

The Effect of *N*-Nitroso-*N*-Ethylurea on Mutagenesis of *Cercospora beticola* SACC.

DOROTA BRILLOVÁ

Institute of Experimental Phytopathology and Entomology,
Slovak Academy of Sciences, Ivanka pri Dunaji*

Abstract. Lethal and mutagenic effects of *N*-nitroso-*N*-ethylurea (NEU) on the parasitic fungus *Cercospora beticola* SACC. was investigated. Mutation frequency increased and the number of surviving individuals (conidia) simultaneously decreased with increasing mutagen concentration and the period of its application.

Treatment of *C. beticola* conidia with NEU induced 14 morphological mutants characterized by changes in pigmentation of air mycelium and substrate, excretion of the pigment into the cultivation medium and colonies morphology. A considerable proportion of morphological mutants lost their sporulation ability both *in vitro* on the sporulation medium and *in vivo* on host leaves and became non-pathogenic. The other morphological mutants (21.4%) were pathogenic on a sensitive sugar beet cultivar Dobrovická A and on other three resistant cultivars (Maribo 1, 2, 3).

A revertant with increased pathogenicity arose from the light non-pathogenic mutant. During the remonosporic isolation of the pathogenic mutants isolates were obtained characterized by a phenotype which originated during the mutagenic process.

Variability of pathogenic fungi and especially the origin of new races have significant influence on relationships between the host plant and the pathogen. It is one cause of the loss of resistance of cultivated plants. New races often originate by mutations which are also responsible for changes in their virulence. *Fungi imperfecti* are especially interesting from this standpoint: one of the main sources of their genetic variability are mutations. These findings and our own experience with isolates of *Cercospora beticola*, where unusually high variability was observed, inspired us to study the genetic variability of this pathogen in detail.

MATERIAL AND METHODS

Conidia of *Cercospora beticola* obtained from a monosporic isolate (provenience Pohronský Ruskov) were used in the present study. The monosporic isolate was cultivated on a potato-glucose-agar medium (PGA). A large amount of conidia was obtained by its further cultivation on medium

Received May 12, 1981, accepted November 30, 1981

* Address: 900 28 Ivanka pri Dunaji, Czechoslovakia.

prepared from beet leaves. Under these conditions the vegetative growth was suppressed and sporulation enhanced. The material was cultivated at 16 °C under natural alternation of day and night periods. Five days old colonies were used for preparation of conidia suspension in distilled water. After evaporation of water the conidia were suspended in a tested concentration of

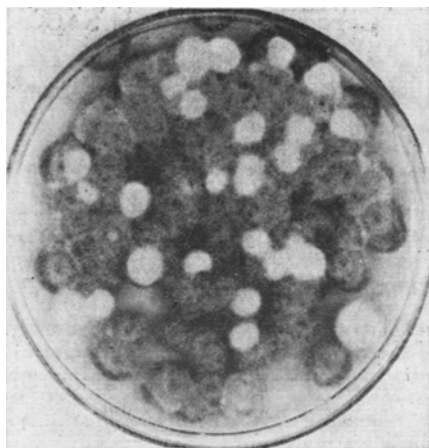


Fig. 1. Formation of white to rose colonies on PGA medium after treatment of *Cercospora beticola* conidia with 0.05 M NEU for 90 min. (Material Brillová, photo Blahutiaková.)

N-nitroso-*N*-ethylurea (NEU) solution for the investigated period of time under constant shaking. Conidia were then three times washed with twice sterilized water and centrifuged at 3 000 rev. min⁻¹ for 15 min.

Conidia density was counted in the Bürker's cell, adjusted to 10 000 pieces . ml⁻¹ and 0.05 ml aliquots were applied in Petri dishes containing PGA medium. Dishes were incubated at 24 °C. NEU was applied in 0.1, 0.05, 0.02, 0.01 and 0.001 M concentration for 30 min. When the dependence of the effect on duration of treatment was investigated 0.05 M NEU solution was applied for 15, 30, 60 and 90 min. The same conidia preparation which was not treated with NEU was used as a control.

The sporulation of isolates was tested *in vitro* on sporulation-inducing medium prepared from beet leaves and *in vivo* on leaves of four sugar beet cultivars (sensitive cultivar Dobrovická A and resistant cultivars Maribo 1, 2, 3). The mycelium suspension of isolates obtained after NEU treatment and of a wild strain cultivated on PGA medium was applied on the leaves. These tests were simultaneously used for estimation of pathogenity and virulence of isolates and wild strain. Evaluating pathogenity and virulence the sporulation frequency and the length of the incubation period were taken into account. Inoculated sugar beet plants were placed into humid chambers and kept under optimum conditions for incubation of the pathogen: 100% relative humidity and 23–25 °C. The course of sporulation was checked at 24 h intervals till the reproductive organs appeared.

TABLE 1

The effect of *N*-nitroso-*N*-ethylurea (NEU) on mutation frequency in *Cercospora beticola* after 30 min treatment

NEU concentration [M]	Total number of evaluated colonies	Surviving individuals (conidia) [%]	Mutations from surviving individuals [%]
0.10	4.125	33.33	1.659
0.05	4.519	51.23	0.929
0.02	7.443	63.50	0.436
0.01	4.115	79.00	0.234
0.001	6.834	91.65	0.141

Remonosporic isolations of sporulating strains were transferred on PGA medium and cultivated at 24 °C.

NEU was synthesized at the Research Institute of Agrochemical Technology, Bratislava.

RESULTS

Increasing concentration of the mutagen decreases percentage of surviving individuals (conidia) and increases percentage of mutations (Table 1). As is shown in Table 2 prolongation of the mutagen treatment has the same effect. When NEU treated conidia were grown on PGA medium together with colonies of the wild strain phenotype (ash-grey to ash-grey-green air mycelium and dark blue-green substrate) white to rosy colonies or sectors were formed (Fig. 1), the frequency of which is shown in Tables 1 and 2. Some ash-grey colonies, which also originated from the NEU-treated conidia were not changed in their pigmentation character (Fig. 1) and had the wild strain phenotype. After transfer of their mycelium mass mosaic colonies

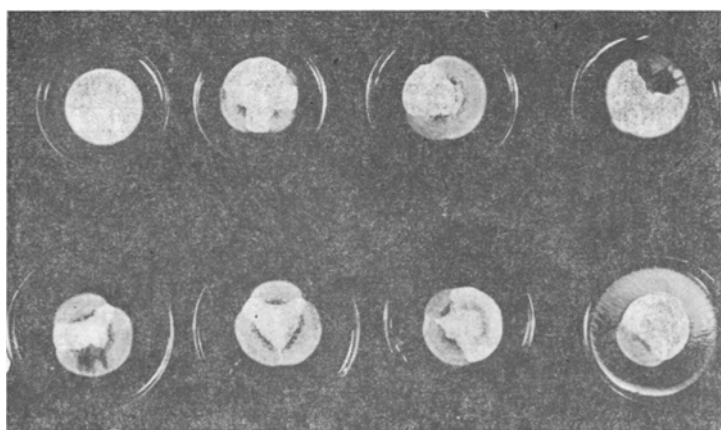


Fig. 2. Formation of coloured sectors on the colonies of the first subculture derived from the *Cercospora beticola* conidia treated with 0.01 M NEU for 30 min. (Material Brillová, photo Blahuťáková.)

TABLE 2

The time-dependence of the effect of *N*-nitroso-*N*-ethylurea (NEU) on mutation frequency in *Cercospora beticola*

NEU concentration [M]	Period of application [min]	Total number of evaluated colonies	Surviving individuals (conidia) [%]	Mutations from surviving individuals [%]
0.05	15	4.926	60.30	0.298
	30	4.519	51.23	0.929
	60	4.732	35.70	1.982
	90	4.859	21.00	3.201

were formed which consisted of sectors characterized by different pigmentation, size and portion of the wild strain. *E.g.* the first subculture of the ash-grey colonies derived from conidia treated with 0.01 NEU gave 20.8% of colonies consisting of differently pigmented sectors (Fig. 2). The same subculture of ash-grey colonies derived from conidia treated with 0.05 M NEU resulted in 43.40% of colonies containing differently coloured sectors (Fig. 3).

Completely mutated colonies were obtained by further cloning of pigmented sectors using hyphal inocula. The effect of applied NEU concentrations on conidia of the pathogen was evaluated according to the visual changes of pigmentation of the air mycelium and substrate, excretion of the pigment into the cultivation medium, ray-like to pleated and lump to porous structure of colonies and velvet or slime surface of colonies. According to these characters the mutants were divided into 14 groups (Table 3) which correspond to 14 species of morphological mutants of *Cercospora beticola*.

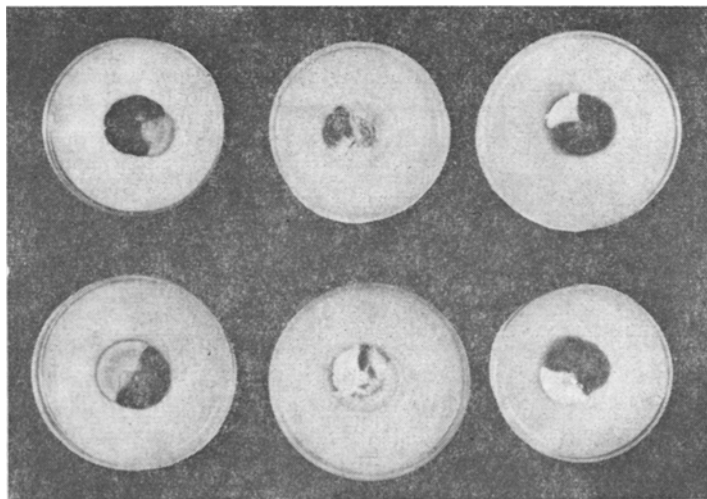


Fig. 3. Formation of coloured sectors on the colonies of the first subculture derived from the *Cercospora beticola* conidia treated with 0.05 M NEU for 60 min. (Material Brillová, photo Blahuťáková.)

TABLE 3
Visual characterization of *Cercospora belicola* mutants induced by *N*-nitroso-*N*-ethylurea

Serial No.	Air mycelium	Substrate	Pathogenicity	Colony habitus	Pigment excreted into medium
1	White	White	Non-pathogenic	Velvet	
2	Light apricot	Light beige	Non-pathogenic	Velvet	
3	Light apricot	Light beige	Non-pathogenic	Velvet	Cyclamen
4	Light apricot-yellow	Straw-yellow	Non-pathogenic	Excreted water drops	
5	Light rose	Cinnamon-orange	Non-pathogenic	High, Velvet	Cinnamon-orange
6	Sulphur-yellow	Cinnamon-brown	Non-pathogenic	Velvet	Cinnamon
7	Light rose	Olive-brown	Non-pathogenic	Ray-like surface	
8	Light apricot	Olive-brown	Non-pathogenic	Velvet	Cyclamen
9	White-yellow	Dark blue-green	Non-pathogenic	Cottonaceous	Brick-red
10	Multicolour: rose-red-yellow-brown-white	Dark blue-green	Non-pathogenic	Fine cottonaceous	Cyclamen
11	Dark brown to black	Dark blue-green	Non-pathogenic	Slime, pleated	
12	Light ash-grey-silver	Blue-green	Pathogenic	Lumpy with water drops	
13	Rose-ash-grey	Dark blue-green	Pathogenic	Ray-like surface	
14	Rose-ash-grey	Dark blue-green	Pathogenic	Pleated	
Wild strain:					
	Ash-grey to grey	Blue-green			

The ash-grey sectors of the wild strain phenotype were sporadically formed on colonies of completely light mutants in the late subcultures (Fig. 4). From these sectors a highly pathogenic mutant was isolated.

NEU also affected the hyphal morphology of mutants. Microscopic analysis showed that the hyphae of mutants characterized by white, apricot, rose and yellow air mycelium are fine ($1.20-1.90\text{ }\mu\text{m}$ thick), consisting of very long

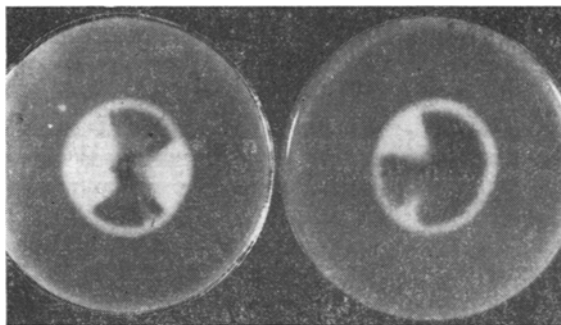


Fig. 4. Formation of dark, reverse mutated sectors of the wild strain phenotype. (Material Brillová, photo Blahutiaková).

cell segments with limited branching. Their cell nuclei are very small in size.

Microscopic observation showed that the majority of morphological mutants form conidiophores and conidia neither on sporulation-inducing medium nor on the leaves of the four tested beet cultivars inoculated with the mycelium suspension of these strains. On the other hand the mycelium suspension of the wild strain formed abundant reproductive organs already 24 h after inoculation which were able to infect the host.

Pathogenicity and virulence tests showed that the mutants which lost their ability to sporulate after NEU treatment also became non-pathogenic (Table 3). All mutants which were able to sporulate and infect were virulent on all four tested sugar beet cultivars.

The mutant derived from the reverted sector was more virulent on tested cultivars than the wild strain. This difference was observed in sporulation capacity where the ratio of conidia of the mutant and the wild strain was $7.5:1$. As compared with the wild strain the incubation period of the mutant was 3–4 days shorter.

Remonosporic isolations confirmed the stability of the phenotypes obtained by mutation. These phenotypes were stable when passaged *via* the host.

DISCUSSION

Mutants with striking pigmentation, morphology and consistency of colonies originated from *C. beticola* conidia after treatment with NEU. It is evident that NEU significantly affects morphogenesis of the pathogen *in vitro*. The mutants differed from the wild strain to such an extent that they look like different species when cultured *in vitro*.

Most of these mutants also differ in their sporulation ability. All mutants with altered colour of air mycelium (white, rose, apricot, yellow or multi-

colour) did not sporulate. Albinism of some mutants is accompanied with changes in spore morphology as *e.g.* in the case of *Alternaria brassicola* (CAMPBELL *et al.* 1968). Disorders in sporulation of mutants described here are, however, more serious and are in some cases responsible for sterility and loss of pathogenicity. Similar changes were also described by REMNEVA and IVANYUK (1968) in the case of *Macrosporium solani*.

Mutants characterized by the loss of their pathogenicity are not important for formation of new races in the host populations. In nature they can only decrease the vitality of the pathogen population and its infection potential. However, the revertant with increased pathogenicity might be of some importance for the origin of new races.

It is known that similar coloured strains can be obtained from phytopathogenic fungi after their exposure to different physical and chemical mutagens. *E.g.* 117 morphological and biochemical mutants of different colour were induced in *Venturia inaequalis* by UV light and nitrous acid (KEITT and BOONE 1954, BOONE and KEITT 1956, BOONE *et al.* 1956). Similar induction of coloured mutants was described in *Cochlyobolus sativus* (TIN-LINE *et al.* 1960), *Verticillium albo-atrum* (HASTIE 1962), *V. dahliae* (NOVOTEL'NOVA and PYSTINA 1968), *Colletotrichum lagenarium* (DUTTA and GARBER 1960), *C. falcatum* (BAJAJ *et al.* 1964), *Fusarium oxysporum* (TUVESON and GARBER 1959), *Kabatiella lini* (LEVITIN and ZACHAROV 1967), *etc.*

Evaluating the variability of *Cercospora* it is necessary to take into account that this fungus belongs to *Fungi imperfecti* which are lacking in the sexual way of reproduction. This eliminates the use of the hybridization method for studies of different aspects of mutation origin and complicates the explanation of some phenomena. In addition to this difficulty *C. beticola* is according to PLOTHO (1952), FRANDSEN (1955) and CANOVA (1959) a haploid and monocaryotic species. It has been proved by CANOVA (1959) that nuclei of conidial cells originate from a single nucleus which is transferred to the originating conidium. This phenomenon eliminates the heterocaryosis which is often assumed to be responsible for the vegetative instability of fungi. The haploid and monocaryotic state of the pathogen may contribute to the early origin of new phenotypes from a certain percentage of conidia after NEU treatment. Formation of sectors, *i.e.* mosaic colonies in the first subculture obtained from colonies originating from NEU treated conidia and showing the wild strain phenotype, may be the result of delayed mutations which often occur after mutation induction with chemomutagens. It is also necessary to take into account that not all conidial cells are in the same cell cycle during the mutation. High formation of coloured sectors in *C. beticola* may be conditioned by G₂ state of certain cells at the time of mutagen application as was described in the case of yeast (JAMES and WERNER 1966, GUGHELMINETTI 1968).

Induced complete and mosaic mutations were studied by a number of authors, *e.g.* NASIM (1967), ABBONDANDOLO and SIMI (1971), JAMES *et al.* (1972), NASIM and GRANT (1973), HANNAN *et al.* (1976), ECKARDT and HAYNES (1977) who postulated several theories for the explanation of this phenomenon. Mechanism of the origin of complete and mosaic colonies of *C. beticola* after NEU treatment cannot be explained on the basis of present experiments. It will be discussed in our forthcoming communication.

REFERENCES

- ABBONDANDOLO, A., SIMI, S.: Mosaicism and lethal sectoring in G₁ cells of *Schizosaccharomyces pombe*. — Mut. Res. **12** : 143—150, 1971.
- BAJAJ, B. S., CHATRATH, M. S., VASUDEVA, R. S.: Mutation in *Colletotrichum falcatum* WENT. the causal organism of red sugarcane. IV. Morphological and biochemical mutants induced by ionizing radiation. — Indian Phytopathol. **4** : 296—303, 1964.
- BOONE, D. M., KEITT, G. W.: *Venturia inaequalis* (CKE) WINT. VIII. Inheritance of color mutant characters. — Amer. J. Bot. **43** : 226—233, 1956.
- BOONE, D. M., STAUFFER, J. F., STAHMANN, M. A., KEITT, G. W.: *Venturia inaequalis* (CKE) WINT. VII. Induction of mutants for studies on genetics, nutrition, and pathogenicity. — Amer. J. Bot. **43** : 199—204, 1956.
- CAMPBELL, R., LARNER, R. W., MADELIN, M. F.: Notes on an albino mutant of *Alternaria brassicola*. — Mycologia **60** : 1122—1125, 1968.
- CANOVA, A.: [Research on Biology and Epidemiology of *Cercospora beticola* Sacc.] In Ital. — Agraria, Roma 1959.
- DUTTA, S. K., GARBER, E. D.: Genetics of phytopathogenic fungi. III. An attempt to demonstrate the parasexual cycle in *Colletotrichum lagenarium*. — Bot. Gaz. **122** : 118—121, 1960.
- ECKARDT, F., HAYNES, R. H.: Induction of pure and sectored mutant clones in excision-proficient and deficient strains of yeast. — Mut. Res. **43** : 327—338, 1977.
- FRANDSEN, N. O.: Untersuchungen über *Cercospora beticola* III. Morphologie des Pilzes. — Zucker **8** : 513—515, 1955.
- GUGLIELMINETTI, R.: The role of lethal sectoring in the origin of complete mutations in *Schizosaccharomyces pombe*. — Mut. Res. **5** : 225—229, 1968.
- HANNAN, M. A., DUCK, P., NASIM, A.: UV-induced lethal sectoring and pure mutant clones in yeast. — Mut. Res. **36** : 171—176, 1976.
- HASTIE, A. C.: Genetic recombination in the hop-wilt fungus *Verticillium albo-atrum*. — J. gen. Microbiol. **27** : 373—382, 1962.
- JAMES, A. P., NASIM, A., McCULLOUGH, R. S.: The nature of replicating instability in yeast. — Mut. Res. **15** : 125—133, 1972.
- JAMES, A. P., WERNER, M. M.: Radiation induced genetic mosaicism and lethal sectoring in yeast. — Mut. Res. **3** : 477—485, 1966.
- KEITT, G. W., BOONE, D. M.: Induction and inheritance of mutant characters in *Venturia inaequalis* in relation to its pathogenicity. — Phytopathology **44** : 362—370, 1954.
- LEVITIN, M. M., ZAKHAROV, I. A.: [The lethal and mutagenic effect of UV rays in the cause of browning and stem break of flax.] In Russ. — Genetika **9** : 65—70, 1967.
- NASIM, A.: The induction of replicating instabilities by mutagens in *Schizosaccharomyces pombe*. — Mut. Res. **4** : 753—763, 1967.
- NASIM, A., GRANT, C.: Genetic analysis of replicating instabilities in yeast. — Mut. Res. **17** : 185 to 190, 1973.
- NOVOTEL'NOVA, N. S., PYSTINA, K. A.: [Induced variability of the fungus causing the verticillium wilt of cotton plants.] In Russ. — Mikol. Fitopatol. **4** : 290—300, 1968.
- PLOTHO, O.: Weitere Untersuchungen zur Zytologie und Morphologie der *Cercospora beticola*. — Zucker **5** : 379—383, 1952.
- REMNEVA, Z. I., IVANVUK, V. G.: [Interspecific inhomogeneity in *Macrosporium solani* ELL. et MART. the cause of early blight of potato.] In Russ. — Mikol. Fitopatol. **3** : 202—208, 1968.
- TINLIE, R. D., STAUFFER, J. F., DICKSON, J. G.: *Cochylobolus sativus*. III. Effect of ultra-violet radiation. — Can. J. Bot. **38** : 275—282, 1960.
- TUVESON, R. W., GARBER, E. D.: Genetics of phytopathogenic fungi. II. The parasexual cycle in *Fusarium oxysporum f. pisti*. — Bot. Gaz. **121** : 74—80, 1959.