code.

The wooden soil has the highest number of fungi: $2–3.8\times10^5$ ufc/ml.

The soil cultivated with maize has the same amount of fungi as agricultural soil: $8\times10^4–2.4\times10^5$ ufc/ml but that one cultivated with acacia and poplar has a lower one: $9\times10^3–1\times10^4$ ufc/ml (fig. 1).

If in the wooden soil prevailed species belonging to *Mucoraceae*, in soils covered with spontaneous vegetation prevailed species belonging to genera *Fusarium*, in that one cultivated with maize, *Penicillium* and in that one with poplar and acacia prevailed *Aureobasidium pullulans*. From soil cultivated with maize, there were isolated fungi specific to this type of crop (*Trichoderma viride, Aspergillus*

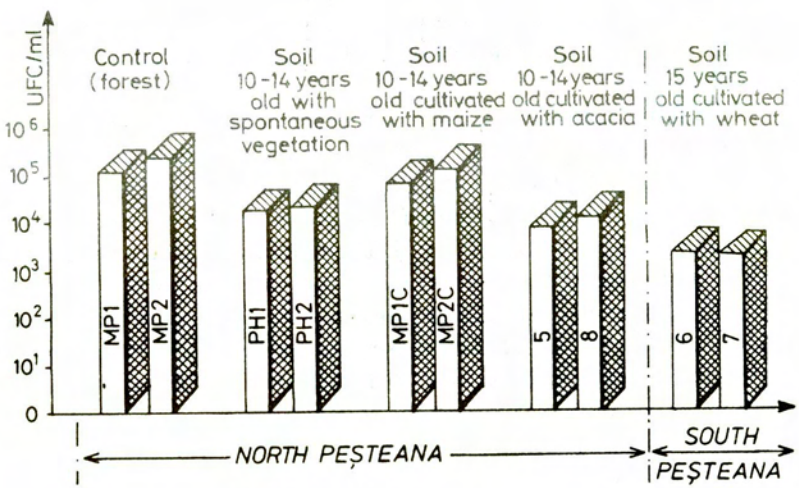

Fig. 1. – Fungi in technogenic soils from North Peșteana and South Peșteana area.

niger) and species belonging to genera *Penicillium, Gliocladium, Fusarium, Myrothecium.*

Although the amount of fungi is reduced in the soil cultivated with acacia and poplar we found a high diversity of genera: *Spicaria, Aureobasidium, Aspergillus, Fusarium, Fusidium, Gliocladium, Verticillium, Alternaria.* All of them are involved in the process of soil making up and in the degradation processes of organic materials.

From South Peșteana was studied only one soil cultivated with wheat (6,7 code) and fungi are not so numerous (3×10^3 ufc/ml, fig. 1). Prevalent genus was *Cladosporium* but other genera were specific to wheat crop (Table 1).

Numerical distribution of fungi has the same low value in soils cultivated with wheat from South Peșteana (6 code) that means a low crop if there are not done particular agricultural works. Also, we found as prevalent genus *Cladosporium* and same *Fusarium* species which might be pathogens.

From Gârla there were taken samples from 10–14 years old soil with spontaneous vegetation (22,21 code) soil cultivated with nut tree (R_1, R_2 code), soil covered with ash and cultivated with alfalfa (10,9 code) or maize (24,23 code).

Table 1

Fungi isolated from technogenic soils of Rovinari branch

Area	Type	Code	Fungi
NORTH PEȘTEANA	control = wooden soil	MP_1	*Phycomycetes, Penicillium sp., Fusarium*
		MP_2	*Penicillium sp., Fusarium sp., Sporotrichum sp., Gliocladium*
	soil with spontaneous vegetation (10–14 years old)	PH_1	*Fusarium sp., Aspergillus sp., Fusidium sp., Verticillium sp.*
		PH_2	*Trichoderma viride, Aspergillus sp. Penicillium sp., Aureobasidium pullulans, Fusarium, Mucoraceae*
	soil cultivated with maize (10–14 years old)	MP_1C	*Penicillium sp., Gliocladium sp.*
		MP_2C	*Fusarium sp., Trichoderma viride, Aspergillus niger, Myrothecium sp.*
	soil cultivated with poplar and acacia	5	*Aureobasidium pullulans, Spicaria sp., Aspergillus fumigatus, Fusarium sp., Alternaria sp., Verticillium sp.*
		8	*Penicillium sp., Fusarium sp., Fusidium sp., Gliocladium sp., Spicaria sp., Verticillium sp.*
SOUTH PEȘTEANA	soil cultivated with wheat	6	*Cladosporium sp., Aspergillus sp., Penicillium sp., Alternaria sp., Rhizopus nigricans, Fusarium sp., Stachybotrys atra, Curvularia sp.*
		7	*Fusarium sp., Cladosporium sp., Penicillium sp., Aspergillus sp., Trichoderma viride*

Table 1 (continued)

Area	Type	Code	Fungi
GÂRLA	soil cultivated with nut trees (10–15 years old)	R_1	*Trichoderma viride, Alternaria sp., Acrothecium sp., Aspergillus sp.,*
		R_2	*Penicilllium sp.* *Penicillium sp., Spicaria sp., Paecilomyces sp., Gliocladium sp., Trichoderma viride, Aspergillus sp., Alternaria sp., Mucor sp.*
	control = soil without ash, with spontaneous vegetation (10–15 years)	22	*Fusarium sp., Penicillium sp., Spicaria sp., Cladosporium sp., Trichurus sp.*
		21	*Penicillium sp., Fusarium sp., Trichoderma.*
	soil with ash cultivated with alfalfa (10–15 years old)	10	*Penicillium sp., Fusarium oxysporum, Alternaria sp., Gliocladium sp., Aspergillus sp., Penicillium sp., Spicaria sp., Mucor sp., Rhizopus sp.*
	soil with ash cultivated with maize (10–15 years old)	24	*Gliocladium sp., Penicillium sp., Aspergillus sp., Verticillium sp., Spicaria sp., Fusidium sp., Myrothecium sp., Phoma sp.*
		23	*Fusarium sp., Cladosporium sp., Penicillium sp., P. funiculosum, Aureobasidium pullulans, Alternaria sp., Stachybotris atra, Trichoderma viride, Cephalosporium sp., Verticillium sp., Aspergillus fumigatus, Myrothecium sp., Stachylidium sp., Stemphylium sp., Rhizopus sp., Chaetomium sp., Epicoccum sp., Helminthosporium sp.*

From the numerical point of view, the highest number of fungi are in the soil cultivated with nut tree, followed by the soil cultivated with alfalfa and maize (fig. 2).

We found a high diversity of genera (Table 1). In the soil cultivated with nut tree prelevant genera are: *Trichoderma, Penicillium* and *Spicaria,* in that one cultivated with maize, *Gliocladium* and in that one cultivated with alfalfa *Penicillium.*

Trichoderma viride is a common fungus found in acid soils but we found it in the soils from North Peşteana with 7.0–8.0 pH. In addition it is a species involved in the humification process. Production of antibiotics has an important role in the protection of roots.

In the soil covered with spontaneous vegetation there is a numerical difference between two layers (0–6.0 cm and 6.0–12 cm) which was not noticed in the soils cultivated with alfalfa or maize. We think that ash has a good influence on the growth of fungi.

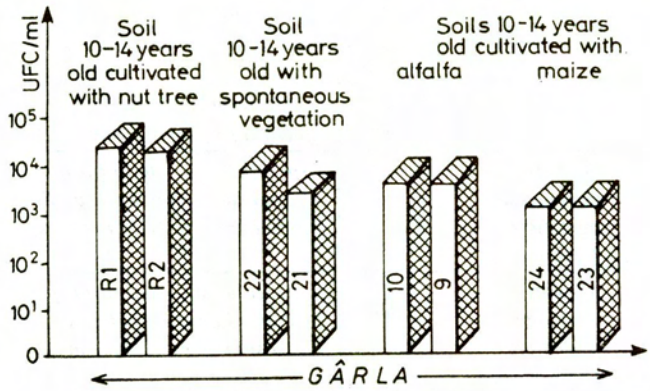

Fig. 2. – Fungi in technogenic soils from Gârla area.

From Berbești branch we studied the hay field soil as a control (BM_1, BM_2) and a soil covered with spontaneous vegetation 15 years old taking samples in May ($B_{1\text{-}a}$, $B_{2\text{-}a}$) and in June ($B_{1\text{-}b}$, $B_{2\text{-}b}$).

The number of fungi of control soil is the nearest to agricultural soil (5.6×10^4–4.0×10^5 ufc/ml, fig. 3) but in soils in May it was much more reduced than in June.

We found genera *Hormiscium* and *Hormodendron* involved in the decomposition of died animal meaning existing of animals in technogenic soils of those soils. From Oltețu area we studied: a hay field as a control (19,20 code) and two technogenic soils from 2 years old (17,18 code) and 15 years old soils (15,16 code, Table 2).

Table 2

Fungi isolated from technogenic soils of Berbești branch

Area	Type	Code	Fungi
BERBEȘTI	control-hay field	BM_1	*Penicillium sp, Aspergillus niger, Aspergillus sp., Trichoderma viride, Mucor sp.*
		BM_2	*Penicillium sp., Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Aspergillus candidus, Fusarium sp., Mucor sp., Trichoderma viride*
	soil with spontaneous vegetation (15 years old)	$B_{1\text{-}a}$	*Mucor sp., Rhizopus nigricans*
		$B_{2\text{-}a}$	*Aspergillus sp., Penicillium sp., Fusarium sp.*
		$B_{1\text{-}b}$	*Fusarium sp., Mycelia sterilia, Alternaria sp., Aspergillus candidus, Cladosporium sp., Hormiscium sp.*
		$B_{2\text{-}b}$	*Penicillium sp., Spicaria sp., Hormodendron sp., Aspergillus candidus, Cladosporium sp., Fusarium sp.*

(*continues*)

Table 2 (continued)

Area	Type	Code	Fungi
OLTEȚU	control-hay field	20 19	*Mucoraceae, Fusarium sp., Penicillium sp.* *Spicaria sp., Aspergillus sp., Alternaria sp.*
	soil with spontaneous vegetation (2 years old)	18 17	*Aspergillus sp., Cladosporium sp., Spicaria sp., Alternaria sp., Spicaria sp., Penicillium sp., Trichoderma viride, Botrytis sp.*
	soil with spontaneous vegetation (15 years old)	16 15	*Aspergillus sp., Alternaria sp., Fusarium sp., Myrothecium sp., Penicillium sp., Cladosporium sp., Mycelia sterilia*

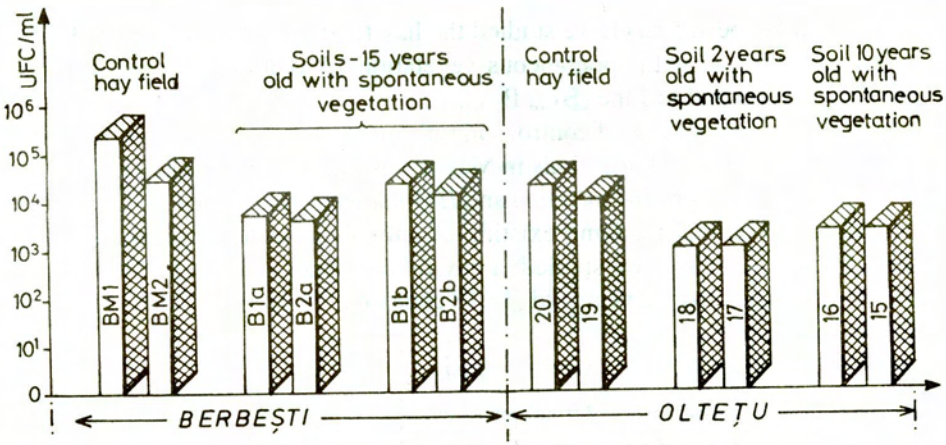

Fig. 3. – Fungi in technogenic soils from Berbești and Olteţu area.

Numerical distribution of fungi in the hay field is the same as in agricultural soil but it is low in technogenic soil meaning a poor evolution to a normal soil (fig. 3). The most frequent genus is *Aspergillus.*

Trichoderma viride, Botrytis and *Penicillium* genera were found in 2 years old soil. If *Botrytis* is known as a pathogenic genus, *P. janthinellum* is a pioneer in young soils and it is involved in the degradation of starch, pectine and cellulose.

CONCLUSIONS

1. Fungi found in soils cultivated with maize are typical of this crop but numerical distribution is lower than in an agricultural soil.

2. Soils with poplar and acacia have a low number and diversity of fungi.

3. Soils covered with ash contain a low number of fungi but of a high diversity.

4. Technogenic soils from Berbești branch go on to an agricultural soil better than those from Oltețu.

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Institute of Biology
Bucharest, Splaiul Independenței 296

IN VITRO MULTIPLICATION OF A RARE SPECIES FROM ROMANIA – *HEDYSARUM GRANDIFLORUM* PALL.

GABRIELA COSTEA, M. COSTEA

The *in vitro* techniques are now used successfully for clonal micropropagation and conservation of biological diversity, especially for endemic or very rare, endangered species. This study was concerned with the conservation and multiplication *in vitro* of *Hedysarum grandiflorum* Pall., a species considered endangered in Romania (4). As explants were used fragments of hypocotyls, tips and cotyledons, cultivated on Murashige-Skoog (1962) basal medium. Subcultures were made on Murashige-Skoog (1962) and Quoirin and Lepoivre (1977) media. The best medium for rooting proved to be ½ Murashige-Skoog medium without hormones. The plants were acclimatized and planted in pots. The comparative anatomic study of plants regenerated *in vitro* and plants collected from the natural environment showed the habitual differences. Consequently, the plants obtained *in vitro* presented very thin cuticle, fewer trichomes, absence of mechanical tissue and less developed vascular tissues.

The importance of *in vitro* cultures for multiplication and conservation of biological diversity was already confirmed. *In vitro* techniques proved to be very useful for the regeneration of true-to-type of the rare and endemic species (7, 8). In Romania *in vitro* techniques were successfully used for the multiplication of *Leontopodium alpinum* Cass. and *Convolvulus persicus* L. (13, 3).

Hedysarum grandiflorum Pall. is a perennial species localized in Romania only near Baltagești (County Constantza), on the hills called "Alah-Bair". According to the "Red list of rare, threatened and endemic plants from Flora of Romania" (4) which used the symbols created by Threatened Plants Committee – TPC of the International Union for Conservation of Nature Resources – IUCN, *Hedysarum grandiflorum* Pall. belongs to the grade E (endangered): taxons in danger of extinction, whose survival is not probable if the causal factors continue to action, or whose populations were impoverished to a critic level, the habitats being reduced drastically.

Because of the importance of biologic diversity conservation, our objectives were to test the possibilities of *in vitro* multiplication and the elaboration of an efficient methodology for a rapid multiplication, beginning with a small lot of seeds.

Also we realized a comparative anatomic study between plants regenerated *in vitro* and plants collected from Alah-Bair.

REV. ROUM. BIOL.–BIOL. VÉGÉT., TOME 42, N^{os} 1–2, P. 97–102, BUCAREST, 1997

MATERIALS AND METHODS

Seeds of *Hedysarum grandiflorum* Pall. harvested from Alah-Bair (County Constantza) were sterilized with $HgCl_2$ 0.1%, during 30 minutes and afterwards rinsed with sterile water three times. The germination of seeds took place on Murashige-Skoog medium. When the plantlets were three weeks old, fragments of hypocotyl (2–3 mm), tips (1–2 mm) and cotyledons (4–5 mm) were gathered and inoculated on Murashige-Skoog basal medium, with 3 mg/l BAP (6-benzylamino-purine) + 0.2 mg/l NAA (2-naphthalenacetic acid) and 1 mg/l BAP + 0.22 mg/l NAA, adding also 30 g/l sucrose and 7 g/l agar. The final pH was adjusted to 5.7–5.8 and then the media were autoclaved for 20 minutes at 120°C and press. = 1 atm.

The subcultures were made on Murashige-Skoog medium with 1 mg/l BAP + + 0.1 mg/l IBA (indole-3-butyric acid) + 0.5 mg/l GA3 (gibberellic acid) and Quoirin and Lepoivre medium with 1mg/l BAP + 0.1 mg/l IBA.

The media tested for rooting were Murashige-Skoog with 0.1 mg/l IAA (indole-3-acetic acid) and 1/2MS without hormones.

The cultures were maintained at 23–25°C with a 16 hours photoperiod. The plantlets were firstly acclimatized in water and then transferred successfully in pots.

For the study of comparative anatomy we used sections realized on stems and leaves belonging to plants regenerated *in vitro* and to plants collected from the natural habitat. The sections were clarified with chloralhydrate and stained with carmine-alaunate and green-iodine. The photos were made on MC-7 microscope.

RESULTS AND DISCUSSIONS

1. *IN VITRO* MULTIPLICATION

On both tested media the explants developed adventive shoots (Fig. 1). The ability to differentiate shoots is higher for tips and hypocotyls – 30% on Murashige-Skoog with 1 mg/l BAP + 0.22 mg/l NAA and 90% on Murashige-Skoog with 3 mg/l BAP + 0.2 mg/l NAA, as compared with cotyledons. After 4–5 weeks of culture on both media, the explants had as a rule 3–4 adventive shoots and many primordia. The fragments of hypocotyl were hypertrofiated and exhibited a green callus with many primordia and 1–2 adventive shoots (Fig. 2). The presence of these shoots, inhibits the primordia development. Therefore, such shoots 5–6 mm in length, were detached and subcultivated on the rooting medium. The remaining cultures were transferred periodically on fresh Quoirin and Lepoivre medium with 1mg/l BAP + 0.1mg/l IBA, which proved to be more efficient for proliferation than the MS medium.

Each explant may produce 25 shoots/ culture and supported three subcultures during 4 months.

The shoots rooted on ½ Murashige-Skoog medium, without hormones, which was superior to Murashige-Skoog medium + 0.1mg/l IAA. The first root primor-

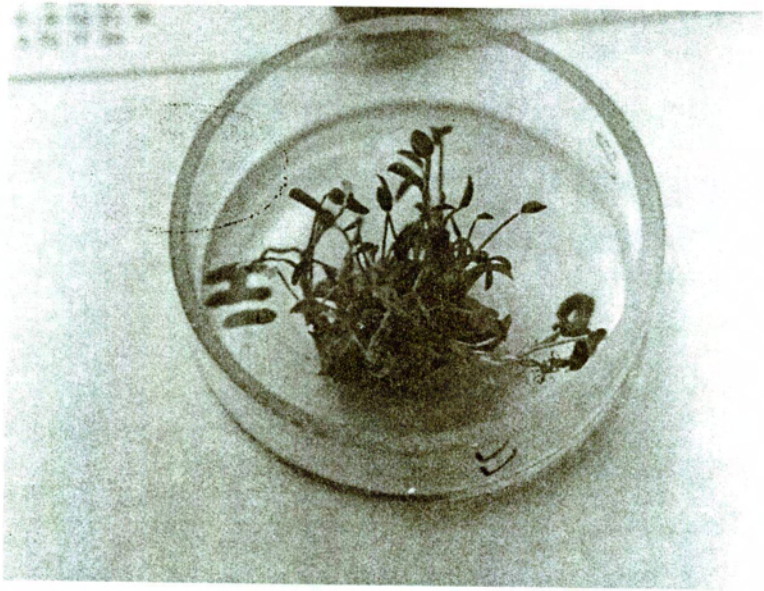

Fig. 1. – Proliferation response of hypocotyl explants – adventive shoots.

dia arise in 2 weeks with a rooting percentage of 80% on ½ Murashige-Skoog. After 4–5 weeks the plants may be acclimatized and cultivated in pots (Fig. 3).

The method described may provide a rapid multiplication starting with a few seeds and consequently with a minimum damage on the endangered population.

2. ANATOMY OF *HEDYSARUM GRANDIFLORUM* PALL. PLANTS REGENERATED *IN VITRO* AND COLLECTED FROM THE NATURAL ENVIRONMENT

a. The leaves

The plants regenerated *in vitro* have simple leaves very much alike to the ones of the plantlets, immediately after germination.

The leaves which belong to plants regenerated *in vitro* have the upper epidermis without trichomes and with many anomocytic stomata. The lower epidermis is provided with many trichomes and stomata very much alike to the ones from the upper epidermis. The hairs consist of 3(2) cells with 2(1) very short basal, thickened cells, and a terminal very long, with thin walls, cell. The cuticle for both epidermis cells is extremely thin. The mesophyll is dorsiventral with the palisade tissue formed by 3–4 cell layers and the spongy mesophyll with 5–7 layers of irregularly shaped cells. In the palisadic tissue there were observed many characteristic secretory elements consisting of 1–3 cells. The vascular tissue consists of colateral bundles not accompanied by mechanical tissue. The veinlet endings are dilated tracheids.

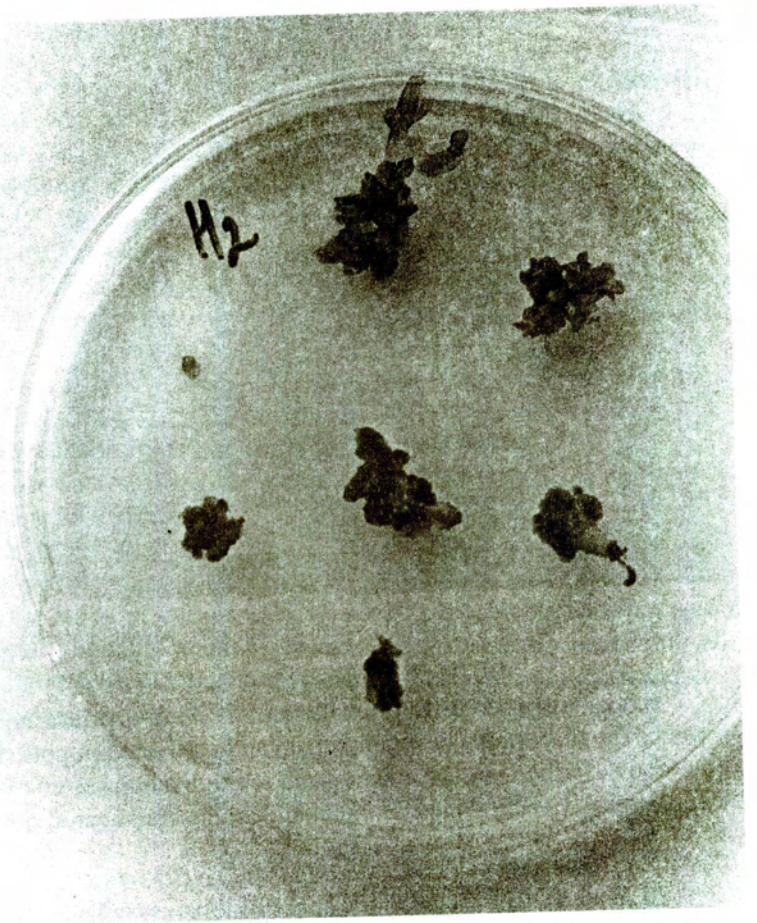

Fig. 2. – Hypertrophiated hypocotyls with buds primordia.

Very rare, from tip explants were obtained leaves with a structure very much alike to hypertrophiated cotyledons, 1000–1200μ thick, with the mesophyll composed almost entirely from palisade tissue.

The plants collected from the natural habitat presented the following structural differences: both epidermis are provided with a thick cuticle, the secretory elements are better developed and the mechanical tissue, represented by collenchyma, is present along the main veins. The secretory elements are supplied with very large cells that spread out from the upper epidermis into the spongy mesophyll, and by smaller cells located above the lower epidermis.

b. The stem

The plants obtained *in vitro* exhibit in transverse sections an epidermis with many stomata and without hairs. The cortex has 5–15 isodiametrical cell layers.

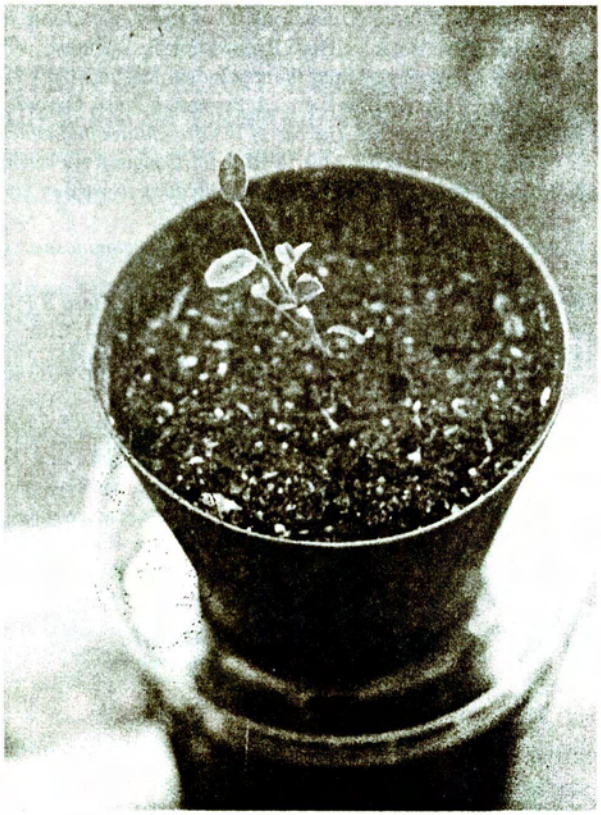

Fig. 3. – Acclimatisated plant transferred in pot.

The mechanical tissue is entirely absent. The endodermis is difficult to establish. The pericycle is formed by strands of fibers located in front of the vascular bundles.

The plants gathered from the natural habitat presented an epidermis with many hairs similar to those from the leaves, the cortex is provided in front of the ribs with well-developed collenchyma, and the vascular tissue is better developed.

As a conclusion, the used media proved efficient for the *in vitro* regeneration of plants.

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Faculty of Agronomy
Bucharest

IN VITRO ACTION OF PLANT EXTRACTS ON *BOTRYTIS* SPECIES FROM ORNAMENTAL PLANTS

M. PÂRVU, TATIANA EUGENIA ȘESAN

The "in vitro" effect of extracts from *Berberis vulgaris L.*, Family Berberidaceae (1% alkaloids) and *Chelidonium majus L.*, Family Papaveraceae (0.25% alkaloids) was studied on the plant pathogenic fungi: *Botrytis cinerea Pers.*, isolated from species of genera *Dahlia, Rosa, Dianthus; B. paeoniae Oudem.*, isolated from *Paeonia spp.* and *B. tulipae Lind.*, isolated from *Tulipa gesneriana Roch.*

The method of inoculation of plant extracts at various concentrations (between 25 and 250 µg / ml) in culture medium was used.

It was stated that: a) the antifungal effect of extracts was proportional to the alkaloids concentration; b) the minimal active concentration differed with micromyceta species and nature of extract; c) the fungicidal effect of plant extracts was obvious in all *Botrytis* species, at a minimal concentration of 250 µg / ml.

The antifungal effect of *B. vulgaris* and *C. majus* extracts on *Botrytis* species under study can constitute a starting point to set up some biological control elements within the systems of integrated protection of ornamentals.

Plant extracts from some cormophyte species exhibit antimicrobial action (4), (7), (10). Use of these still becomes actual in crop protection as an alternative, non-polluting means to inhibit plant pathogenic fungi damaging agricultural crops.

One of plant pathogenic fungus genera rising hard problems to agriculture and horticulture is the polyvorous genus *Botrytis*, an object of study for mycologists, plant pathologists and practitioners. A series of new results are known, referring to attempts to inhibit *Botrytis* species and particularly *B. cinerea Pers.*, by applications of plant extracts.

Thus, Esterio et al., 1992 (2) demonstrated efficacy of the plant extract BC-1000 obtained from grapefruit seeds at a concentration of 1,500 ppm against the fungus *B. cinerea* isolated from table grapes, similar to that of fungicides vinclozolin and benomyl.

Jirátko & Veselá, 1992 (5) have obtained extracts of *Chelidonium majus L.* and *Pastinaca sativa L.*, which proved to be highly active *in vitro* against *B. cinerea* isolated from grapevine, inhibiting this pathogen to an extent of 90%.

Jirátko, 1994 (6) noticed total inhibition of *B. cinerea* on detached haricot bean leaves under the action of extracts from *Petroselinum hortense Hoffm.* and *P. sativa*, from the growing tip of *Lycopersicum esculentum Mill.* and *Cinnamomum*, when the inoculum has been applied 72 hours after plant extract application.

REV. ROUM. BIOL.–BIOL. VÉGÉT., TOME 42, N^{os} 1–2, P. 103–110, BUCAREST, 1997

Total extracts from *Berberis vulgaris L.* and *C. majus* have antibiotic effects on a great number of pathogens (1), (3), (8).

The active ingredients of these plants are berberin alkaloids from *B. vulgaris* and chelidonin from *C. majus* (7), (9). Chelidonin occurs at rates of 0.2–1.4% in roots and 0.012–0.8% in vegetative underground organs of *C. majus* (3). The raw drug product Herba Chelidonii contains 0.351% alkaloids, while Radix Chelidonii 1.519% (8). Activity of total alkaloids from *C. majus* has been tested on pathogenic bacteria from genera *Staphylococcus, Streptococcus, Escherichia, Pseudomonas* and *Candida* fungus pathogenic to man (3).

In our work we pursued to establish *in vitro* the efficient dose of total extracts from *B. vulgaris* and *C. majus* able to inhibit growth, sporulation and sclerotia formation in some *Botrytis* species isolated from ornamental plants.

MATERIAL AND METHODS

The biological material used consisted of 3 isolates belonging to three *Botrytis* species: *B. cinerea Pers.* from *Rosa, B. paeoniae Oudem.* from *Paeonia* and *B. tulipae Lind.* from *Tulipa gesneriana Rock.*

The plant extracts from *B. vulgaris*, Family *Berberidaceae* (1% alkaloids) and *C. majus*, Family *Papaveraceae* (0.25% alkaloids) have been obtained by the

Table 1

In vitro action of *Chelidonium majus* extract on *Bortytis cinerea*

Variant / Alkaloid concentration µg / ml	Colony diameter (mm)	Sporulation[1)]	Sclerotia[2)]	Inhibition %
250	0.00***	–	–	100.0
225	5.02***	–	–	92.8
200	10.02***	–	–	85.7
175	18.04***	–	–	74.2
150	25.04***	–	–	64.2
100	45.02***	–	–	35.7
25	65.02***	+	++	7.1
– (Check)	70.00	++	++++	–

*** Significantly different from the control at $P < 0.05$

	[1)] **Sporulation**	[2)] **Sclerotia**
++++	abundant;	> 60 sclerotia / plate;
+++	dense;	41–60 sclerotia / plate;
++	moderate;	21–40 sclerotia / plate;
+	poor;	1–20 sclerotia / plate;
–	absent;	no sclerotia

volumetric method (3), (10) from plants collected and processed according to special literature (7), (10). The extracts have been introduced in the culture medium Czapek-agar after autoclavation. Various alkaloid concentrations have been prepared (250–25 μg/ml) by the method of dilutions.

Each variant with 5 replications in Petri dishes 70 mm in diameter has been sown in the central point with conidial suspensions of the three *Botrytis* species, in dilutions of 10^{-5}. The Petri dishes have been maintained at the optimum growth temperature of *Botrytis* isolated (22°C) for 12 days.

The action of plant extracts in the experimental variants upon the *Botrytis* isolates was evaluated with the following parameters: colony diameter (mm), sporulation and sclerotia formation (Tables 1–4), compared to a check variant not containing plant extracts.

Data have been treated by analysis of variance (ANOVA).

RESULTS

The total extract from *C. majus* exerted antifungal action against *B. cinerea* in ornamental plants (Table 1, Figs. 1, 2, 3), this being dependent on the concentration of alkaloids included in the nutritive medium. Increase of alkaloid concentration paralleled enhanced inhibition of the test-fungus. Thus, for values of 200–250 μg alkaloids/ml of medium, percentage of growth and sporulation inhibition ranged between 85.7 and 100.0; for values between 100 and 175 μg/ml, percentage of inhibition of test-fungus development declined to 35.7–74.2, whereas in the variant with the least values (25 μg/ml) the inhibition percentage was very low (7.1).

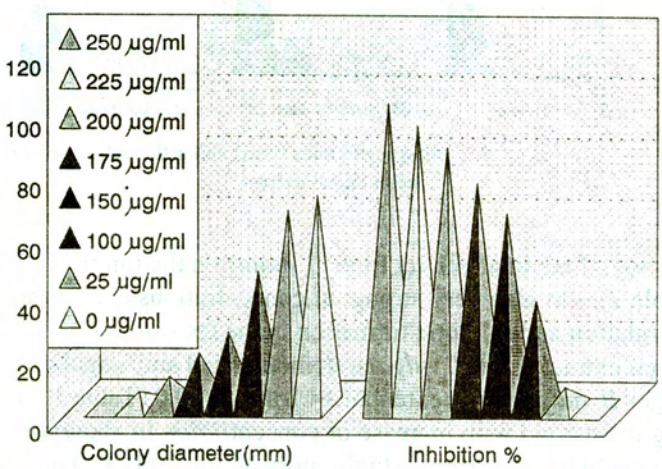

Fig. 1 – *In vitro* action of *Chelidonium majus* extract on *Botrytis cinerea*.

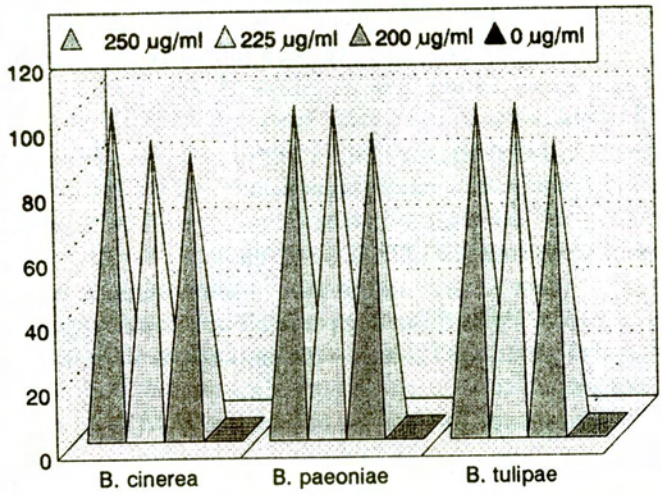

Fig. 2 – Inhibition (%) of *Botrytis spp.* by *Chelidonium majus* extract.

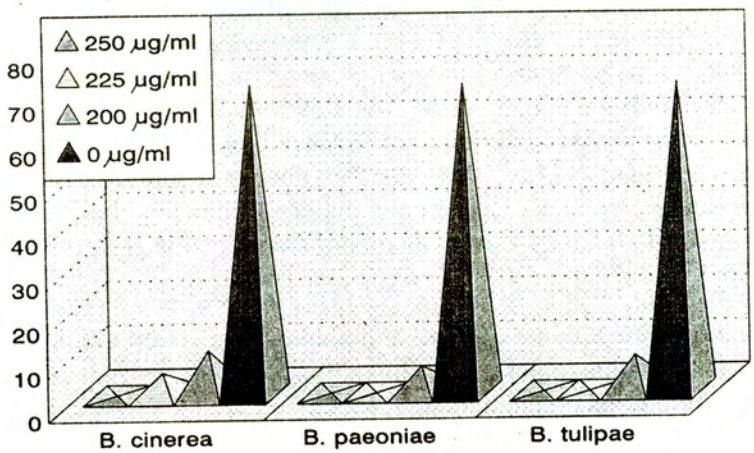

Fig. 3 – Diameter of *Botrytis spp.* colonies (mm) under the action of *Chelidonium majus* extract.

The action of the total extract from *C. majus* on the fungi *B. paeoniae* and *B. tulipae* (Table 2) was also very strong; at concentrations exceeding 225 µg / ml, growth, sporulation and sclerotia formation in the test-fungi was totally inhibited.

The total extract from *B. vulgaris* demonstrated antifungal action, inhibiting the test-fungus *B. tulipae* to an extent of 14.2 up to 100.0 (Table 3, Figs. 4, 5, 6), its action being intensified with increase of concentration in alkaloids introduced in the nutritive medium. The strongest inhibitory action (100.0%) has been recorded in the variant with the highest alkaloid concentration (250 µg/ml). In variant with

Table 2

In vitro action of *Chelidonium majus* extract on *Botrytis spp.*

Species	Alkaloid concentration µg /ml	Colony diameter (mm)	Sporulation[1)]	Sclerotia[2)]	Inhibition %
B. cinerea	250	0.01***	–	–	100.0
	225	5.02***	–	–	90.0
	200	10.02***	–	–	85.7
	– (check)	70.00	++	++++	–
B. paeoniae	250	0.01***	–	–	100.0
	225	0.01***	–	–	100.0
	200	6.02***	–	–	91.4
	– (check)	70.00	+	++++	–
B. tulipae	250	0.01***	–	–	100.0
	225	0.01***	–	–	100.0
	200	8.08***	–	–	88.4
	– (check)	70.00	+++	++	–

*** Significantly different from the control at $P < 0.05$

	1) **Sporulation**	2) **Sclerotia**
++++	abundant;	> 60 sclerotia / plate;
+++	dense;	41–60 sclerotia / plate;
++	moderate;	21–40 sclerotia / plate;
+	poor;	1–20 sclerotia / plate;
–	absent;	no sclerotia

Table 3

In vitro action of *Berberis vulgaris* extract on *Botrytis tulipae*

Variant / Alkaloid concentration µg / ml	Colony diameter (mm)	Sporulation[1)]	Sclerotia[2)]	Inhibition %
250	0.00***	–	–	100.0
225	6.04***	–	–	91.4
200	10.02***	–	–	85.7
175	18.02***	–	–	74.2
150	23.04***	–	–	67.1
100	45.04***	+	–	35.6
25	60.02***	++	+	14.2
– (Check)	70.00	+++	++	–

*** Significantly different from the control at $P < 0.05$

	1) **Sporulation**	2) **Sclerotia**
++++	abundant;	> 60 sclerotia / plate;
+++	dense;	41–60 sclerotia / plate;
++	moderate;	21–40 sclerotia / plate;
+	poor;	1–20 sclerotia / plate;
–	absent;	no sclerotia

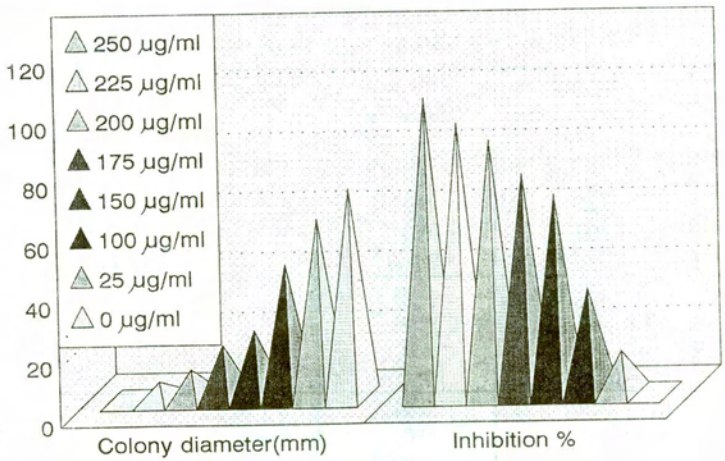

Fig. 4 – *In vitro* action of *Berberis vulgaris* extract on *Botrytis tulipae*.

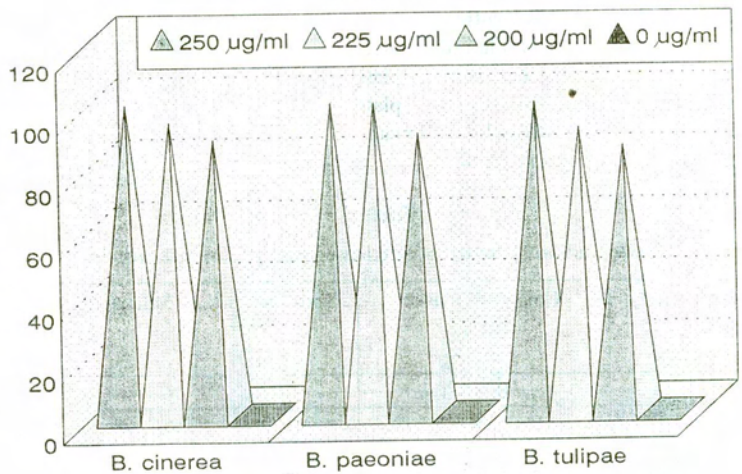

Fig. 5 – Inhibition (%) of *Botrytis spp.* by *Berberis vulgaris* extract.

200–250 µg alkaloid/ml of medium inhibition was likewise strong, between 85.7 to 91.4%, however by 8.6–14.3% more reduced, compared to the maximum dose. For values of 150–175 µg/ml, inhibition percentage was 67.1–74.2, and in that with 100 µg/ml only 35.6. The lowest inhibitory capacity of the fungus *B. cinerea* (14.2%) was noted with the least dose of alkaloids (25 µg/ml).

The highest antifungal effect (100.0% inhibition) against *B. paeoniae* (Table 4) has been obtained in variants with 225–250 µg alkaloids/ml of medium.

The highest inhibition of *B. tulipae* (Table 4) was recorded in the variant with 250 µg/ml; with 200–225 µg/ml the inhibitory action had values lower by 8.6–14.3%.

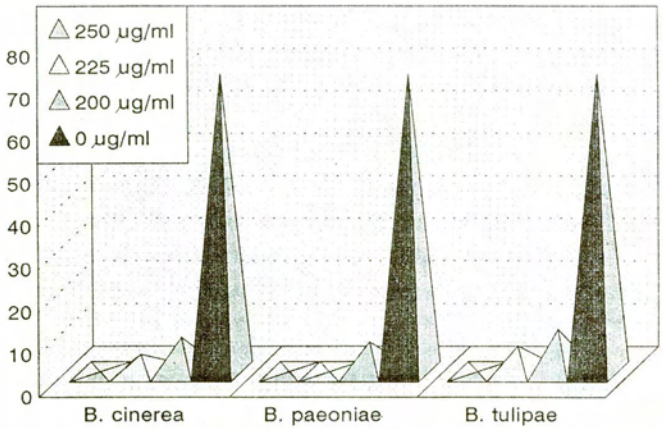

Fig. 6 – Diameter of *Botrytis spp.* colonies (mm) under the action of *Berberis vulgaris* extract.

Table 4

In vitro action of *Berberis vulgaris* extract on *Botrytis spp.*

Species	Alkaloid concentration µg /ml	Colony diameter (mm)	Sporulation[1)]	Sclerotia[2)]	Inhibition %
B. cinerea	250	0.01***	–	–	100.0
	225	4.04***	–	–	94.2
	200	8.06***	–	–	88.5
	– (check)	70.00	++	++++	–
B. paeoniae	250	0.01***	–	–	100.0
	225	0.01***	–	–	100.0
	200	7.04***	–	–	90.0
	– (check)	70.00	+	++++	–
B. tulipae	250	0.01***	–	–	100.0
	225	6.04***	–	–	91.4
	200	10.02***	–	–	85.7
	– (check)	70.00	+++	+	–

*** Significantly different from the control at $P < 0.05$

	[1)] **Sporulation**	[2)] **Sclerotia**
++++	abundant;	> 60 sclerotia / plate;
+++	dense;	41–60 sclerotia / plate;
++	moderate;	21–40 sclerotia / plate;
+	poor;	1–20 sclerotia / plate;
–	absent;	no sclerotia

CONCLUSIONS

– The *in vitro* antifungal effect of total extracts from *C. majus* and *B. vulgaris* against the three *Botrytis* species (*B. cinerea, B. paeoniae* and *B. tulipae*) isolated

from ornamental plants (*Dahlia, Rosa, Dianthus, Paeonia, Tulipa gesneriana*) were directly proportional to the alkaloids concentrations introduced in the culture medium;

– The highest active concentration of alkaloids varied with micromycete species tested and with the nature of extract;

– The *in vitro* fungicidal effects of plant extracts appeared against all *Botrytis* species at a minimum concentration of 250 μg alkaloids/ml of medium;

– The antifungal properties of plant extracts from *C. majus* and *B. vulgaris* against *Botrytis* species searched can serve as starting point in establishing some elements of biological control within the systems of plant ecological protection.

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Babeș-Bolyai University
Faculty of Biology
Str. Hașdeu 8, 3400 Cluj-Napoca

EPICOCCUM PURPURASCENS. III. *IN VITRO* RELATIONSHIPS WITH OTHER ANTAGONISTIC FUNGI

TATIANA EUGENIA ȘESAN, MARIA OPREA

There were studied the relationships between the antagonistic fungus *Epicoccum purpurascens* and 19 isolates belonging to other 6 antagonistic fungal genera (*Trichoderma, Trichothecium, Gliocladium, Chaetomium, Coniothyrium, Verticillium*) in order to establish their compatibility for biocontrol in complex.
All micromyceta under study proved to be non-antagonistic or indifferent to *E. purpurascens*, expressing the possibility to be applied together for biocontrol of some plant pathogenic fungi.

They are discussed more and more at present by the *Biocontrol Group of Fungal and Bacterial Pathogens* of the *International Organization of Biological and Integrated Control against Pests and Plant Pathogens*, regarding the future of plant fungal economically significant pathogens biocontrol with antagonistic fungi about the rational, ecological application of biological control agents (BCA), claiming use of many BCA together [2], [4], [5].

In this line it is important in practice to establish accurate relationships between fungi used as biological control agents (BCA's), to this end non-antagonistic relationships and particularly the synergistic ones between various BCA's being useful.

This paper reveals *in vitro* relationships between the antagonistic fungus *Epicoccum purpurascens* [3], [6], [7] and other 6 antagonistic fungal species used till now to assess compatibility between different BCA's in a complex, more efficient application within the systems of non-chemical control.

MATERIAL AND METHODS

It was used as biological material one *E. purpurascens* isolate originating in wheat kernels [6], [7], which was tested versus 19 isolates belonging to 6 genera of other antagonistic fungi (Table 1).

In order to reveal *in vitro* relationships between *E. purpurascens* and other test-antagonistic fungi, the method of double cultures has been used [1], [7]. Scoring was done by calculating the ratio (coefficient) **x** between the inner (i) and outer

Table 1

Antagonistic test-fungi used in tests

Species and isolate	Abbreviations	Provenance
Trichoderma viride	Td 5	– tomato roots (ing. Z. Beratlief);
	Td 49	– bean seed – Dâmbovița district;
	Td 50	– idem;
	Td grâu	– wheat kernels – Timiș district;
	Td China	– China – Collection of R.I.P.P. Bucharest (dr. doc. Ana Hulea & Maria Oprea);
Trichothecium roseum	Tt 1	– pea seed – Bacău district;
	Tt 2	– bean seed – Bacău district;
Gliocladium roseum	Gl 1	– soybean seedlings – Cluj district;
	Gl 2	– bean seedlings – Argeș district;
	Gl 3	– bean seedlings – Argeș district;
Verticillium tenerum	Vert. ten.	– bean seed – R.I.C.T.P. Fundulea Călărași district;
Chaetomium sp.	Chaet.	– bean seed – R.I.C.T.P. Fundulea Călărași district;
Coniothyrium minitans	CR	– Cluj (Șesan & Crișan, 1988);
	CS	– Sweden (Prof. B. Gerhardson);
	C 15; C 18	– The Netherlands (dr. M. Gerlagh – IPO-DLO
	C 102;	Wageningen)
	IVT 1; IVT 5	

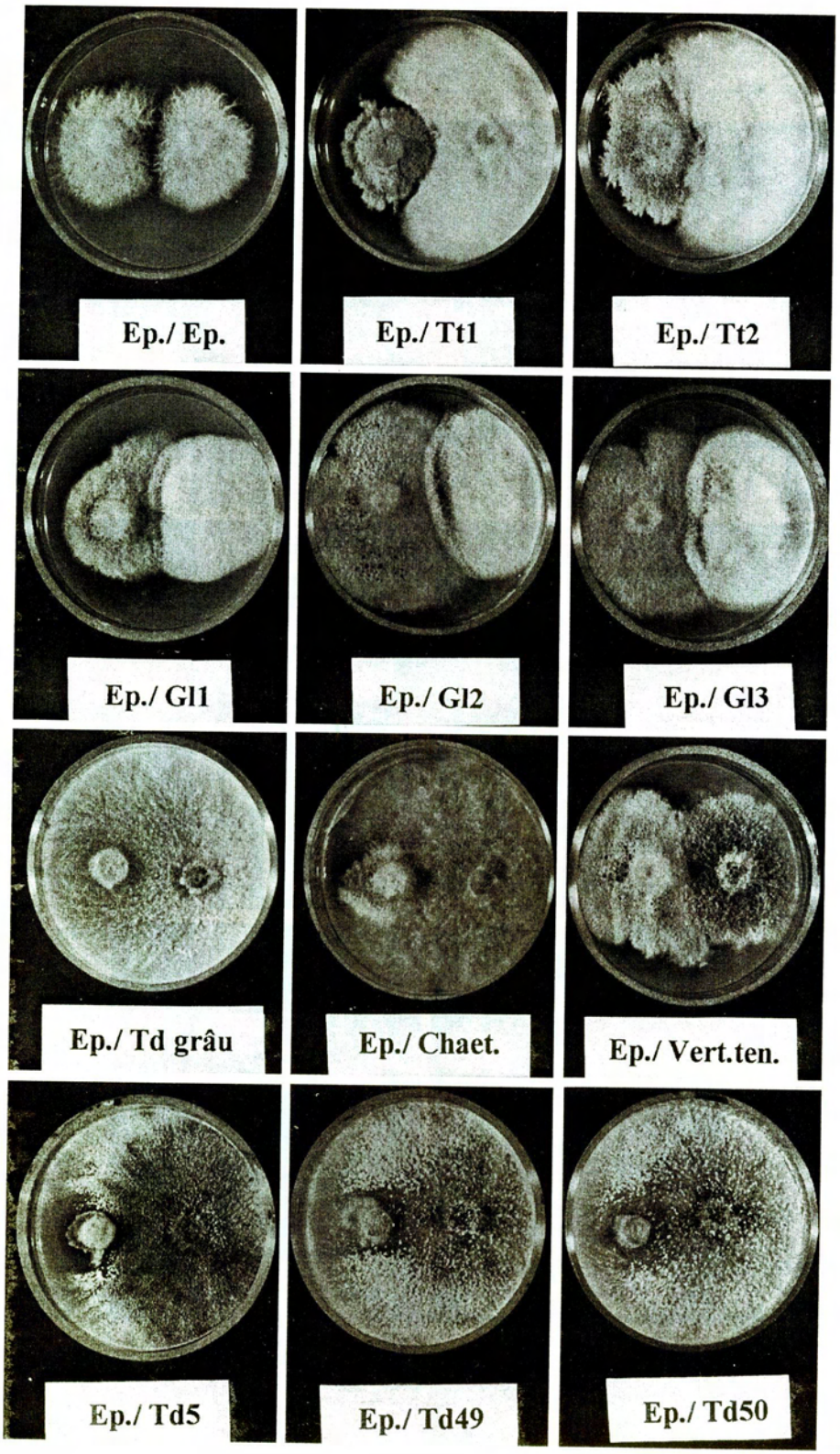
(e) radius of the test-fungus (A) and the antagonist *E. purpurascens*, with the formula **x** = iA / iB x eB / eA. Tests were organized in variants with 4 replications each, data being by the analysis of variance.

RESULTS AND DISCUSSION

From the data presented in Table 2 and Plates I–II it was revealed that among 19 isolates of test-antagonistic fungi belonging to 6 species, the majority (17) proved not be antagonistic to *E. purpurascens*, among them 4 indifferent *C. minitans* isolates (CR, CS, C 102, IVT 1) (**x** =1.000) and 13 (Td 5, Td 49, Td 50, Td wheat, Td China, Chaet., C 18, IVT 5, Tt 1, Tt 2, Gl 1, Gl 2, Gl 3) with an unsignificantly higher inhibitory capacity than *E. purpurascens* antagonism.

Having in view that all isolates from fungal antagonists of genera: *Trichoderma, Trichothecium, Gliocladium, Chaetomium, Coniothyrium, Verticillium*

Plate I. – *In vitro* relationships between *Epicoccum purpurascens* (Ep.) and some antagonistic → fungi: *Trichothecium roseum* (Tt 1, Tt 2), *Gliocladium roseum* (Gl 1, Gl 2, Gl 3), *Trichoderma viride* (Td wheat = grâu, Td 5, Td 49, Td 50), *Chaetomium sp* (Chaet.), *Verticillium teneraum* (Vert. ten.).


Ep./ Ep.
Ep./ Tt1
Ep./ Tt2
Ep./ Gl1
Ep./ Gl2
Ep./ Gl3
Ep./ Td grâu
Ep./ Chaet.
Ep./ Vert.ten.
Ep./ Td5
Ep./ Td49
Ep./ Td50

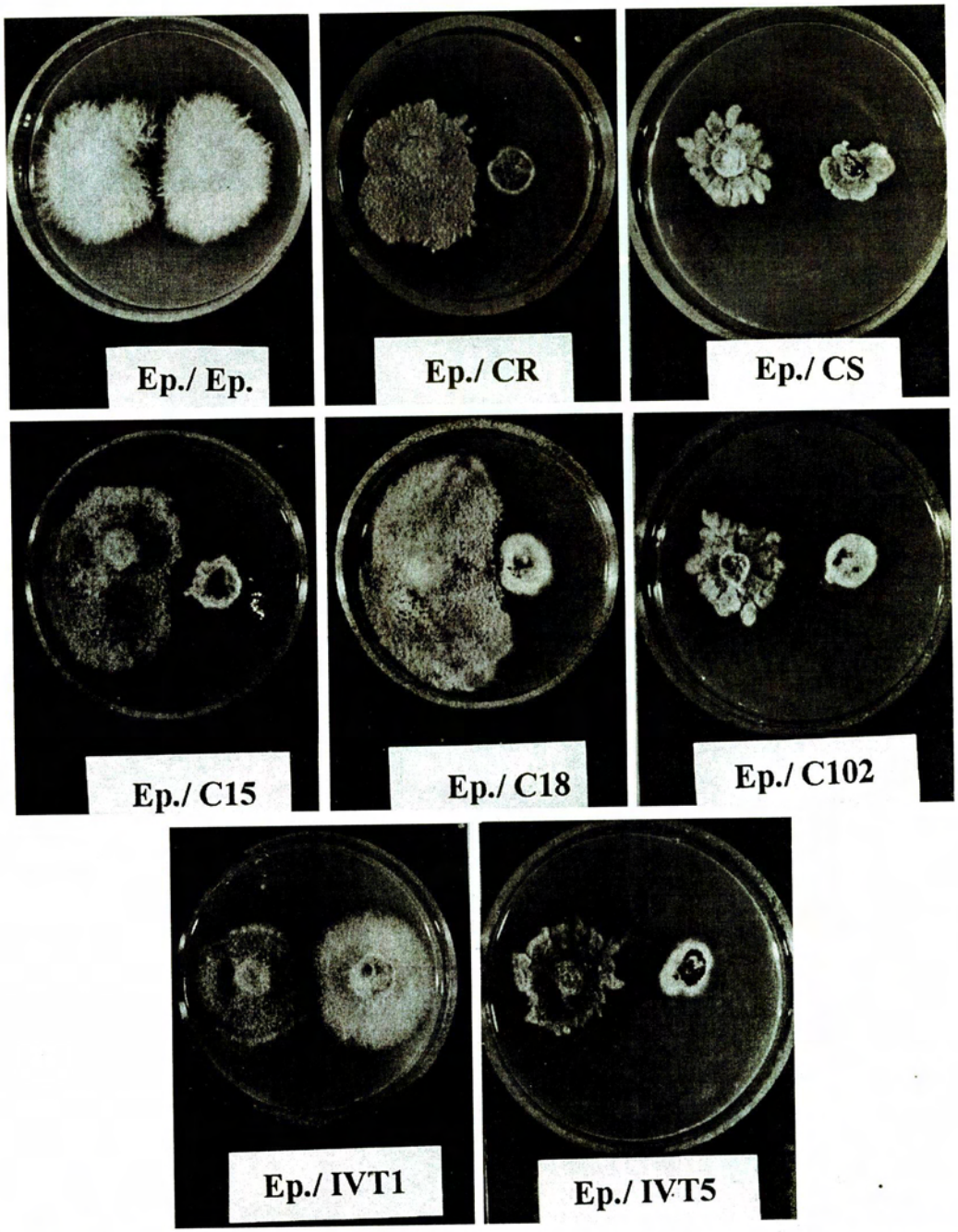


Plate II. – *In vitro* relationships between *Epicoccum purpurascens* (Ep.) and some isolates of *Coniothyrium minitans* (CR, CS, C 15, C 18, C 102, IVT 1, IVT 5).

Table 2

In vitro relationships between *E. purpurascens* and other antagonistic fungi, expressed as the coefficient **x**, after 5 days

Antagonistic isolate	**x**	Difference from check	Scoring relationships[a)]
Td 5	1.062	+ 0.062	N
Td 49	1.015	+ 0.015	N
Td 50	1.077	+ 0.077	N
Td wheat	1.214***	+ 0.214	N
Td China	1.014	+0.014	N
Tt 1	1.087*	+ 0.087	N
Tt 2	1.063	+ 0.063	N
Gl 1	1.169***	+ 0.169 .	N
Gl 2	1.019	+ 0.019	N
Gl 3	1.077	+ 0.077	N
Vert. ten.	0.890000	– 0.110	SA
Chaet.	1.070	+ 0.070	N
CR	1.000	0.000	I
CS	1.000	0.000	I
C 15	0.956	– 0.044	SA
C 18	1.071	+ 0.071	N
C 102	1.000	0.000	I
IVT 1	1.000	0.000	I
IVT 5	1.079	+ 0.079	N

LD 5% = 0.080; LD 1% = 0.108; LD 0.1% = 0.142

a) **x** = 1 – absence of reciprocal influences between fungi (I = indifferent); **x** > 1 – antagonism absent (N); **x** < 1 – the strongest antagonism, the lower values are (A); SA = slight antagonism.

tested behaved either indifferent or non-antagonistic to *E. purpurascens*, conclusion can be drawn that these can be used together as an BCA to control various plant patogenic fungi.

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Research Institute for Plant Protection,
71592 Bucharest, Bdul Ion Ionescu de la Brad nr. 8

CONTRIBUTIONS TO THE BIOLOGY OF CELLULOSOLYTIC FUNGI. INFLUENCE OF SOME CARBON, MINERAL NITROGEN AND AMINOACID SOURCES ON THE DEVELOPMENT OF *BOTRYOTRICHUM PILULIFERUM* SACC. & MARCH.

AL. MANOLIU, LĂCRĂMIOARA ANTOHE

The authors present the results of their study on the influence of different carbon sources (mannitol, arabinose, fructose, lactose, maltose, cellobiose, cellulose, carboxymethylcellulose, starch) and glucose as reference, mineral nitrogen sources (potassium nitrate, calcium nitrate, sodium nitrate, ammonium nitrate, ammonium carbonate, ammonium phosphate, ammonium chloride) and aminoacids (L-serine, L-isoleucine, valine, α-alanine, asparagine, methionine, L-leucine) on the *Botryotrichum piluliferum* Sacc. & March. The evolution of the carbon, nitrogen and aminoacids sources assimilation degree was followed on the growth dynamics of culture. Scoring colony growth has been carried out by recording the average diameter of a colony at various times (48–290 hours). The most favourable conditions for optimal growth were: a) carbon sources: mannitol, carboxymethylcellulose, lactose, starch and cellulose; b) mineral nitrogen sources: calcium nitrate, sodium nitrate, ammonium carbonate; c) aminoacids: asparagine, serine, valine, alanine.

Botryotrichum piluliferum was described by Saccardo and Marchal in 1885, as a coprofile species, being subsequently identified – quite frequently – in air, on the ground, on woven fabrics, cellophane, paper etc., and – occasionally – on dry herbaceous plants (6). A. Blochwitz published some data on the morphology, growing, physiology and ecology of this species (2). In Romania, the fungus was collected from frescs (10), (13) and soil (11). In 1961 J. Daniels described the telemorph *Chaetomium piluliferum* species (4). Among the investigations performed on the telemorph's biology, special mention should be made of the utilisation of starch, pectin, xylan and carboxymethylcellulose by various strains belonging to this species, cellulose and lignin decomposition (8), evidencing of cellulases C_1 and C_x (4), (18), chitin decomposition (12). In Romania the *Chaetomium piluliferum* species has been recently identified on *Vitis vinifera* strains (9). The present study-part of our more extended investigations on the biology of cellulosolytic fungi – discusses the influence of various sources of carbon, mineral nitrogen and aminoacids on the development of the *Botryotrichum piluliferum* species. The literature, issued in Romania, mentions some recent studies on the development of micromycetes as

REV. ROUM. BIOL.–BIOL. VÉGÉT., TOME 42, N^{os} 1–2, P. 115–126, BUCAREST, 1997

a function of the carbon (1), (15), mineral nitrogen and aminoacids sources (14), (15), (16) from the culture media.

MATERIALS AND METHOD

The investigations have been performed on the *Botryotrichum piluliferum*, collected from old religious books of the Vorona Monastery, the county of Botoșani.

In the laboratory, the fungus was cultivated on medium containing: 4 g – yeast extract; 10 g malt extract; 4 g glucose; 15 g agar; 1000 ml distilled water, in Petri plates, in 10 cm diameter, incubation being developed at a temperature of 28°C; this medium was considered as reference. In the experiments dealing with the influence of the various carbon sources on the growing rhythm, glucose from the reference medium has been replaced with other carbohydrates, such as: mannitol, arabinose, fructose, lactose, maltose, cellobiose, cellulose, carboxymethylcellulose, starch, while, for putting into evidence the role played by the various nitrogen and aminoacids sources on the development of the fungus, 2 g from each of the following mineral compounds with nitrogen: potassium nitrate, calcium nitrate, sodium nitrate, ammonium nitrate, ammonium carbonate, ammonium sulphate, ammonium phosphate, ammonium chloride, have been added to the reference medium, as well as with the following aminoacids: L-serine, L-isoleucine, valine, α-alanine, asparagine, methionine, L-leucine. The media containing one of the above-mentioned compounds have been inoculated with disks – 0.8 cm in diameter cut up from a 7 days old culture of *Botryotrichum piluliferum*; each variant has been experimented in 5 replications. Growing was appreciated through measuring of the diameter of fungus colony at 48 hours, 72 hours, 96 hours, 120 hours, 144 hours, 168 hours, 192 hours, 216 hours, 240 hours, 264 hours and, respectively, 290 hours. The last measurements were performed when the colony of the fungus occupied the whole surface of the Petri plates, with all variants taken into study.

RESULTS AND DISCUSSIONS

The data expressing the influence of the various carbon sources upon the growing rhythm are plotted graphically in Figures 1 and 2; they permitted the observation that 48 hours after inoculation, the best growing was recorded on the medium containing mannitol, the colony of the fungus diameter being of 2.65 cm, and the lowest, on the medium containing cellobiose – 1.80 cm; the values obtained in the culture media containing the other carbohydrates were the following: carboxymethylcellulose – 2.43 cm, starch – 2.38 cm, fructose – 2.26 cm, maltose – 2.23 cm, arabinose – 2.16 cm, lactose – 2.11 cm, cellulose – 2.00 cm; on the reference medium, the growing rhythm was of 1.88 cm, that is, lower than for most variants.

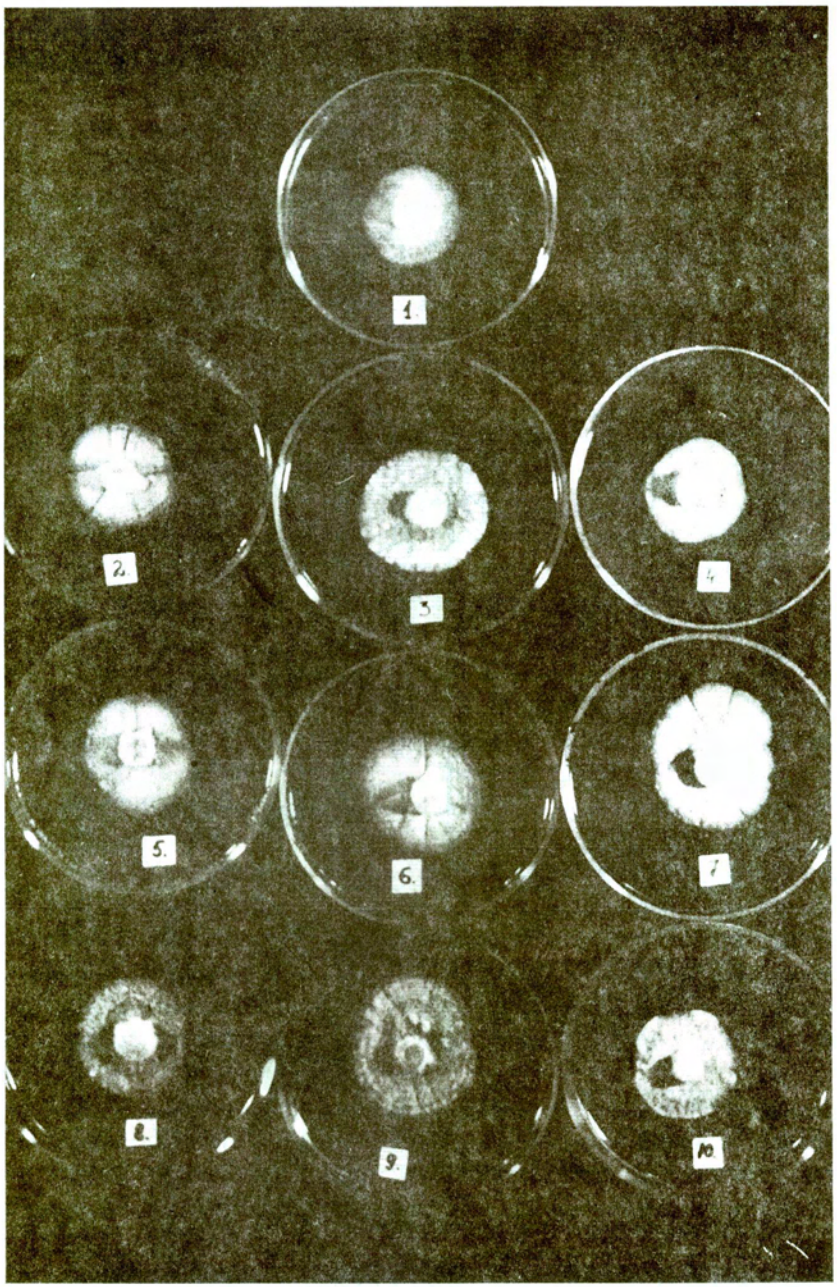
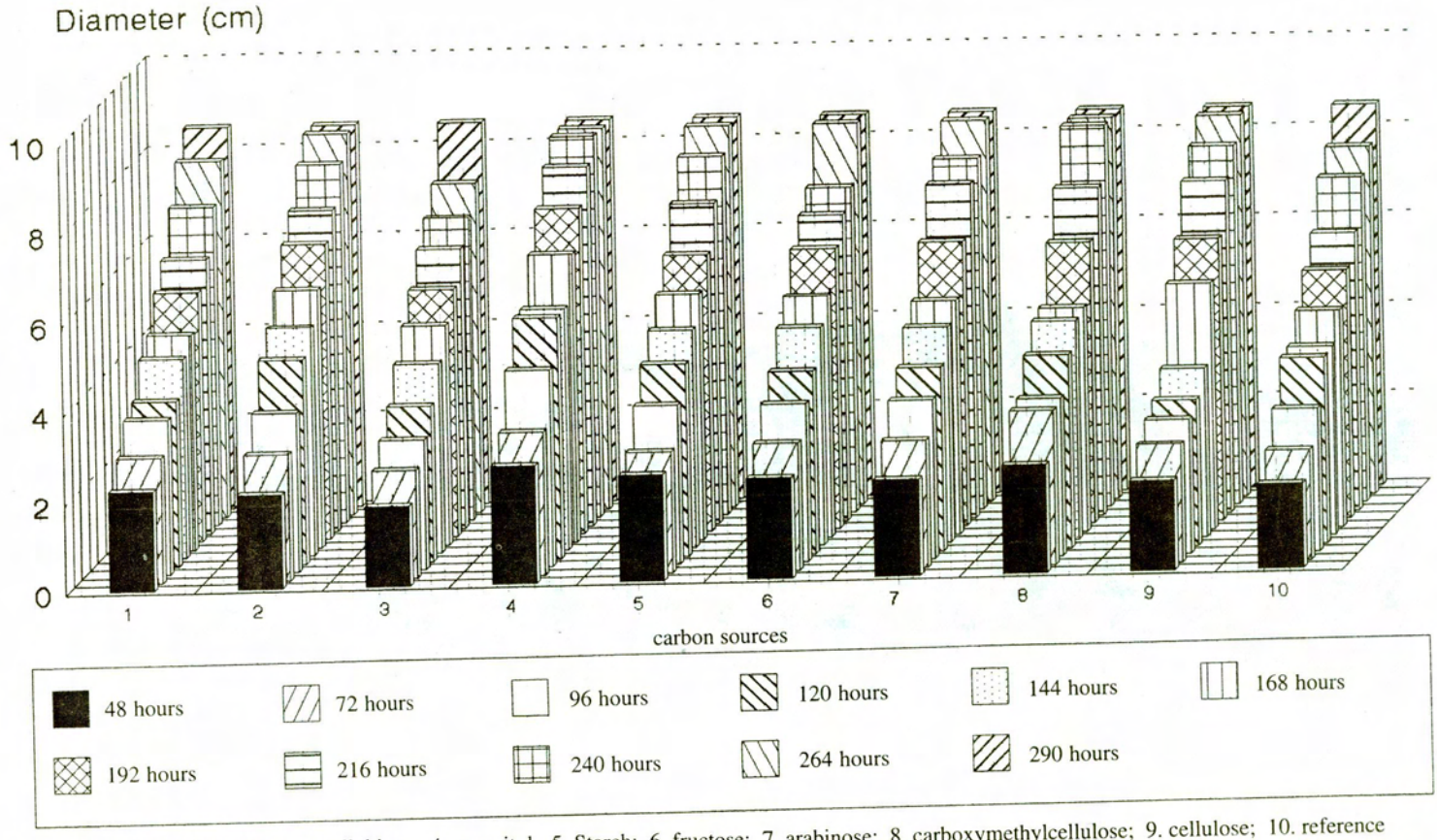


Fig. 1. – The development of *Botryotrichum piluliferum* on the media with carbon sources at 120 hours after inoculation. 1. Reference, 2. Maltose, 3. Lactose, 4. Cellobiose, 5. Starch, 6. Mannitol, 7. Fructose, 8. Arabinose, 9. Carboxymethylcellulose, 10. Cellulose.



1. maltose; 2. lactose; 3. cellobiose; 4. mannitol; 5. Starch; 6. fructose; 7. arabinose; 8. carboxymethylcellulose; 9. cellulose; 10. reference

Fig. 2. – Development of *Botryotrichum piluliferum* on media with various carbon sources.

At 72 hours, the fungus is growing best on the medium containing carboxymethylcellulose – 3.44 cm, followed by that with mannitol – 3.20 cm, significantly differences being recorded versus the culture media containing arabinose and fructose – 2.85 cm each, lactose – 2.83 cm, maltose – 2.80 cm, starch – 2.70 cm, cellulose – 2.64 cm, cellobiose – 2.40 cm; the growing rhythm recorded on the reference medium – 2.46 cm, was higher only to variant containing cellobiose. After 96 hours, the fungus utilized optimally mannitol, which meant a considerably higher growing rhythm – 4.40 cm, comparatively with the other variants – the reference included –, characterized by a more reduced growth, as follows: lactose and arabinose – 3.56 cm each, starch – 3.55 cm, fructose and carboxymethylcellulose – 3.53 cm each, maltose – 3.46 cm, reference – 3.20 cm, cellulose – 3.20 cm, cellobiose – 2.90 cm. The same difference between the growing rhythm on the variant with mannitol comparatively with the media containing the other carbon sources, is maintained, too, 120 hours after inoculation: mannitol – 5.40 cm, lactose – 4.56 cm, carboxymethylcellulose – 4.32 cm, starch – 4.30 cm, reference – 4.15 cm, arabinose – 4.11 cm, fructose – 4.08 cm, maltose – 3.70 cm, cellobiose – 3.48 cm, cellulose – 3.27 cm. Although in the time interval between 120 and 144 hours, the colony of the fungus growth on the medium containing mannitol ceased, remaining at a value of the colony's diameter of 5.40 cm, this value was not attained by the growing rhythm of the other variants: lactose – 5.10 cm, carboxymethylcellulose – 4.90 cm, starch and fructose – 4.86 cm each, arabinose – 4.82 cm, maltose – 4.46 cm, cellobiose – 4.23 cm, reference – 4.22 cm, cellulose – 3.77 cm. In the following time intervals 160 hours, 192 hours and 216 hours, the best growing rhythm was again recorded on the medium containing mannitol, followed in a non-uniform manner by the variants containing the other carbon sources. At 240 hours after inoculation, the fungus occupied the whole surface of the Petri plates (8.50 cm) on the medium with mannitol and with carboxymethylcellulose; after 264 hours, the whole Petri plates were covered by the fungus mycelium, for the variants containing lactose, starch, fructose, arabinose and cellulose, as well. The last measurements were made 290 hours after inoculation; they corresponded to the moment in which the colonies of the fungus occupied the whole surface of the Petri plates at last variants considered for the study: maltose, cellobiose and reference.

All these results show that the best carbon sources for the development of *Botryotrichum piluliferum* fungus were the following: mannitol, carboxymethylcellulose, lactose, starch and cellulose, which confirms the literature data on the favorable influence of cellulose (1), starch (16), lactose and mannitol (15) upon the development of the micromycetes.

The information on the influence of various mineral nitrogen sources on the fungus' development is present in Figures 3 and 4, which show that the addition of these compounds in the culture medium induces non-uniform modifications of the growing rhythm, comparatively with the variant taken as a standard. Thus, 48 hours

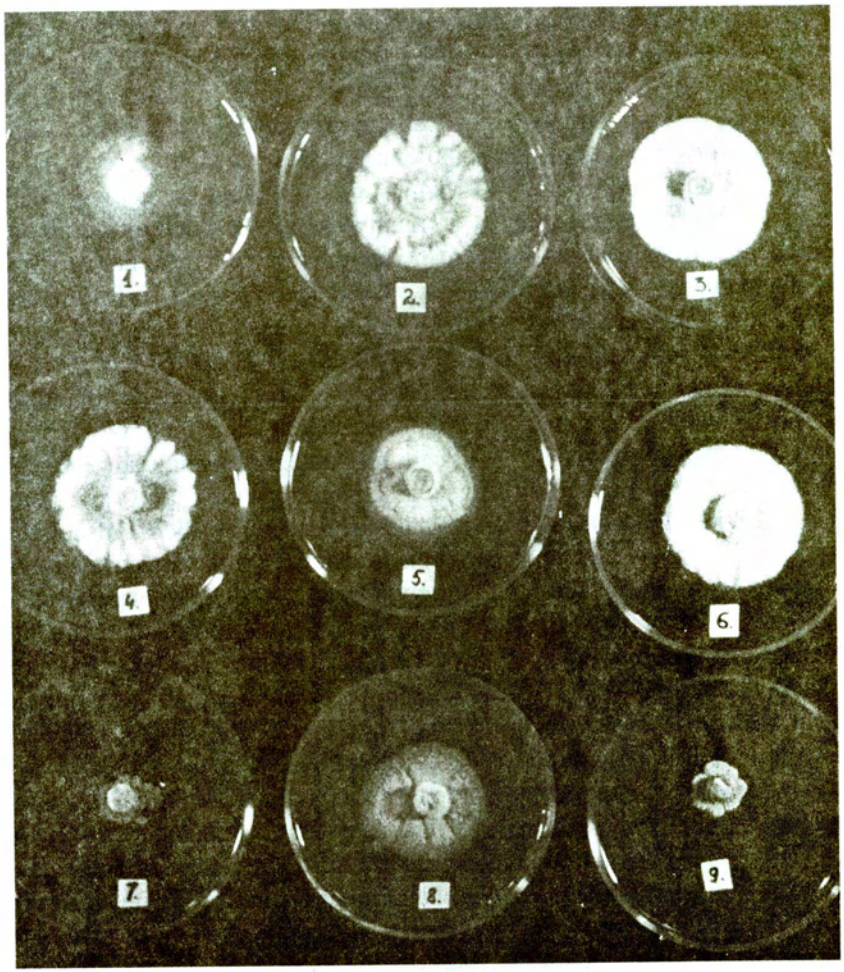
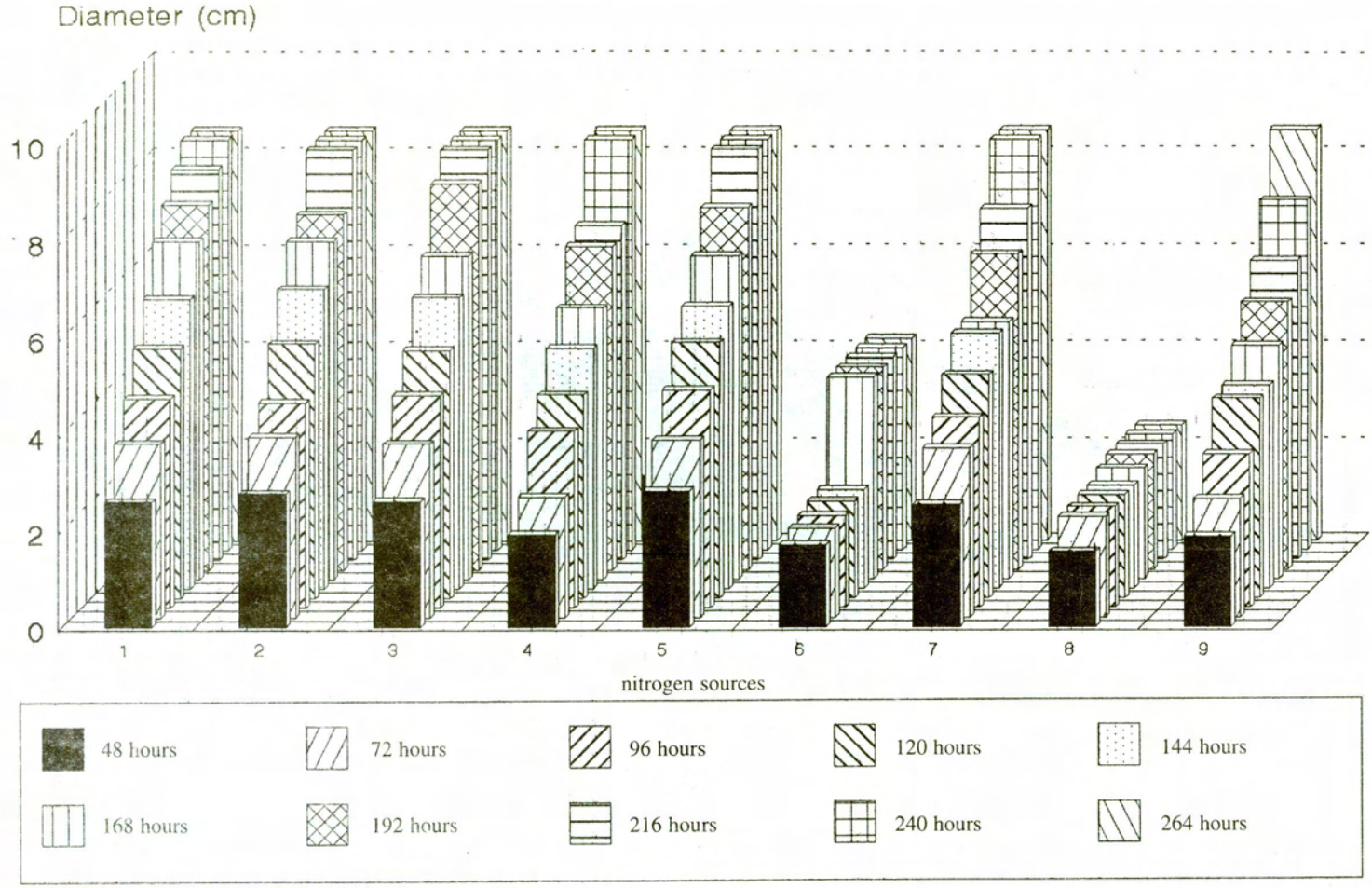


Fig. 3. – The development of *Botryotrichum piluliferum* on the media with mineral nitrogen sources at 120 hours after inoculation. 1. Reference, 2. Potassium nitrate, 3. Calcium nitrate, 4. Sodium nitrate, 5 Ammonium nitrate, 6. Ammonium carbonate, 7. Ammonium sulphate, 8. Ammonium phosphate, 9. Ammonium chloride.

after inoculation, the fungus attained a maximum of the growing rhythm on the medium containing ammonium carbonate – 2.83 cm and a minimum value on that with ammonium chloride – 1.57 cm, all variants with the other carbon sources, the reference included: calcium nitrate – 2.78 cm, sodium nitrate – 2.62 cm, potassium nitrate – 2.61 cm, ammonium phosphate – 2.56 cm, ammonium nitrate – 1.93 cm, reference – 1.88 cm, ammonium sulphate – 1.71 cm laying between the above mentioned extreme values. After 72 hours, the fungus evidenced the best growing rhythm on medium with calcium carbonate – 3.76 cm, followed at a small differ-



1. potasium nitrate; 2. calcium nitrate; 3. sodium nitrate; 4. ammonium nitrate; 5. ammonium carbonate; 6. ammonium sulphate; 7. ammonium phosphate; 8. ammonium chloride; 9. reference

Fig. 4. - Development of *Botryotrichum piluliferum* on media with mineral nitrogen sources.

ence, by the variants containing ammonium carbonate – 3.70 cm, ammonium and sodium nitrate – 3.62 cm each, ammonium phosphate – 3.53 cm, and, respectively, at higher differences from the culture media with ammonium nitrate – 2.51 cm, reference – 2.46 cm, ammonium chloride – 2.10 cm, ammonium sulphate – 1.86 cm. After 96 hours, the dynamics of the growth rhythm followed approximately the same order as that recorded for the previous time interval, as follows: calcium carbonate – 4.53 cm, sodium nitrate – 4.42 cm, potassium nitrate – 4.36 cm, calcium nitrate – 4.28 cm, ammonium phosphate – 3.96 cm, ammonium nitrate – 3.68 cm, reference – 3.20 cm; on the culture media containing ammonium sulphate and ammonium chloride, the growth rhythm was much slower, the fungus colonies taking values ranging between – 2.10 cm, respectively, 1.92 cm. In the following intervals the diameter of colonies was 5.32 cm on the medium containing calcium carbonate and 2.02 cm on that with ammonium sulphate, at 120 hours, 6.22 cm for the variant with calcium carbonate and 2.15 cm with ammonium chloride, at 144 hours, 7.00 cm for the variants with potassium nitrate and calcium nitrate and 2.30 cm at variant with ammonium chloride, 168 hours after inoculation. After 192 hours, the colonies of the fungus had the following diameters: sodium nitrate – 8.00 cm potassium nitrate – 7.53 cm, ammonium carbonate – 7.50 cm, calcium nitrate – 7.36 cm, ammonium nitrate – 6.70 cm, ammonium phosphate – 6.54 cm, reference – 5.54 cm, ammonium sulphate – 4.20 cm (the same diameter as the recorded at 168 hours), ammonium chloride – 2.40 cm.

At 216 hours after inoculation, the colonies of the fungus occupied the whole surface of the Petri plates (8.50 cm) at the variants containing calcium nitrate, sodium nitrate, calcium carbonate, while – on the media containing ammonium sulphate and ammonium chloride, the fungus' growth ceased; on these two last variants, stopping of the growth was observed after 240 hours, as well, while, on the medium with potassium nitrate, ammonium nitrate and ammonium phosphate, the colonies of the fungus occupied the whole surface of the Petri plates; with the reference variant, the fungus occupied the whole surface of Petri plate at 264 hours after inoculation, on the media containing ammonium sulphate and ammonium chloride, the diameter of the colonies of the fungus remained the same at 168 hours, and respectively, 192 hours. These data show that, out of the 8 sources of mineral nitrogen employed, the highest fungus growth was assured by the media containing calcium nitrate, sodium nitrate and ammonium carbonate, and the weakest one the media with ammonium sulphate and ammonium chloride. Similar results have been obtained by other authors, too, on the development of microfungi on media containing calcium nitrate and ammonium nitrate (14), (15), ammonium sulphate (14), and ammonium carbonate (16).

Figures 5 and 6 plot the results of describing the influence of the aminoacids on the growth rhythm, according to which, 48 hours after inoculation, on all media containing these compounds, growing was higher than on the reference variant. After 72 hours, the fungus evidenced a maximum growth rhythm on the medium

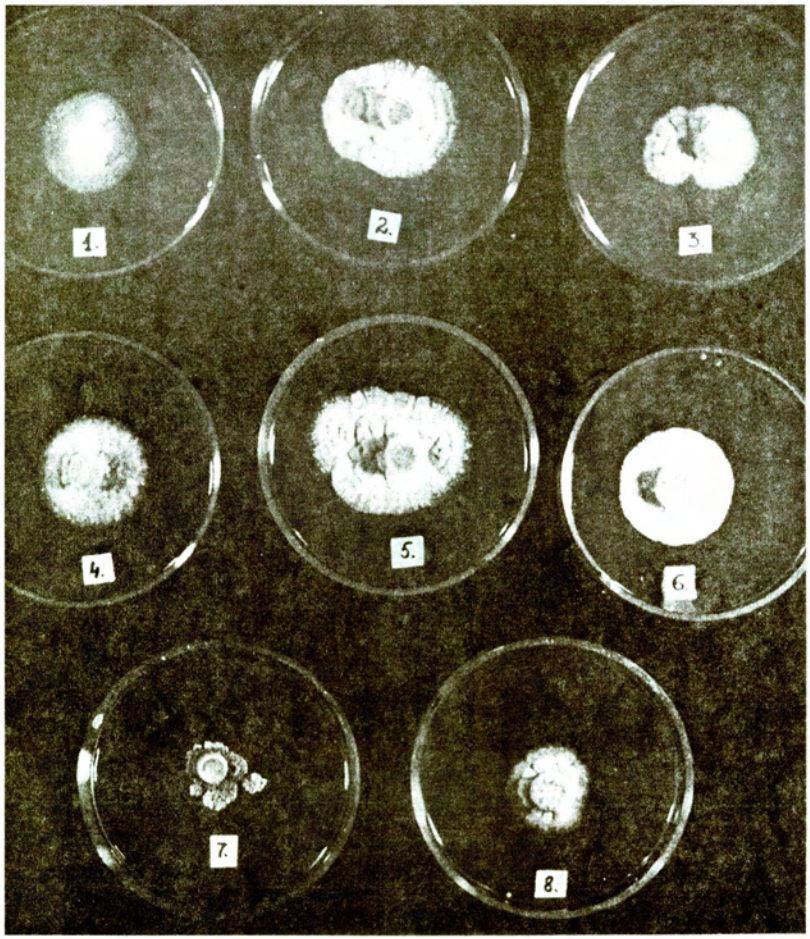
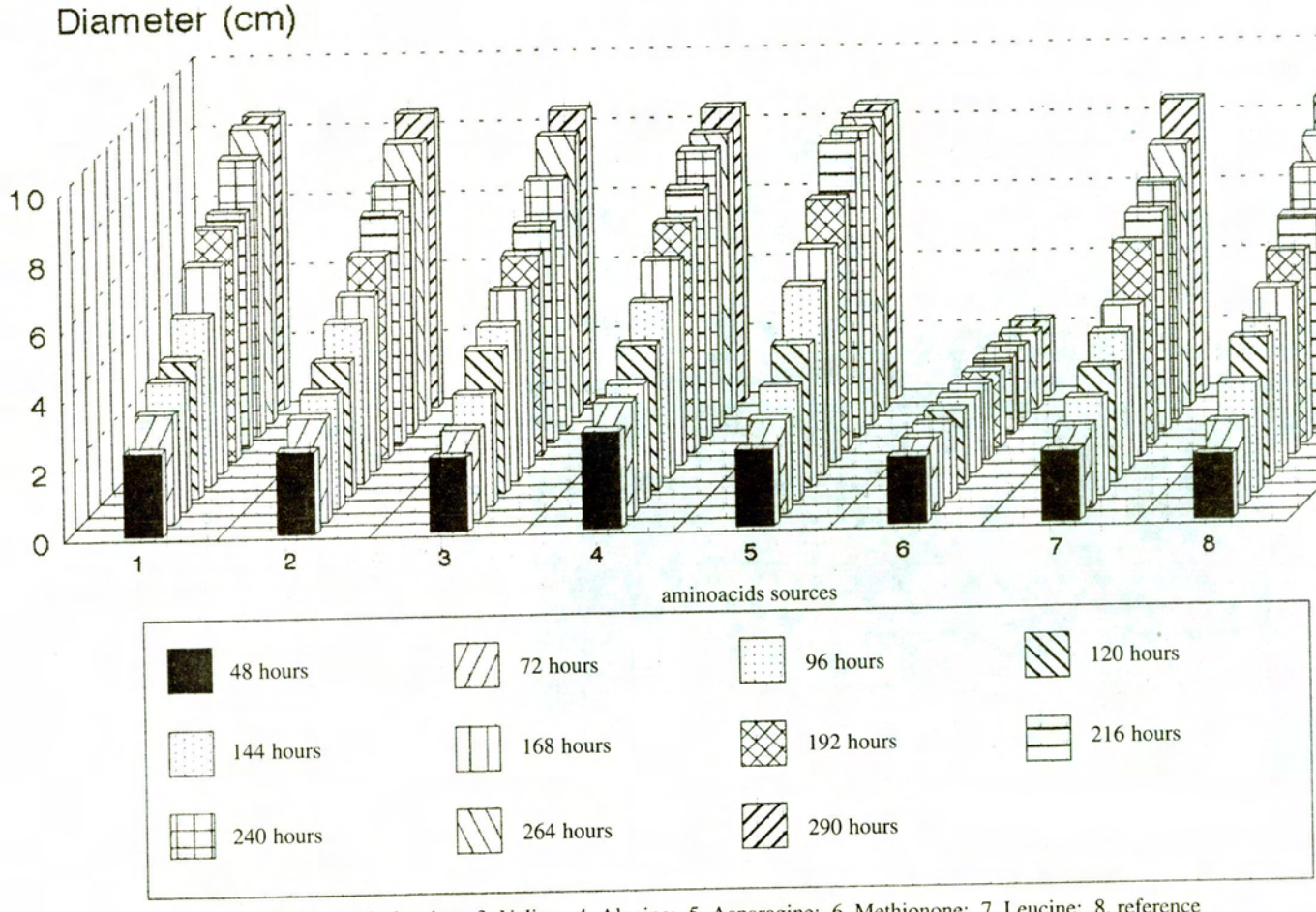


Fig. 5. – The development of *Botryotrichum piluliferum* on the media with aminoacids sources at 120 hours after inoculation. 1. Reference, 2. Serine, 3. L-Isoleucine, 4. Valine, 5. α-Alanine, 6. Asparagine, 7. Methionine, 8. L-Leucine.

containing alanine – 3.32 cm, followed, in a decreasing order (yet, with values higher than that of the reference – 2.46 cm) by the variants with serine – 3.18 cm, isoleucine – 3.00 cm, asparagine – 2.72 cm, valine – 2.60 cm, as well as leucine – 2.40 cm and methionine – 2.13 cm (values lower than the reference).

At 96 hours after inoculation, the growing of the fungus rhythm on the media containing serine, alanine, isoleucine, asparagine and valine was higher than that of the reference, while the values recorded on media with methionine and leucine were lower than the reference. Further on (at 120 hours, 144 hours, 168 hours, 192 hours) the growth rhythm is non-uniformly influenced by the presence of aminoacids, the observation being made that it was only 144 hours after inoculation that fungus



1. Serine; 2. Isoleucine; 3. Valine; 4. Alanine; 5. Asparagine; 6. Methionone; 7. Leucine; 8. reference

Fig. 6. – Development of *Botryotrichum piluliferum* on media with various aminoacid sources.

development on the medium containing methionine was stopped, the diameter of the colony remaining up to the end of the experiment (290 hours) at 2.20 cm.

After 216 hours, the growing rhythm was higher than that of the reference 6.20 cm on the medium with asparagine, occupying the whole Petri plate while, with the other variants, it took the following values: alanine – 7.26 cm, serine – 6.80 cm, isoleucine – 6.67 cm, leucine – 6.34 cm, valine – 6.30 cm. After 240 hours, the growing rhythm was non-uniform, after 264 hours – the fungus occupied the whole Petri plate and on the medium containing serine, as well, and, after 290 hours – on all media – reference included, the fungus occupied the whole Petri surface.

The data discussed in the present study show that *Botryotrichum piluliferum* grows best on the media containing asparagine, serine, valine and alanine while methionine inhibits the growth of the fungus. Similar results discussing the stimulating effect of aminoacids have been obtained by other authors, as well (14), (15), (16), (18); also, Veronica Tudosescu mentioned the inhibiting role played by methionine in the development of certain fungi (18).

CONCLUSIONS

1. The optimum carbon sources for the growth of *Botryotrichum piluliferum* fungus were: mannitol, carboxymethylcellulose, lactose, starch and cellulose.

2. From the sources of mineral nitrogen utilised in the experiment, the most favourable ones for the fungus' growth were: calcium nitrate, sodium nitrate and ammonium carbonate, while ammonium sulphate and ammonium chloride inhibited the development of the fungus.

3. Aminoacids have a stimulating effect on the growth of the fungus with the exception of methionine, which caused ceasing of development, 144 hours after inoculation.

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