Heterokaryosis and Alkaloid Production in *Claviceps purpurea*

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Strain 275 FI of *Claviceps purpurea*, which produces large amounts of peptide alkaloids in submerged culture, is a heterokaryon; after several generations on agar media, it segregates the single components. These components (by us labeled V, C, and W) are stable and produce almost no alkaloids under described conditions of submerged culture. The mycelium of strain 275 FI consists of hyphae with multinucleate cells and does not produce conidia. Strains V, C, and W form numerous anastomoses when grown together on agar. By combining strains V and C, a heterokaryon similar to 275 FI in appearance has been obtained. This new strain produces amounts of alkaloids much larger than those produced by V and C separately or in associated submerged culture. We conclude, therefore, that in strain 275 FI the heterokaryotic condition is favorable to the production of alkaloids. Several conidia-producing cultures of *C. purpurea* of various origins, as well as sclerotia of the same species, have been examined. The results demonstrated that the heterokaryotic condition is rare in the cultures, but it is frequent in the mycelium from sclerotia. Since it is known that the production of alkaloids is typical of the sclerotal phase in *C. purpurea*, it is suggested that this capacity is related to the heterokaryosis of the producing strains.

A strain of *Claviceps purpurea* labeled 275 FI produces, under submerged culture, remarkable amounts of a mixture of peptide alkaloids, mainly consisting of ergotamine (1). In the accompanying paper (2), it is reported that the ability of strain 275 FI to produce alkaloids is correlated with the simultaneous utilization of large amounts of both sucrose and citric acid. In the same paper, three other strains (labeled V, C, and W), obtained from strain 275 FI, were described. They are virtually unable to produce alkaloids and are characterized by a reduced capacity for utilizing citric acid sucrose (strain V), or (strain C), or both (strain W).

**Materials and Methods**

Strain 275 FI of *C. purpurea* produces in submerged culture 1,100 to 1,400 µg (per ml) of a mixture of alkaloids, mainly ergotamine (1). The media and the culture methods are the same as described (2).

Giant colonies were obtained by seeding the center of (12-cm diameter) petri dishes containing 20 ml of medium T2 with a fragment taken from a slant of the strain under study. The plates were incubated at 28°C for 7 to 10 days, and then were kept at room temperature for 15 to 20 more days.

Since no conidia are produced by strain 275 FI, isolation cultures were prepared by grinding, in a Potter homogenizer, a portion of mycelium added with sterile distilled water. The homogenized suspension was then filtered through a silk cloth, suitably diluted, and distributed on petri dishes containing medium T2, which were then incubated at 28°C for 10 to 12 days. The seed suspension consisted of small fragments of hyphae only. The nuclear staining reactions were performed by the Feulgen reaction. Anastomoses were detected directly under the microscope in 6- to 7-day-old cultures on thin layers of medium T2 smeared on slides and incubated in a wet chamber at 28°C.

**Results**

Heterokaryotic nature of strain 275 FI and relation to capacity for producing alkaloids. The giant colonies of strain 275 FI, obtained on medium T2 as above described, are 8 to 9 cm in diameter and show a compact consistency and white color with pinkish hues. About 30% of the giant colonies show sharply distinct sectors that originate from a point of the colony distant from the center. These sectors were also observed when single fragments of hyphae were employed for the preparation of giant colonies; this fact, together with their late appearance in the colonies, is sufficient to discard the hypothesis that the sectors may be due to the use of an inoculum consisting of a mixture of strains.

Three kinds of sectors were observed. The first
one was deep purplish-violet in color, the second cream-white, and the third, much less frequent than the others, pure white. From each kind of sector, transfers were made to slants of medium T2. Cultures from the purplish-violet sectors were labeled V, those from the cream-white sectors were labeled C, and those from white sectors, W.

The three types of cultures proved to be quite unproductive when tested in submerged culture. Only the V strain produced measurable quantities (about 100 μg/ml) of alkaloids. Also, no production was obtained in submerged culture by using various mixtures of the three strains. Giant colonies were prepared from strains V, C, and W; in no case were sectors observed. These results indicate that strain 275 Fl is a heterokaryon and that strains V, C, and W are its components.

It is known that one of the most efficient ways to reach the heterokaryotic condition in fungi is the formation of anastomoses. We therefore examined slides (prepared as above) of cultures of the single strains and their mixtures for the presence of anastomoses. Only a few were observed on slides of the single strains, whereas the anastomoses were very abundant in the mixed cultures, particularly of strains V and C (Fig. 1).

Heterokaryosis is obviously related to a multinucleate condition of the hyphal cells. Observations of hyphae of strain 275 FI, after Feulgen staining, demonstrated that each cell usually contains five nuclei. It is also known that the septa of the hyphae of ascomycetes permit the passage not only of cytoplasm but also of nuclei. The lack of conidia in strain 275 FI strengthens the hypothesis that it is a heterokaryon. Conidia, in fact, are usually uninucleate and therefore unable to maintain the heterokaryotic state. We conclude that strain 275 FI is a heterokaryon in which interaction at the nuclear level of strains unable to produce significant amounts of alkaloids leads to the ability to produce remarkable yields of these substances.

**Synthesis of the heterokaryon by strains V and C.** To verify the above conclusions, we tried to obtain a strain similar to 275 FI by synthesizing a heterokaryon with strains V, C, and W. For this purpose we seeded petri dishes containing medium T2 with a mixture of equal amounts of hyphal fragments of the three strains suspended in water. These plates were incubated at 28°C for 15 days. Each time the colonies of strain V and C made an evident area of overgrowth appeared at the contact line between the colonies (Fig. 2). This was never observed with strain W. Assuming that the

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**Fig. 1. Anastomosis between hyphae in mixed cultures on medium T2 of strains V and C of Claviceps purpurea.**

**Fig. 2. Overgrowth at the contact line between colonies of strains V and C on medium T2.**
overgrowth observed at the contact zone between strain V and C was due to heterokaryosis, we further investigated these two strains.

A thick suspension of a 1:1 mixture of hyphal fragments of strains V and C was inoculated on plates containing medium T2 and incubated at 28°C for 10 days. About 1 cm² of the resultant mycelial mat, which thoroughly covered the surface of the plates, was withdrawn, homogenized, filtered as previously described, and used for the preparation of isolation plates with medium T2. Analogous control plates with the same medium were also prepared with suspensions of hyphal fragments of the single cultures of strains V and C. Plates prepared with single cultures gave rise to colonies corresponding to V and C. Those prepared with the suspension of mycelium of the two mated strains, however, gave rise to three types of colonies; one was similar to V, one to C, and one was intermediate between these two strains and very similar in appearance to strain 275 FI. These three types of cultures were labeled, respectively, neo-V, neo-C, and neo-275 FI, and were employed for the preparation of giant colonies. The giant colonies originating from mycelia of neo V and neo-C did not sector; those made with neo-275 FI displayed late sectors, quite similar in their features to strain V or C. In other words, neo-275 FI behaves as strain 275 FI.

Thirty colonies of the three types of cultures (neo-V, neo-C, neo-275 FI), about 1 cm in diameter, were withdrawn (along with their under-layer of agar) from the plates, transferred into test tubes containing 2 ml of water, and thoroughly ground with a glass rod. To each of these suspensions, 4 ml of the van Urk reagent was added. This reagent gives a blue color with the indole alkaloids. No color was detected with colonies of neo-C; those of neo-V occasionally showed a faint coloration; the great majority of neo-275 FI colonies gave a deep blue reaction.

Ten other colonies of the three types were tested for their ability to produce alkaloids in submerged culture, by transplanting the colonies directly from the isolation plates into medium TG and then into the production medium, T25. The results obtained, compared with those observed with colonies isolated from strain 275 FI, are reported in Table 1.

Cultural characteristics, behavior of the giant colonies, and inability to produce alkaloids allow us to conclude that strains neo-V and neo-C are identical to strains V and C, respectively. Strain neo-275 FI, obtained by mating strains V and C, exhibited cultural characteristics similar to those of strain 275 FI, gave rise to strains V and C from giant colonies as does strain 275 FI, and produced a considerable amount of alkaloids.

<table>
<thead>
<tr>
<th>Table 1. Production of alkaloids in submerged culture⁴</th>
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<tr>
<td>Colonies from strain 275 FI</td>
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<tr>
<td>neo-V</td>
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<td>1,100</td>
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<td>1,200</td>
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<td>1,320</td>
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<td>1,040</td>
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⁴ Ten colonies of 275 FI and of each of the derived strains were tested. Results are expressed micrograms per milliliter.

Concluded, therefore, that strain neo-275 FI is a heterokaryon formed by strains V and C and that the heterokaryotic condition is favorable to the production of alkaloids.

Heterokaryosis in other strains of C. purpurea.

Investigations were carried out to establish whether the heterokaryosis demonstrated in strain 275 FI was an isolated phenomenon or a feature common to other strains of C. purpurea. We microscopically examined conidia of five strains of C. purpurea isolated from sclerotia of various origins. The conidia, stained by the Feulgen reaction, were all uninucleate, as reported by Tonolo et al. (6). Rochelmeyer (5) found that all the conidia of young cultures of C. purpurea are uninucleate, whereas up to 10% of the conidia of older cultures are binucleate or trinucleate. As a rule, the presence of uninucleate conidia in a fungus is incompatible with heterokaryosis. It is possible, however, that a heterokaryon which breaks with formation of conidia could be re-established through anastomoses between the hyphae arising from conidia germinating together. But it is obvious that the conservation of the heterokaryotic condition depends on many factors, such as the mass dispersion of the conidia, the presence of an environment equally favorable to the germination of the conidia of all of the components of the heterokaryon, and the formation of numerous anastomoses. We prepared many giant colonies of subcultures of strains producing conidia, using mycelium which had not yet sporulated as inoculum, and indeed we found a few cases in which the formation of late sectors occurred. Unfortunately, we are not able to report the percentages. It is impossible to exclude, therefore, the possibility that hetero-
Karyotic phenomena may occur in conidia-producing strains of *C. purpurea*. At any rate, it is clear that, in strains with evident capacity to form uninucleate conidia, the heterokariosis is a very unstable condition.

We prepared giant colonies directly from cultures obtained from sclerotia of *C. purpurea*. The sclerotia were first washed in water, then in a 0.5% solution of HgCl₂, and finally thoroughly rinsed in sterile water. They were aseptically broken, and the two resulting pieces were placed in the center of petri dishes containing medium T2 and incubated at 28°C. The humidity of the plates was maintained by periodic additions of sterile water. Of the 110 sclerotia employed, only 22 produced suitable cultures. The others did not develop mycelium at all, or were contaminated by molds or bacteria. The 22 cultures were incubated until typical giant colonies were formed. Only 4 of these cultures did not give rise to sectors; 16 showed one or more evident sectors, and 2 gave unclear results.

Two sclerotia, labeled 74 and 101, were studied in more detail. Both of the cultures obtained from these sclerotia served for double isolations on medium T2; the first was made by utilizing the clusters of unsporulated mycelium which first appeared on the sclerotium, and the second by using subcultures of the same mycelium on medium T2 incubated at 28°C for 8 days (abundantly sporulated). The mycelium not yet sporulated was in the meantime employed to seed plates of medium T2 for obtaining giant colonies. Both strains gave rise to giant white colonies with sectors that were violet with strain 74 and violet-brown with strain 101. In the isolation plates from the mycelium not yet sporulated, strain 74 usually produced white colonies with occasional bulky violet colonies (not more than 3 to 5%); from the sporulated mycelium, the bulky violet colonies represented about 35%. Strain 101 produced primary white colonies from the unsporulated mycelium, and about 28% of the colonies from the sporulated mycelium were bulky violet-brown. With this strain, furthermore, a flat type of colony, yellowish white, appeared (15%). We thus think that sclerotia 74 and 101 were heterokaryons that segregated the components in the conidia and in the giant colonies, strengthening the opinion that high frequency of giant colonies forming late sectors in a strain is a result of its heterokaryotic nature.

We conclude that, whereas the conidia-forming cultures of *C. purpurea* are not frequently heterokaryotic, a high percentage of sclerotia are composed of heterokaryotic mycelium.

**Discussion**

Strain 275 FI of *C. purpurea*, which is able to produce in submerged culture large amounts of peptide alkaloids, is a heterokaryon. Under conditions of saprophytic culture, this heterokaryon can segregate its components. We isolated and studied three of these component strains and observed that singly, or in associated culture, they are essentially unable to produce alkaloids.

Furthermore, we were able to synthesize a heterokaryon by combining two of these three strains, and we confirmed that the heterokaryotic condition is favorable to the production of alkaloids.

In the accompanying paper (2), we showed that the capacity of producing alkaloids is correlated with that of utilizing large amounts of both sucrose and citric acid.

From the genetic point of view, we think that the nuclear interaction between strains V and C can give rise to a strain such as 275 FI. This strain, in fact, displays characteristics similar to those of combined strains V and C as far as sucrose and citric acid utilizations are concerned.

As for the presence of component W in the heterokaryotic strain 275 FI, we cannot give any conclusive explanation. In the accompanying paper (2), we suggested that this strain might be a "normal form" from which strains V and C originated by mutation. It is evident, however, that strain W can theoretically also be formed by recombination of strains V and C.

The fact that a heterokaryon can produce substances not produced by the single components has been reported (4). It is also known that the heterokaryotic condition is common in fungi (3), although no reference to heterokaryotic forms in the genus *Claviceps* has previously been reported.

We determined that heterokaryosis is frequent in sclerotia of *C. purpurea*, but it is not common in cultures which produce conidia. Since, in the genus *Claviceps*, the production of large amounts of alkaloids is typical of the sclerotial phase, we suggest that alkaloid production is related to heterokaryosis of the producing strains.

**Acknowledgments**

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**Literature Cited**