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Degeneration phenomenon of alkaloid-producing strains of *Claviceps paspali*

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Passaging of *Claviceps paspali* alkaloid-producing strains leads to degenerative morphological changes which seem to be responsible for a reduction of alkaloid production. It was shown that cultures of *Claviceps paspali* consist of two types of colonies on a PDA medium: small-brown and large-white ones, the first existing mainly in early passages and the latter appearing in successive passages. Apparently, the appearance of white colonies, which might be the result of a mutational process, is responsible for the loss of alkaloid production. The possibility of regeneration through selection of brown colonies is discussed.

It is well-known that *Claviceps paspali* strains produce ergot-alkaloids by submerged fermentation cultures (Pacifici *et al.* 1962, 1963, Kobel *et al.* 1964, Agurell 1966a, b, c), since Arcamone *et al.* (1960, 1961) have described the production of lysergic acid derivatives by a strain of *Claviceps paspali* Stevens and Hall.

Claviceps paspali strains, which produce 6 Δ methyl 8.9 ergolene carboxylic acid by fermentation, are commercially exploited (Kobel *et al.* 1964). The submerged fermentation cultures for the alkaloid production are inoculated with conidial suspensions from agar slant cultures, as described later. During the passaging on the agar medium, the strains undergo morphological changes which appear to be responsible for a reduction of alkaloid production.

This study is an investigation of the degeneration phenomenon in alkaloid-producing strains of *Claviceps paspali*, which has been previously briefly reported (Kobel 1969), and it discusses the possible reasons for this phenomenon.

Material and methods

Growth of *Claviceps paspali* in agar slant cultures: For growth purposes and conidia production two different media are required (Kobel *et al.* 1964). It is possible for *Claviceps paspali* to grow in form of white mycel on medium I, a modified potato dextrose agar (PDA), but no conidia are produced on this medium. After massive transfer of mycel pieces from culture I to medium II (containing 500 ml beer-wort, 250 ml cornsteep extract, 20 g agar, pH 4.5), the fungi are able to sporulate. Conidia suspensions from these sporulating cultures are then used for inoculation of the submerged cultures.

Alkaloid production in submerged cultures: Primary submerged shaking cultures (4.5% malt extract medium) in which the inoculated conidia germinate are transferred to the main fermentation cultures, in which alkaloids are produced. The medium used for the submerged fermentation cultures has been described previously (Kobel *et al.* 1964), and consists mainly of sorbitol, succinic acid, KH_2PO_4, $MgSO_4$, $FeSO_4$ and $ZnSO_4$. After 10—14 days of growth at 24 °C under constant shaking, the submerged fermentation cultures become redbrown-violet and alkaloids are colourimetrically determined in the filtrates using

the VAN URK reagent according to the method of ALLPORT and COCKING (1932). Testing morphological changes appearing during the passaging of agar slant cultures: In order to determine the morphological changes occurring during passaging of *Claviceps paspali*, the cultures were plated on PDA plates (medium I), and the morphology of single colonies was observed.

Results and discussion

Correlation between alkaloid production and culture morphology

During the passaging of *Claviceps paspali* strains on growth medium II (sporulating medium) gradual morphological changes were observed in the cultures, namely, they developed an overgrowth of white non-sporulating mycel. It was also observed that these morphological changes were connected with the loss of alkaloid production. Therefore, an experiment was undertaken for the purpose of specifically following the morphological changes and their correlation to alkaloid production.

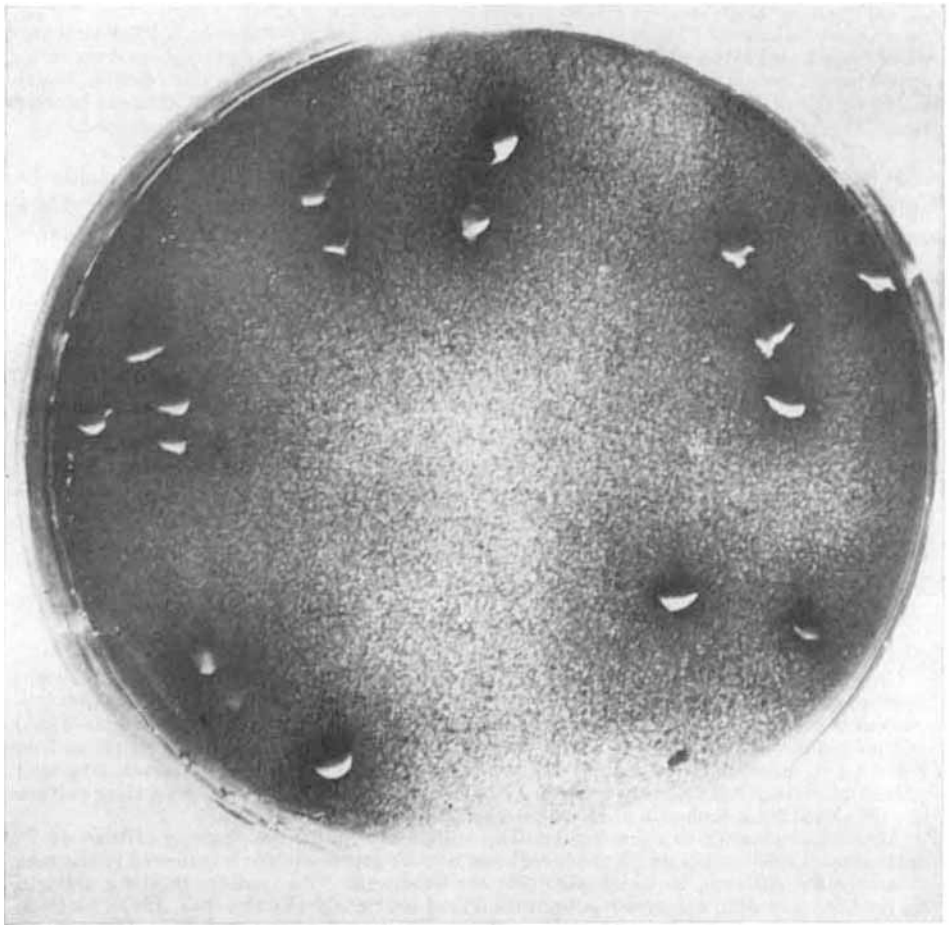

Fig. 1. Colonial morphology — a criterion for degeneration. — a) 1st generation: 100% small-brown colonies

A freshly isolated single spore *Claviceps paspali* strain was passaged on medium II up to ten generations. Conidial suspensions of each generation were plated on PDA plates and alkaloid production was tested in submerged fermentation cultures. As may be seen in Fig. 1, two types of colonies were observed: small, compact, brown and white, large, diffuse colonies. Cultures of early generations were composed mainly of brown colonies and each passage increased the relative percentage of the white colonies until only white colonies appeared. Table 1 represents the relative percentages of the brown and white colonies and the yield of alkaloids of each generation. As can be seen, the increase in the percentage of white colonies is followed by a gradual decrease in alkaloid yield and ends finally with a total loss of alkaloid production.

Appearance of white colonies — criterion for degeneration

We consequently presumed that the degeneration must be related to the appearance of the white colonies. Therefore, the next step was to compare the strains which were isolated from brown colonies to those isolated from white

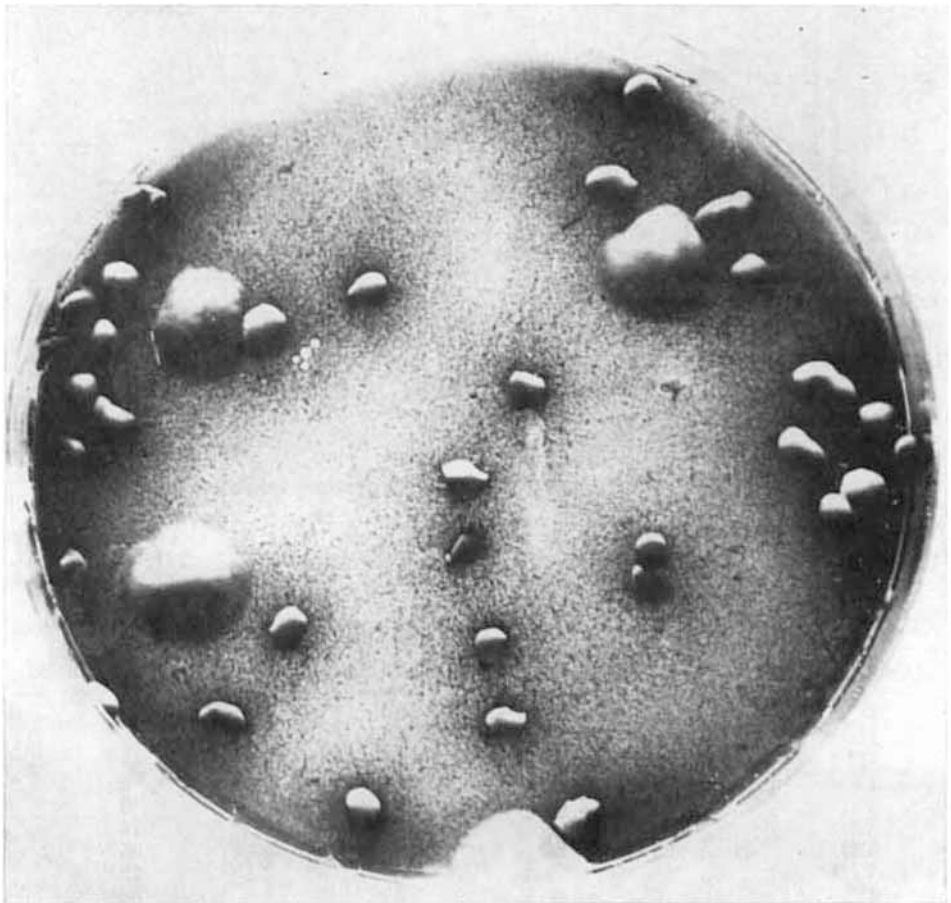

Fig. 1b. 6th generation: 86% small-brown colonies, 14% large-white

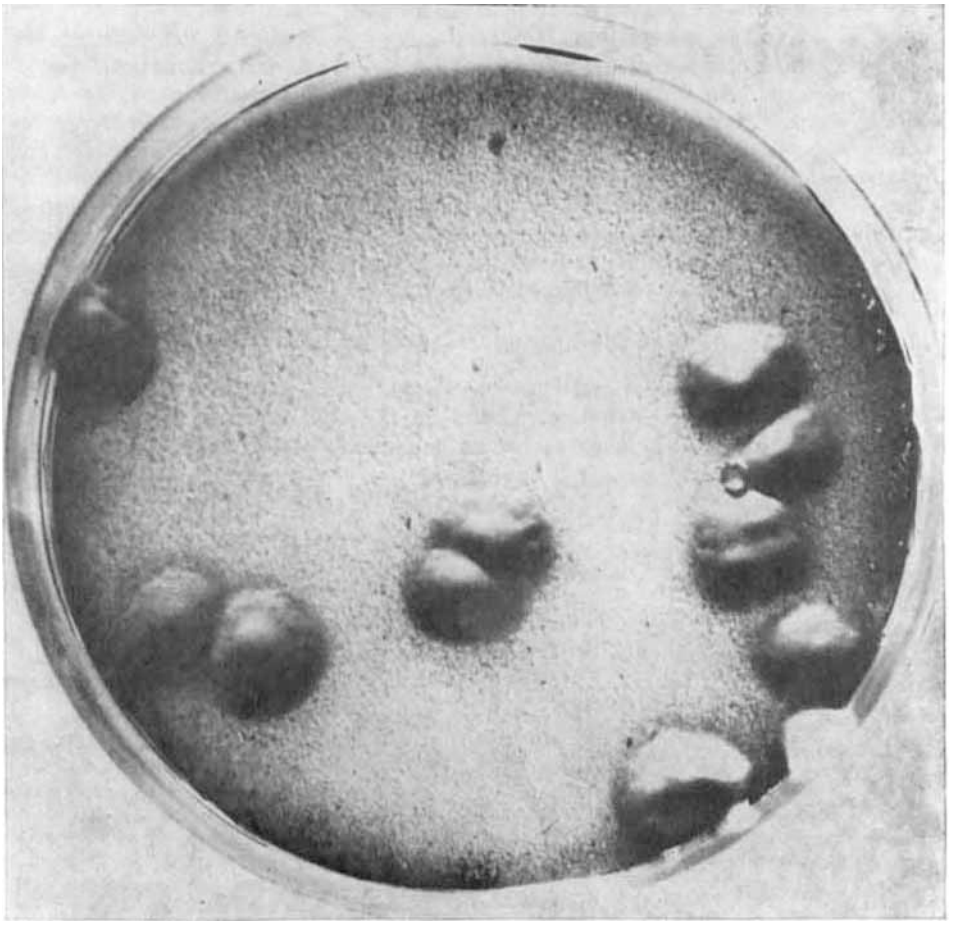

Fig. 1c. 10th generation: 100% large-white colonies

Table 1
Correlation between alkaloid production and culture morphology

Generation	Alkaloid yield mg/l	% of brown colonies	% of white colonies
I	—	100	0
II	2060	99	1
III	1910	99	1
IV	1860	98	2
V	1850	91	9
VI	1390	86	14
VII	370	65	35
VIII	40	28	72
IX	0	12	88
X	0	0	100

colonies. From each of the eight generations, 30 colonies (brown and white) were isolated, grown on medium II, and then checked for alkaloid production and replated on PDA plates. Table 2 summarizes the results of this experiment, showing that (a) the alkaloid yield is not influenced by the generation from which the colonies were isolated, and (b) that alkaloid yield is clearly influenced by the morphology of the colony. Cultures grown from brown colonies of the eight generation yielded the same alkaloid values as those originating from second generation.

Table 2
Influence of morphology of colony from which strains are isolated on their alkaloid production and colonial morphology pattern

Gen. from which colony was isolated	Morphology of the originally isolated colony	Alkaloid yield mg/l	Pattern of morphology of the new colonies	
			% of brown col.	% of white col.
I	brown	1870	97.0	3.0
	white	0	0	100.0
II	brown	1860	92.6	7.4
	white	340	0	100.0
III	brown	1880	94.7	5.3
	white	760	0	100.0
IV	brown	1940	95.5	4.5
	white	210	0	100.0
V	brown	1950	96.0	4.0
	white	750	0	100.0
VI	brown	2010	96.1	3.9
	white	420	0	100.0
VII	brown	1940	93.6	6.4
	white	430	0	100.0
VIII	brown	1960	94.0	6.0
	white	300	0	100.0

The plating on PDA plates revealed that strains isolated from brown colonies of all generations show a similar pattern of morphology, namely, they consist of brown and white colonies. In contrast, strains isolated from white colonies consisted of white colonies only, regardless of the generation. In order to determine whether strains isolated from later generations degenerate more rapidly than those isolated from earlier generations, the following experiment was performed. Brown and white colonies were isolated from each of eight generations and followed for twelve generations for alkaloid production and relative percentage of brown and white colonies. Judging from Table 3, showing the results of this experiment, the generation from which the colonies were isolated had no effect on alkaloid production or colony morphology distribution. Strains isolated from brown colonies of early and late generations undergo the same degenerative phenomenon in the same period of time.

Morphological changes — a result of mutational process

It seems that the white colony is a stable form whereas the brown is labile; that transformation from brown to white takes place gradually and this

Table 3
Alkaloid production and degeneration velocity in dependence of the generation of the culture from which the brown colony was isolated

Generation of the original colony	I	II	III	IV	V	VI	VII	VIII
Passage II:								
Alkaloids mg/l	2000	1750	2200	2150	2150	1930	1750	2010
% brown col.	94.3	91.3	87.5	95.0	94.5	93.5	96.0	97.0
Passage III:								
Alkaloids mg/l	2090	1960	1940	1710	1760	1890	1280	1840
% brown col.	95.8	91.6	74.8	92.8	86.1	98.3	91.6	89.2
Passage IV:								
Alkaloids mg/l	2080	2040	1920	2040	1910	970	1230	1750
% brown col.	94.3	88.4	39.3	88.8	83.7	90.3	96.2	91.1
Passage V:								
Alkaloids mg/l	2030	1820	1960	2080	2050	830	1210	1160
% brown col.	100.0	95.0	44.0	90.0	54.0	94.5	81.8	94.5
Passage VI:								
Alkaloids mg/l	1800	1540	2080	2020	1940	880	1050	1050
% brown col.	92.2	95.5	18.5	93.6	56.8	97.4	82.4	95.8
Passage VII:								
Alkaloids mg/l	1890	1350	1990	1660	2089	780	940	1020
% brown col.	83.2	98.2	5.5	93.3	57.9	87.8	56.7	97.6
Passage VIII:								
Alkaloids mg/l	2090	1930	1780	1790	1750	1510	240	1230
% brown col.	97.0	89.3	20.2	84.8	56.5	94.3	38.7	74.5
Passage IX:								
Alkaloids mg/l	1600	1730	1760	1750	1880	1100	150	780
% brown col.	25.0	92.2	9.2	86.1	46.2	91.8	6.9	49.3
Passage X:								
Alkaloids mg/l	460	1560	1540	1680	1080	830	50	460
% brown col.	11.1	80.0	12.5	64.7	39.3	67.1	2.6	5.3
Passage XI:								
Alkaloids mg/l	0	1360	1070	1490	1020	370	10	510
% brown col.	0	53.5	0	12.5	11.1	24.6	0	25.0
Passage XII:								
Alkaloids mg/l	0	0	0	0	0	0	0	0
% brown col.	0	28.5	0	7.5	11.1	0	0	0

might be the result of a mutational process. *Claviceps paspali* was therefore treated with UV and aethylenimine to establish whether the mutagenic treatment enhances the appearance of the white colonies. As may be seen from Table 4, the mutagenic treatment actually results in a rise of white colony percentage. Despite the high mutation rate of the brown form to the stable white form, brown colonies are still predominantly isolated from natural sources, and this fact can easily be explained by the observation (KOBEL 1969) that only conidia of brown colonies are virulent for the natural host (*Paspalum dilatatum*). Selection of brown colonies under laboratory conditions thus causes the same regenerating effect on alkaloid producing strains as a passage on the natural host.

Table 4
The influence of mutagenic treatment on percentage of white colonies

Mutagenic treatment	Survival rate	% of white colonies
	100 %	18.6
U.V. irradiation 20 sec	83 %	22.4
U.V. irradiation 40 sec	11 %	25.3
U.V. irradiation 60 sec	0.53 %	27.0
U.V. irradiation 90 sec	0.0007%	35.6
U.V. irradiation 120 sec	0.0002%	43.0
U.V. irradiation 150 sec	0.00008%	53.0
	100 %	22.1
Aethylenimine 30 min	67 %	23.1
Aethylenimine 60 min	27.2 %	21.0
Aethylenimine 120 min	7.8 %	26.6
Aethylenimine 150 min	0.14 %	33.7
Aethylenimine 180 min	0.015%	46.0

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