Mutants with Increased Sensitivity to Canavanine in the Fungus *Coniochaeta velutina*

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Mutants of *Coniochaeta velutina* with increased sensitivity to canavanine were derived from wild-type, *pyr-3* (presumably defective in carbamoyl-phosphate synthase for pyrimidine synthesis), *arg* (blocked in steps prior to arginine formation) and *pro* (blocked in steps prior to glutamate semialdehyde formation) strains, and were classified into eight distinct groups by genetic mapping. Four canavanine-sensitive genes (*cns*) were allelic or very tightly linked to known *arg* loci. The pairs of genes involved were *cnsA* and *arg-3* (arginine requirer), *cnsB* and *arg-7* (citrulline requirer), *cnsC* and *arg-2* (citrulline requirer) and *cnsG* and *arg-1* (ornithine requirer). Another (*cnsF*) was mapped in linkage group III.

A relationship was shown between the level of intracellular arginine and the extent of canavanine sensitivity; *cns* strains contain less intracellular arginine than the less sensitive wild-type. In most cases, this was shown to result from mutations affecting the enzyme(s) involved in arginine metabolism. *cnsA* and *cnsG* were thought to be affected in arginine- and ornithine-synthesizing enzymes, respectively. *cnsB* and *cnsC* exhibited a requirement for citrulline when the two genes were combined and were suggested to be loci specifying the two polypeptide chains of carbamoyl-phosphate synthase for arginine synthesis. *cnsH* was the locus controlling ornithine carbamoyltransferase. The metabolic lesions of the other *cns* mutants (*cnsD*, *cnsE* and *cnsF*) were unclear.

Of the eight *cns* mutants, six could suppress *pyr-3M* and/or *pro* mutations: *cnsG* was able to suppress *pyr-3M*; *cnsB*, *cnsE*, *cnsD* and *cnsF* were effective in suppressing *pro*; *cnsH* suppressed both *pyr* and *pro*. The mechanism of the *pyr* and *pro* suppressions was explained on the basis of metabolic cross-feeding.

INTRODUCTION

The study of mutants in micro-organisms resistant or sensitive to amino acid analogues has led to a greater understanding of the control mechanism of metabolic pathways and the nature of analogue sensitivity (Moyed, 1964; Richmond, 1965). A well investigated system is the regulation of arginine biosynthesis in *Escherichia coli* (Maas, 1961) and *Saccharomyces cerevisiae* (Bechet *et al.*, 1970) elucidated by studies with canavanine-resistant mutants. In general, an overproduction of arginine due to loss of a control mechanism and an impairment of arginine uptake have been shown to account for their resistance to canavanine. The other mode of resistance found in micro-organisms such as *Pseudomonas* (Kalyankar *et al.*, 1958) and *Streptococcus* (Kihara & Snell, 1957) was attributed to a canavanine-detoxifying system which degrades canavanine to a non-toxic product.

In filamentous fungi, knowledge of canavanine resistance or sensitivity and its relation to the regulatory mechanism of the arginine pathway is limited to *Neurospora crassa* and *Aspergillus nidulans*. In *Neurospora*, resistance to canavanine was shown to be by a gene

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coding for the production of a canavanine-cleaving enzyme and at least three other genes modifying the uptake system for canavanine or arginine (Bauerle, 1963; Bauerle & Garner, 1964; Logan, 1969). In *Aspergillus*, certain proline suppressor mutants which were sensitive to canavanine were shown to be affected in enzymes related to arginine metabolism (Weglenski, 1967).

This study extends a similar type of investigation using the ascomycete fungus *Coniochaeta velutina*. Previously we have reported *cns* (canavanine-sensitive) strains that were revertants of an arginine mutant (Tan & Cooke, 1969). The *cns* mutant behaved like a wild-type but was sensitive to canavanine. We suggested that canavanine sensitivity may be governed by the level of endogenous arginine. Wild-type *Coniochaeta* appears to produce sufficient arginine to counteract the inhibitory effect of canavanine whereas the *cns* strains have reduced arginine production due to the mutation.

This paper describes the genetical location and growth characteristics of a large sample of *cns* mutants produced by various methods, their roles on the pyrimidine, arginine and proline pathways and the nature of canavanine sensitivity.

METHODS

Strains. The strains of *Coniochaeta velutina* used were the wild-type, strains auxotrophic for pyrimidine (*pyr*), arginine (*arg*) or proline (*pro*) and canavanine-sensitive (*cns*) strains. The induction and nutritional requirements of the auxotrophic mutants have been described earlier (Tan, 1968; Cooke *et al.*, 1969; Paterson, 1969; Paterson *et al.*, 1969). Canavanine-sensitive mutants were obtained from the wild-type strains following *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment (Paterson, 1969; Paterson *et al.*, 1969). In addition, *cns* strains were produced from *pyr-3*, *arg-2*, 3, 7 and *pro-1*, 2 mutants by u.v. irradiation (Tan, 1971). The treated conidia from these strains were plated on 25 to 30 ml of solid glucose minimal medium, GMA (Cooke *et al.*, 1969). After 5 to 6 d incubation at 25 °C, the fungal colonies were replica-plated on to GMA containing 1 µg canavanine ml⁻¹. Colonies which grew very poorly or not at all on canavanine medium after 5 to 6 d incubation were classified as *cns* mutants.

Media. GMA, with or without appropriate supplement (L-arginine, citrulline, ornithine, proline, lysine and uracil from Fisher Scientific Co. and L-canavanine from Sigma), was used for growth experiments and characterization of recombinants. The concentrations of these supplements were 1 µg ml⁻¹ unless stated otherwise. Liquid Vogel minimal medium, VM (Vogel, 1956), was used to culture mycelium for enzyme assays and amino acid determinations. The crossing medium was 1.2% (w/v) Difco malt extract (Cooke *et al.*, 1969).

Genetic analysis. This was done as described by Cooke *et al.* (1969). To detect linkage between *cns* mutations, or between *cns* and auxotrophic mutations, a rapid screening technique similar to that described by Markert (1952) was employed. If a prototroph frequency from a cross was significantly lower than 20%, linkage was suspected and a random ascospore analysis using the individual isolation method or the replica-plating method was done. As complementation tests in heterokaryons were not possible, a prototroph frequency lower than 0.1% was considered a close approximation of allelism (or very tight linkage).

Growth conditions. Growth experiments were done on 25 ml of appropriately supplemented GMA in Petri dishes. Approximately equal conidial inocula were transferred to a point on the test plates and incubated at 25 °C. Diameters of colonies were measured 5 to 7 d after inoculation.

For enzyme assays and amino acid determination, mycelium was grown in a 2.8 l flat-bottomed Erlenmeyer flask containing 1 l VM. Flasks were inoculated with a heavy conidial suspension in sterile distilled water and were incubated at 25 °C for 3 to 4 d on a rotary shaker (model G10, New Brunswick Scientific Co.) operating at 70 rev. min⁻¹.

Preparation of cell-free extracts. The mycelium was blotted dry and mixed with 5 vol. buffer (0.2 M-Tris/HCl buffer, pH 8, for ornithine carbamoyltransferase and arginase; 0.2 M-phosphate buffer, pH 8, for ornithine aminotransferase) containing 0.143 M-mercaptoethanol. The mixture was sonicated for 5 min (at 80 W, tone 0, using a Fisher Ultrasonic Generator; Fisher Scientific Co., Ottawa, Canada) and centrifuged at 1750 g for 20 min at 4 °C. The supernatant was used immediately for enzyme assays.

Enzyme assays. The assays for ornithine carbamoyltransferase [OCT; carbamoyl-phosphate:L-ornithine carbamoyltransferase; EC 2.1.3.3], arginase [L-arginine amidinohydrolase; EC 3.5.3.1] and ornithine aminotransferase [OAT; L-ornithine:2-oxo-acid aminotransferase; EC 2.6.1.13] were similar to those used by Weglenski (1967). For OCT assay, the incubation mixture contained 10 µmol L-ornithine, 100 µmol carbamoyl phosphate, 100 µmol Tris/HCl buffer (pH 8.5) and 0.15 ml enzyme extract, in a total volume of

0.55 ml. For arginase assay, the reaction mixture contained 10 μ mol L-arginine.HCl (pH 9.5), 1 μ mol $MnCl_2$, 20 μ mol Tris/HCl buffer (pH 9) and 0.3 ml enzyme extract, in a total volume of 0.75 ml. For OAT assay, the reaction mixture contained 10 μ mol ornithine.HCl, 10 μ mol 2-oxoglutarate (pH 7), 20 μ mol potassium phosphate buffer (pH 8.5) and 0.4 ml enzyme extract, in a total volume of 0.6 ml. The incubation periods for the OCT, arginase and OAT assays were 15 to 20, 90 and 40 to 60 min, respectively, at 30 °C. Reactions were stopped by adding 0.25 ml 10 % (w/v) trichloroacetic acid (TCA) for OCT and arginase assays and 0.3 ml TCA for OAT assays. The protein precipitate was removed by centrifugation and the supernatants from the OCT, arginase and OAT mixtures, respectively, were tested for citrulline, urea and glutamate semialdehyde (acetaldehyde was used as a standard instead of glutamate semialdehyde) (Knivett, 1954; Albrecht *et al.*, 1962).

Protein was measured by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as a standard.

Determination of free arginine in mycelium. One part of acetone-dried mycelium was mixed with six parts of distilled water and autoclaved at 121 °C for 1 h. After centrifuging at 7400 g for 30 min to remove the cell debris, samples (0.1 to 0.4 ml) of the supernatant were used for arginine determination by the Sakaguchi reaction (Van Pilsum *et al.*, 1957).

Estimation of arginine excreted into culture medium. A bioassay method, basically similar to that for *Neurospora*, was used to test for trace amounts of arginine excreted by the fungus into culture media (Doermamn, 1945). The test medium was filtered through a Millipore filter (0.45 μ m pore size) which excluded conidia from the filtrates. The filtrates were incorporated into solid VM and assayed for arginine using an *arg-3* strain. GMA plates containing known concentrations of arginine were used as controls.

RESULTS

*Genetic analysis of *cns* mutants*

Eight genetically distinct groups of *cns* mutants, designated as *cnsA*, *cnsB*, *cnsC*, *cnsD*, *cnsE*, *cnsF*, *cnsG* and *cnsH*, were identified by means of numerous pairwise crosses (Table 1). From more than 100 crosses made, there were 18 *cnsB* isolates, 15 *cnsC*, 14 *cnsG*, 4 *cnsH* and 2 *cnsA*; *cnsD*, *cnsE* and *cnsF* had only one isolate each.

The finding of *arg* revertants which were canavanine-sensitive prompted an investigation into the relationships between *cns* and *arg*. The eight distinct *cns* mutants were crossed with the nine known *arg* mutants. The pairs *cnsB/arg-7*, *cnsC/arg-2* and *cnsG/arg-1* were shown to be allelic or very tightly linked since an extremely low prototroph frequency (< 0.1 % among about 2000 offspring tested) was observed in each combination. The cross between *cnsA* and *arg-3* was infertile, but *cnsA* may be allelic to *arg-3* since it was produced by u.v. irradiation of an *arg-3* strain. *cnsD*, *cnsE*, *cnsF* and *cnsH* were neither allelic nor linked to the nine known *arg* mutants.

Mutants carrying *cnsC* and *cnsF* were mapped on two of the three known linkage groups. The other *cns* mutants did not show linkage with the markers of any known groups.

Like *arg-2*, *cnsC* is linked to *his-1* in linkage group II. From nine crosses involving *his-1* and different *cnsC* alleles, a total of 1980 canavanine-sensitive, 1991 histidine-requiring, 208 wild-type and 145 canavanine- and histidine-requiring offspring was obtained (8 % recombination). This is similar to the recombination frequency of *arg-2* with *his-1* (Cooke *et al.*, 1969).

The *cnsF* mutation was mapped in linkage group III which contains *pyr-3*, *pyr-2* and *pyr-1* loci (6.2 to 13.4 % recombination between *pyr-2* and *pyr-3*; 21.3 ± 0.5 % recombination between *pyr-3* and *pyr-1*; 23.4 ± 0.9 % recombination between *pyr-2* and *pyr-1*). When *cnsF* was crossed to three different alleles of *pyr-3*, recombination frequencies between 23.0 ± 4.1 % and 29.0 ± 3.7 % were obtained from a total of 755 offspring tested. When *cnsF* was crossed to *pyr-1* in the same linkage group, 189 recombinants were found among 391 offspring tested (48 % recombination), suggesting that *pyr-3* is closer to *cnsF* than to *pyr-1*. No data on recombination between *cnsF* and *pyr-2* are available. On the basis of these two-point results, the order could be either *cnsF pyr-2 pyr-3 pyr-1* or *cnsF pyr-3 pyr-2 pyr-1*.

Table 1. *Strains used for genetic analyses and subsequent investigations*

Strain	Isolation no.	Origin	Mutagen used†
Wild-type	o_1z , o_4z , H1*	—	—
<i>cnsA</i>	1	<i>arg-3</i> (z64)	u.v.
<i>cnsB</i>	1	Wild-type (o_1z)	NG
	2	Wild-type (o_1z)	NG
	3	<i>arg-7</i> (M29)	u.v.
	Others	<i>arg-7</i> (M102)	u.v.
<i>cnsC</i>	1	Wild-type (o_4z)	NG
	2	Wild-type (o_4z)	NG
	3	<i>arg-2</i> (M122)	u.v.
	Others	<i>arg-2</i> (z135)	u.v.
<i>cnsD</i>	1	<i>pro-1</i> (M317)	u.v.
<i>cnsE</i>	1	<i>pro-2</i> (M324)	u.v.
<i>cnsF</i>	1	Wild-type (o_1z)	NG
<i>cnsG</i>	1	Wild-type (o_4z)	NG
	2	<i>pyr-3</i> (M608)	u.v.
	Others	<i>pyr-3</i> (M608)	u.v.
<i>cnsH</i>	1	<i>pyr-3</i> (M608)	u.v.
	Others	<i>pyr-3</i> (M608)	u.v.

* H1 is a conidial isolate slightly more sensitive to canavanine inhibition than the original wild-type culture, o_1z .

† NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; u.v., ultraviolet light.

Fig. 1

Fig. 1. Effect of different concentrations of canavanine on the growth of the wild-type strain o_1z (Δ) and *cnsB1* ($\blacksquare$), *cnsB2* ($\circ$), *cnsC1* ($\square$) and *cnsF1* ($\blacktriangle$) mutants. Growth was expressed as the mean diameter of eight colonies measured 5 d after inoculation.

Fig. 2

Fig. 2. Reversal of canavanine ($1 \mu\text{g ml}^{-1}$) inhibition by different amounts of arginine for the wild-type strains o_1z (Δ) and H1 ($\blacktriangle$), and for the mutants *cnsC3* ($\bullet$) and *cnsG1* ($\circ$). Growth was expressed as the mean diameter of six colonies measured 7 d after inoculation.

Quantitative inhibition by canavanine and its reversal

The responses of certain *cns* and wild-type strains to different concentrations of canavanine are shown in Fig. 1. There was no inhibition at a concentration below $5 \times 10^{-3} \mu\text{g ml}^{-1}$, but *cns* strains were strongly inhibited by canavanine at concentrations greater than $5 \times 10^{-1} \mu\text{g ml}^{-1}$. In the presence of $1 \mu\text{g ml}^{-1}$ canavanine, the wild-type strains were inhibited by 8.2 %, while *cnsB1*, *cnsB2*, *cnsC1* and *cnsF1* mutants showed 45.2, 100, 73.3 and 100 % inhibition, respectively. Other *cns* mutants carrying different alleles of the same gene may also be inhibited to different degrees. At $10 \mu\text{g ml}^{-1}$ the wild-type strains were inhibited

Table 2. *Effects of amino acids and analogues on the growth of wild-type O₄Z and cnsB1, cnsC3 and cnsG1 mutants*

Addition(s) to minimal medium*	Inhibition of growth (%)†			
	Wild-type	<i>cnsB</i>	<i>cnsC</i>	<i>cnsG</i>
Canavanine	5.9	59.4	50.0	80.7
Ornithine	0	0	0	-25.8
Citrulline	0	-6.3	-4.7	-21.0
Arginine	-2.9	-9.1	-6.3	-29.0
Lysine	0	0.6	1.6	1.9
Canavanine+ornithine	5.9	54.8	50.8	35.5
Canavanine+citrulline	2.9	18.2	25.0	32.3
Canavanine+arginine	-2.9	3.0	0	0.6
(2)	-2.9	0	-9.4	-6.5
(3)	-2.9	0	-9.4	-16.1
Canavanine+lysine	5.9	50.7	34.4	54.8
(2)	5.9	32.4	25.8	25.8
(3)	2.9	18.2	22.6	22.6

* All supplements were added at 1 $\mu\text{g ml}^{-1}$, except (2) and (3) contained arginine or lysine at 2 and 3 $\mu\text{g ml}^{-1}$, respectively.

† Growth inhibition was estimated after 5 d incubation as $[100 \times (\text{Difference between colony diameters on minimal and test media})/(\text{Colony diameter in minimal medium})]$. Negative values indicate stimulation of growth.

by about 13 %, while all the *cns* mutants tested were completely inhibited. It appears that 1 $\mu\text{g canavanine ml}^{-1}$ is the minimum concentration for distinguishing *cns* mutants from the wild type.

The inhibitory effects of canavanine could be reversed by a number of amino acids (Table 2). Arginine was the most effective in reversing inhibition, followed by citrulline, lysine and ornithine in decreasing order, except that ornithine was more effective than lysine in counteracting canavanine inhibition of the *cnsG1* mutant. Both arginine and citrulline stimulated the growth of all strains tested, ornithine stimulated *cnsG1* and lysine inhibited *cns* strains slightly.

Figure 2 shows the amounts of arginine required to reverse the effect of canavanine on certain *cns* and wild-type strains. Arginine began to counteract canavanine inhibition at 1×10^{-5} $\mu\text{g ml}^{-1}$ for one of the wild-type strains and at 10^{-3} $\mu\text{g ml}^{-1}$ for *cnsC* and *cnsG* mutants. The reversal of inhibition of the other wild type was gradual. For complete reversal, approximately 5×10^{-3} to 1×10^{-1} $\mu\text{g arginine ml}^{-1}$ was required for the wild-type strains, and 1 $\mu\text{g arginine ml}^{-1}$ for the *cns* mutants. The amount of arginine required for complete reversal of canavanine inhibition therefore appears to be related to the degree of canavanine sensitivity.

The inhibition of *cns* strains also decreased with the passage of time. *cnsG*, an allele of an ornithine-requiring mutant *arg-1*, had a longer lag phase than the wild-type strains in both canavanine (3 $\mu\text{g ml}^{-1}$) medium and minimal medium. On minimal medium, its growth rate was 3.25 mm d⁻¹ but on canavanine medium it had an initial rate of 1 mm d⁻¹ and then increased to the normal rate after 7 d. The wild-type strain grew at about 3.5 mm d⁻¹ from the beginning on both types of medium.

Effects of cns mutations on OCT, OAT and arginase activities

Ornithine carbamoyltransferase (OCT), arginase and ornithine aminotransferase (OAT) were assayed in some *cns* and related strains (Table 3). Of the *cns* mutants examined, only *cnsH* failed to show detectable OCT activity (≤ 10 % wild-type OCT). The other mutants

Table 3. *Specific activities of OCT, arginase and OAT in wild-type, pro, pyr, cns and suppressed strains*

Genotype of strain	Specific activities*		
	OCT	Arginase	OAT
Wild-type o ₁ z	3.1	0.015–0.223	0.045–0.261
Wild-type o ₄ z	5.3	0.021–0.232	0.115–0.271
<i>pro-1</i> †	4.2	0.170	0.275
<i>pro-2</i> †	6.3	0.022–0.150 (0.129–0.329)‡	0.142 (0.275)‡
<i>pyr-3M</i>	3.4	—	—
<i>cnsB1</i>	3.2	0.027	0.056–0.230
<i>cnsB1 pro-1</i>	3.0	0.039	0.046
<i>cnsD1</i>	4.3	0.006	0.055–0.189
<i>cnsD1 pro-1</i>	4.1	0.087	0.150–0.255
<i>cnsE1</i>	3.5	0.051	0.120
<i>cnsE1 pro-2</i>	7.5	0.025–0.284	0.265
<i>cnsF1</i>	3.8	0.003	0.025
<i>cnsF1 pro-1</i>	—	—	—
<i>cnsG1</i>	4.6	—	—
<i>cnsG1 pyr-3M</i>	4.	—	—
<i>cnsH1</i>	ND	—	—
<i>cnsH1 pro-1</i>	ND	—	—
<i>cnsH1 pyr-3M</i>	ND	—	—

ND, Not detectable; —, not tested.

* Activities are expressed as $\mu\text{mol product formed h}^{-1} (\text{mg protein})^{-1}$ based on the average or range for at least two experiments.

† *pro-1* and *pro-2* were cultured in 1 l Vogel minimal medium supplemented with 5 mg proline or arginine.

‡ Activities of strains grown in arginine supplemented medium.

showed OCT specific activities which were not significantly lower than in the wild-type strains.

The results of arginase and OAT assays were variable for the strains tested, especially for wild-type and *pro-2* in arginine-free medium. However, the range of activities could be used as a basis for comparison. The specific activities of these enzymes in *cnsB1*, *cnsD1*, *cnsE1* and *cnsF1* mutants were slightly lower than or similar to those in wild-type. The *pro* mutants showed higher activities of arginase and OAT in arginine medium than in proline medium.

Effects of cns on other cns, arg, pro and pyr mutations

Effects of cns double mutations. Certain *cns* double mutants showed a requirement for arginine. This is illustrated in a number of crosses between *cnsB* and *cnsC*, and between *cnsB* and *cnsE* (Table 4). In crosses between these *cns* mutants (e.g. *cnsB* and *cnsC*), four classes of genotypes [*cnsB*, *cnsC* (parental groups), wild-type and *cnsB cnsC* (recombinants)] would be expected with equal frequency assuming no linkage between the two genes involved. If the double mutants *cnsB cnsC* were arginine-requiring, instead of canavanine sensitive like the *cns* single mutants, a ratio of 2:1:1 for canavanine-sensitive, wild-type and arginine-requiring offspring would be obtained. However, this ratio often varied because of different degrees of viability of the mutant spores, particularly those exhibiting the arginine-requiring phenotype. To confirm that the arginine-requiring offspring were *cns* double mutants, the arginine-requiring strains were back-crossed to the wild-type. These crosses produced not only arginine-requiring and wild-type offspring but also canavanine-sensitive offspring, indicating that the arginine-requiring offspring were indeed *cns* double mutants. In crosses between other *cns* mutants, no arginine-requiring offspring were found among at

Table 4. Production of arginine-requiring offspring in crosses of *cns* mutants

Cross*	No. of offspring			Total
	Canavanine-sensitive	Wild-type	Arginine-requiring	
<i>cnsB</i> × <i>cnsC</i> (11 replicate crosses)	1740	985	325	3050
+ + × <i>cnsB4 cnsC</i> (2 replicate crosses)	395	258	104	757
<i>cnsB</i> × <i>cnsE</i> (2 replicate crosses)	134	77	26	237
+ + × <i>cnsB cnsE</i> (2 replicate crosses)	133	82	23	238

Expected ratio 2:1:1

* Each replicate cross involved *cns* strains carrying different alleles of the same gene.

least 300 analysed in each cross. Like *arg-2* (allele of *cnsC*) and *arg-7* (allele of *cnsB*), the arginine-requiring *cns* double mutants involving *cnsB*, *cnsC* and *cnsE* grew well on media supplemented with citrulline or arginine at 1 µg ml⁻¹. The OCT activity of the double mutants was normal.

Effects of cns on arg mutations. Strains carrying *arg-1* and *arg-4* responded to ornithine, citrulline or arginine. When *cnsH* was associated with *arg-1* or *arg-4*, however, the double mutant only responded to citrulline or arginine, suggesting that *cnsH* prevents the utilization of exogenous ornithine. At a high concentration of ornithine (40 mg ml⁻¹) the double mutants grew poorly, indicating that a very limited amount of exogenously supplied ornithine could be used in these strains. An alternative explanation for the poor growth on this high concentration of ornithine is that the ornithine may be contaminated with traces of arginine or citrulline.

Effects of cns on pro mutations. Certain *cns* mutants were able to suppress the nutritional requirements of *pro* mutants when combined with them. Of the *cns* tested, *cnsB*, *cnsD*, *cnsE*, *cnsF* and *cnsH* belonged to this group (Table 5). When *cns* and *pro* were crossed, four genotypes [*cns*, *pro*, *cns pro* and wild-type] were expected with equal frequency assuming no linkage between the two genes involved. If there were no suppression, the double mutant *pro cns* should have a phenotype similar to the *pro* single mutant, which was also canavanine-sensitive. If suppression occurs, the double mutant should be indistinguishable phenotypically from *cns*. Thus, if one assumes no linkage or inviability, the ratio of canavanine-sensitive to wild-type to proline-requiring offspring would be 1:1:2 without suppression but 2:1:1 with suppression. Because of different degrees of viability in the mutant spores, the segregation of the expected phenotypes did not strictly follow in a 2:1:1 ratio. However, the segregation ratios in most crosses, and the appearance of proline-requiring offspring from back-crosses between presumed double mutants and the wild-type, support the view that certain *cns* mutations did suppress the nutritional requirements of the *pro* mutants.

Some of the *cns* and their *pro*-suppressed strains grew well in the presence of citrulline or arginine, but grew slowly in the absence of these supplements.

Effects of cns on pyr mutations. Strains carrying *cnsG* or *cnsH* in combination with certain *pyr-3* alleles (designated *pyr-3M*) were pyrimidine-independent and sensitive to both canavanine and arginine (Table 6). The double mutant *cnsG pyr-3M* was also sensitive to ornithine and, to a greater extent, citrulline and arginine. This inhibitory effect could be overcome by an exogenous supply of uracil. The double mutant *cnsH pyr-3M* was not sensitive to ornithine (Fig. 3).

Double mutants carrying other combinations of *cns* with *pyr-1*, *pyr-2* or *pyr-3* were all pyrimidine-requiring and canavanine-sensitive, indicating that other *cns* mutants were not

Table 5. *Suppression of pro mutants by cns mutants*

Cross	No. of offspring			Total
	Canavanine-sensitive	Wild-type	Proline-requiring	
<i>cnsB1</i> × <i>pro-1</i>	226	120	103	449
<i>cnsB1</i> × <i>pro-2</i>	261	150	60	471
<i>cnsD1</i> × <i>pro-1</i>	116	69	65	250
<i>cnsD1</i> × <i>pro-2</i>	47	35	22	104
<i>cnsE1</i> × <i>pro-1</i>	241	169	110	520
<i>cnsE1</i> × <i>pro-2</i>	175	97	30	302
<i>cnsF1</i> × <i>pro-1</i>	150	81	60	291
<i>cnsF1</i> × <i>pro-2</i>	172	87	43	302
<i>cnsH1</i> × <i>pro-1</i>	122	74	54	250
<i>cnsH1</i> × <i>pro-2</i>	83	46	31	160
+ + × <i>cnsB1 pro-1</i>	208	106	95	409
+ + × <i>cnsD1 pro-1</i>	149	96	85	330
+ + × <i>cnsE1 pro-2</i>	101	48	25	174
+ + × <i>cnsF1 pro-1</i>	113	89	47	249
+ + × <i>cnsH1 pro-2</i>	86	54	33	173

Expected ratio 2:1:1

Table 6. *Suppression of pyr-3M by cns mutants*

Cross*	No. of offspring				Total
	<i>cns</i>	<i>pyr</i>	+ +	<i>cns pyr</i> †	
<i>cnsG</i> × <i>pyr-3M</i> (2 replicate crosses)	116	106	239	131	592
+ + × <i>cnsG pyr-3M</i> † (3 replicate crosses)	94	83	91	79	347
<i>cnsH</i> × <i>pyr-3M</i> (2 replicate crosses)	44	26	67	23	160
+ + × <i>cnsH pyr-3M</i> † (2 replicate crosses)	48	25	66	21	160

* Each replicate cross involved *cns* strains carrying different alleles of the same gene.

† Pyrimidine-independent and sensitive to canavanine or arginine.

Fig. 3. Effect of different concentrations of ornithine (O), citrulline (Δ) and arginine (●) on the growth of (a) *cnsH1 pyr-3M* and (b) *cnsG1 pyr-3M* mutants. Growth was expressed as the mean diameter of four colonies measured 6 d after inoculation.

Table 7. Free arginine contents of mycelia of wild-type and *cns* strains with different degrees of canavanine inhibition

Genotype of strain	Free arginine [mg (g dry mycelium) ⁻¹]		Inhibition by 2 µg canavanine ml ⁻¹ (%)
	3 d-old	4 d-old	
Wild-type o ₁ z	8.6	5.5	2.9
Wild-type o ₄ z	6.0	5.3	8.8
<i>cnsB3</i>	3.4	2.3	95.5
<i>cnsC1</i>	2.2	1.0	93.5
<i>cnsD1</i>	3.9	3.9	94.0
<i>cnsE1</i>	—	2.8	80.0
<i>cnsG1</i>	—	2.6	88.0
<i>cnsH1</i>	2.5	2.2	100.0

—, Not tested.

able to nullify the effect of the *pyr* mutations. Furthermore, *cnsG* and *cnsH* were unable to act as suppressors for some *pyr-3* alleles, designated as *pyr-3MN* or *pyr-3N*. This locus is thought to code for a bifunctional enzyme.

Effect of *cns* mutations on content of free arginine

The free arginine contents of 3 and 4 d-old mycelia of two wild-type (o₁z and o₄z) and six *cns* strains grown in arginine-free medium are summarized in Table 7. The wild-type strains were only inhibited by 2.9 to 8.8 % in medium supplemented with 2 µg canavanine ml⁻¹, whereas the mutants were at least 80 % inhibited. The free arginine content in the wild-type strains [5.3 to 8.6 mg (g dry mycelium)⁻¹] was much higher than in the *cns* strains tested. Mycelia from *cnsB3*, *cnsC1*, *cnsD1*, *cnsE1*, *cnsG1* and *cnsH1* strains contained 39.5 to 56.7 %, 18.2 to 36.7 %, 45.4 to 73.6 %, 50.9 to 52.8 %, 47.3 to 49.1 % and 29.1 to 41.5 %, respectively, of the wild-type amount of arginine.

Attempts to detect arginine by the Sakaguchi reaction in media in which *Coniochaeta* strains had grown were unsuccessful. A more sensitive bioassay method was therefore used to estimate arginine. The arginine tester strain, *arg-3*, grew slowly on the culture medium of the wild-type strain and the amount of arginine excreted into the medium was calculated to be about 6 µg (g dry mycelium)⁻¹. No growth occurred in medium in which *cns* mutants had been grown showing that no arginine could be detected by this method.

DISCUSSION

Nature of canavanine sensitivity. In *Coniochaeta*, canavanine sensitivity has been shown to result from the impairment of arginine synthesis. Of the eight distinct groups of *cns* mutants, five were presumably blocked in steps leading to arginine biosynthesis (see Fig. 4 and Table 8). The others were also shown to be affected in arginine metabolism. All the *cns* mutants tested had less free arginine in their mycelia and in their culture media than the wild-type strains. In addition, certain groups of *cns* double mutants (*cnsB cnsC* and *cnsB cnsE*) were citrulline-requiring, whereas the original *cns* single mutants were citrulline-independent. Certain slow-growing *cns* and *pro*-suppressed strains required arginine for good growth. These findings are consistent with the hypothesis that the level of intracellular arginine determines the sensitivity or resistance to canavanine in *Coniochaeta*.

Although the cause of a difference in intracellular arginine levels may vary, canavanine sensitivity of some micro-organisms can be explained by the above hypothesis. Weglenski (1967) has reported that wild-type *Aspergillus*, which possessed more intracellular arginine

Table 8. *Summary of genetic locations, proposed enzymic lesions, suppression or interaction effects of cns loci*

Locus	Presumed to be allelic with	Suppression of	Proposed enzymic lesions	Comments
<i>cnsA</i>	<i>arg-3</i>	—	Arginine-synthesizing enzyme(s)	<i>arg-3</i> is an arginine requirer
<i>cnsB</i>	<i>arg-7</i>	<i>pro-1, 2</i>	Carbamoyl phosphate synthase – arginine synthesis	<i>arg-7</i> is a citrulline requirer; <i>cnsB cnsC</i> and <i>cnsB cnsE</i> require citrulline
<i>cnsC</i>	<i>arg-2</i>	—	Carbamoyl phosphate synthase – arginine synthesis	<i>arg-2</i> is a citrulline requirer; mapped on linkage group II; <i>cnsB cnsC</i> requires citrulline
<i>cnsD</i>	—	<i>pro-1, 2</i>	—	—
<i>cnsE</i>	—	<i>pro-1, 2</i>	—	<i>cnsB cnsE</i> requires citrulline
<i>cnsF</i>	—	<i>pro-1, 2</i>	—	Mapped on linkage group III
<i>cnsG</i>	<i>arg-1</i>	<i>pyr-3M</i>	Ornithine-synthesizing enzyme(s)	<i>arg-1</i> is an ornithine requirer; <i>cnsG pyr-3M</i> is sensitive to ornithine, citrulline and arginine; these sensitivities could be overcome by uracil
<i>cnsH</i>	—	<i>pro-1, 2</i> and <i>pyr-3M</i>	Ornithine carbamoyl-transferase	<i>cnsH pyr-3M</i> is sensitive to citrulline and arginine; these sensitivities could be overcome by uracil; <i>cnsH arg-1</i> and <i>cnsH arg-4</i> could not respond to ornithine, in contrast to <i>arg-1</i> and <i>arg-4</i>

than a *pro* suppressor strain, was less sensitive to canavanine. Schwartz & Maas (1960) reported that canavanine inhibited the growth of *E. coli* almost completely in the presence of low intracellular arginine while normal growth was observed in the presence of high intracellular arginine. Strains of *E. coli* (Maas, 1961) and *Saccharomyces* (Bechet *et al.*, 1970) which produce excess arginine through a loss of regulation of arginine synthesis are more resistant to canavanine than the wild-type. The hypothesis, however, has not been used to explain canavanine sensitivity or resistance in *Neurospora* (Bauerle, 1963; Logan, 1969).

In view of the evidence presented for *cns* mutations in *Coniochaeta*, we can rule out the following mechanisms as possible explanations for the genetic defects: (i) an alteration of uptake system(s); (ii) an alteration of RNA synthetase or related protein-synthesizing machinery; (iii) a loss of control of arginine biosynthesis in response to canavanine or arginine; and (iv) a canavanine-detoxifying system. However, some of these mechanisms may explain other *cns* mutants which have not yet been isolated in this fungus.

Pyrimidine, arginine and proline pathways. The effects of *cns* mutations on the pyrimidine and proline mutants revealed similarities in the pyrimidine, arginine and proline pathways between *Coniochaeta* and *Neurospora*. In the pyrimidine pathway, a complex locus, *pyr-3*, presumably specifying the bifunctional protein carbamoyl-phosphate synthase (CPS-P) and aspartate carbamoyltransferase (ACT), was detected. *cnsH* was able to distinguish the suppressible *pyr-3M* (presumably CPS-P defective), the non-suppressible *pyr-3N* (presumably ACT defective) and *pyr-3MN* (presumably defective in CPS-P and ACT). These *pyr* mutants, however, mapped at a single locus known as *pyr-3*. A similar locus has also been described in *Neurospora* (Suyama *et al.*, 1959; Davis & Woodward, 1962; Woodward & Davis, 1963). In the arginine pathway, *cns* mutants such as *cnsB*, *cnsC* and *cnsH* have helped to clarify the metabolic positions of *arg-2* and *arg-7*. These two *arg* mutations cause blocks prior to citrulline synthesis in the pathway. It has now been confirmed that they appear to be loci specifying carbamoyl-phosphate synthase (CPS-A) in the arginine pathway because both these citrulline-requiring mutants are distinct from *cnsH* (OCT defective strain)

Fig. 4. Proposed metabolic relationships between the pyrimidine, arginine and proline pathways of *Coniochaeta*. Genes shown in parentheses are allelic or very tightly linked to those shown directly above them. Enzymes: CPS-A, carbamoyl-phosphate synthase – arginine synthesis; CPS-P, carbamoyl-phosphate synthase – pyrimidine synthesis; ACT, aspartate carbamoyltransferase; OAT, ornithine aminotransferase; OCT, ornithine carbamoyltransferase. * and ** indicate carbamoyl phosphate for arginine synthesis and pyrimidine synthesis, respectively.

in their genetic locations and OCT activity. Like *Neurospora* (Davis, 1967, 1972) the two *arg* loci were considered to produce two polypeptide subunits which in turn constituted CPS-A. This is also supported by the fact that *cnsB* and *cnsC* (alleles of *arg-7* and *arg-2*, respectively) when combined as the double mutants are phenotypically citrulline-requiring and exhibit normal OCT activity. If the products of *cnsB* and *cnsC* represent partially defective polypeptides coded by *arg-7* and *arg-2*, respectively, it is conceivable that a non-functional distorted enzyme would be produced in the double mutants *cnsBcnsC*.

pro strains exhibited higher activity of arginase and OAT in the presence of arginine than in the proline media. This probably reflects the fact that both arginase and OAT are inducible enzymes as they are in *Neurospora* (Davis *et al.*, 1970) and *Aspergillus* (Weglenski, 1967).

The auxotrophic behaviour of *pyr-3M*, *arg-2* and *arg-7* suggests that there are two separate carbamoyl phosphate (CAP) pools, one specific for the pyrimidine pathway (CAP_{pyr}) and one specific for the arginine pathway (CAP_{arg}). The two CAP pools are not easily accessible to the alternative pathways unless mutations which bring about 'dechannelling' occur. This phenomenon has been observed in *Neurospora* (Davis, 1967; Reissig *et al.*, 1967) but it differs from that of *Saccharomyces* in which the CAP_{arg} and CAP_{pyr}, although governed by different enzymes, are accessible to both pathways (Lacroute *et al.*, 1965).

The effect of *cnsH* on the phenotype of ornithine requirers parallels a similar situation observed by Davis (1968) in *Neurospora*. He postulated that there was a competitive effect of OCT and OAT for a common substrate ornithine and differential utilization of endogenous and exogenous ornithine. In wild-type *Neurospora*, OCT has higher affinity for endogenous ornithine than OAT; thus the endogenous ornithine is used mainly for the OCT reaction and is practically unavailable for the OAT reaction. In contrast, exogenous ornithine is used mainly for OAT reactions. A similar situation may exist in *Coniochaeta*.

From the above findings and some earlier observations (Tan, 1968; Paterson *et al.*, 1969) a proposed metabolic relationship between the pyrimidine, arginine and proline pathways, with relevant genes and enzymes, has been formulated for *Coniochaeta* (Fig. 4).

Mode of pyr and pro suppression. In *Coniochaeta*, *cnsG* and *cnsH* are two distinct groups

of *pyr-3M* suppressors. *cnsG* was affected in ornithine synthesis while *cnsH* was affected in OCT reaction. The former could suppress *pyr-3M* mutants while the latter could suppress both the *pyr-3M* and *pro-1* or *pro-2* mutants. Suppressors with the above metabolic defects have been described in *Neurospora* (Mitchell & Mitchell, 1952; Davis, 1962; Wieland & McDougall, 1969) and *Coprinus* (Cabet *et al.*, 1965). The *cnsG* mutation is believed to result in a reduction of ornithine which limits citrulline formation. This would lead to an accumulation of CAParg, which, in excess, could be used by ACT for pyrimidine synthesis. Addition of ornithine, citrulline or arginine would be expected to prevent this accumulation thus causing the observed reduction in growth. The *cnsH* mutation would cause an accumulation of CAParg and ornithine due to a reduction of OCT reaction. This excess CAParg could be used in the pyrimidine pathway while ornithine could be used for proline synthesis. Addition of arginine or citrulline would reduce ornithine synthesis but the addition of ornithine would not cause inhibition as observed.

cnsB, *cnsD*, *cnsE* and *cnsF* are new *pro* suppressors of *Coniochaeta* which, to our knowledge, have not been reported in other filamentous fungi. *cnsB*, presumably being affected in CPS-A, would produce less CAParg which would limit the OCT reaction. As a result, endogenous ornithine would accumulate and become available for proline synthesis via OAT. On the other hand, *cnsD*, *cnsE* and *cnsF* may be regarded as mutations affecting the regulation of certain enzymes or the property of enzyme compartment in the arginine pathway. These types of mutations would also allow the endogenous ornithine to be channelled to the proline pathway. Further study on these *cns* mutants may provide new information on the regulation of arginine biosynthesis in *Coniochaeta* and possibly filamentous fungi as a whole.

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