VARIABILITY AND NUTRITION OF *CLAVICEPS PURPUREA* IN CULTURE

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Supachai Ratanopas

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FOREWORD

The format adopted for this thesis deviates from the conventional in that the materials, methods and results are presented in the form of three publications, the formats of which comply with the requirements of the Canadian Journal of Botany. A general discussion follows, and the thesis terminates with a bibliography and appendices. This format has been approved by the Council of the Faculty of Graduate Studies of the University of Manitoba.

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GENERAL ABSTRACT

Ratanopas, Supachai. Ph.D., The University of Manitoba, October, 1976. Variability and Nutrition of Claviceps purpurea in Culture. Major Professor: Claude C. Bernier.

Cultural characteristics of 143 isolates of Claviceps purpurea (Fr.) Tul. from various hosts in the Canadian Prairies were assessed and attempts were made to determine relationships between cultural characters and host origin and virulence of the isolates. Cultural characteristics varied considerably and were classified into 10 groups on the basis of type of growth (raised or flat), surface characteristics (smooth, folded or wrinkled) and color of the colony (white or colored). None of the cultural characteristics appeared to be related to host origin of the isolates or to the virulence of 49 selected isolates. Small, globose conidial masses were observed in cultures of a few isolates and more prominent conidial horns were observed inconsistently in cultures of one isolate. Sectoring occurred with equal frequency in cultures derived from monoascospores as well as from sclerotia indicating that it is probably due to mutation. Variability was observed among 20 monoascospore isolates from a single sclerotium suggesting that variability might be due to recombination of genetically different

nuclei during the sexual stage as well as to mutation.

The development of conidial horns by isolate R-37C of C. purpurea as well as factors affecting their formation were investigated further. On media containing asparagine and a high concentration of sucrose (T2 medium) cultures of this isolate developed either conidial horns, small globose conidial masses or a thick plectenchymatic mycelial mat. Globose conidial masses and conidial horns appeared to originate from microscopic conidial masses. Twenty-day-old horns disintegrated readily in water whereas older horns did not. In mature horns the conidia lost their oval shape and became rounded and compacted.

Extracts prepared by suspending young conidial horns in water for 15 minutes contained fructose, glucose, sucrose and one unidentified sugar suggesting that sugars might be involved in the adhesion of conidia in the horns. However, no conclusions could be made regarding the similarity of the sugars in conidial horn extracts and honeydew.

Conidial structures occurred more consistently and abundantly on media adjusted to pH 7 and prepared with either tap water or deionized water containing mineral salts similar to those in tap water. Thick plectenchymatic mycelial mats appeared to resemble tissue of ergot sclerotia. Small, black, hemispherical structures were occasionally observed among conidial horns and were

also composed of plectenchymatic tissue.

In a third study, the influence of selected amino acids on growth and conidial production of C. purpurea was investigated. L-asparagine, L-glutamine and to a lesser extent L-proline were good sources of nitrogen for mycelial growth of five ergot isolates but supported appreciable conidial production in two isolates only (F-2C and PM-2C). All five isolates grew well on L-tryptophan but none of them produced conidia. DL-methionine, DL-valine, DL-alanine and to a lesser extent DL-isoleucine were poor sources of nitrogen for both growth and sporulation of all isolates. Mycelial growth of all the isolates on asparagine was good even at 50 ppm nitrogen but two isolates only (F-2C and PM-2C) produced conidia at levels of nitrogen lower than 500 ppm. β-Alanine and D-alanine were relatively poor sources of nitrogen for growth and sporulation of all five isolates whereas L-alanine was about as good as L-asparagine. In the presence of high levels of L-asparagine, sporulation of several isolates was enhanced by β-alanine and DL-alanine and reduced by D-alanine. At low levels of L-asparagine, β- and D-alanine were highly inhibitory to both growth and sporulation of isolate F-2C and PM-2C. The amino acid analog DL-p-fluorophenylalanine (FPA) was inhibitory to both growth and sporulation of isolates F-2C and PM-2C. The degree of inhibition depended on the concentration of L-asparagine and FPA in the medium.

GENERAL INTRODUCTION

Claviceps purpurea (Fr.) Tul., a fungus in the class Ascomycetes, attacks ovaries of various cereals and grasses. These include rye, wheat, barley, oats and many wild and cultivated grasses. The disease is recognized by honeydew exudate, and by black, hard sclerotia produced on the heads of infected hosts instead of kernels.

On susceptible hosts, sclerotia are usually larger than the kernels and honeydew is produced abundantly. However, smaller sclerotia (kernel size) and less honeydew are produced by the fungus on cultivars of spring wheat than on cultivars of rye or triticale (Platford and Bernier 1976). Furthermore, the production of very small sclerotia (smaller than the size of the kernel) and small amounts of honeydew in two wheat cultivars was considered as an expression of host resistance (Platford and Bernier 1970; 1976). Ergot isolates were found to produce small amounts of honeydew and small sclerotia (smaller than the size of the kernel) on both susceptible and resistant wheat cultivars, and were considered to be low in virulence (Ratanopas 1973).

Amino acid content of plants has been related to their susceptibility and resistance to diseases in several studies (Kuc et al. 1959; Ross 1968; Strech and Cappellini 1965; van Andel 1966). The recent finding that alanine occurs in high concentration in developing rye, triticale and wheat seeds (Corbett et al. 1974; Dexter and Dronzek

1975) coupled with the fact that honeydew production is reduced when florets are infected at or after anthesis (Ratanopas 1973) suggests that this amino acid might have an influence on sporulation. L-asparagine and L-glutamine were found to be of equal value as sources of nitrogen for growth of C. purpurea (Taber and Vining 1957) and L-asparagine has been widely used (Amici et al. 1967 a; Gjerstad and Ramstad 1955; Grein 1967; Johansson 1964a; Kirchhoff 1929; Mantle and Tonolo 1968; Strnadová and Kybal 1974). However, the utilization of glutamine for growth and sporulation bears reexamination since it has been reported to predominate in cereal seeds (MacMaster et al. 1971).

Since reduced sporulation is an expression of resistance in the host as well as of low virulence in the pathogen it appeared that knowledge of nutritional requirements for growth and sporulation in culture might lead to a better understanding of the phenomenon of reduced sporulation on the host.

Among 59 ergot isolates tested for their pathogenicity on four wheat cultivars, Ratanopas (1973) found that a large number of isolates were less virulent on two cultivars known to be resistant, whereas many of them showed high virulence on the other two cultivars known to be susceptible (Platford and Bernier 1970). Variability in cultures of C. purpurea, collected in Europe, has been reported to occur on a sucrose-asparagine medium (Amici

et al. 1967a; Grein 1967; Strnadová and Kybal 1974). However, no attempts have been made to establish cultural types or to determine relationships of cultural characters to host origin or virulence of isolates.

The objectives of these studies were to investigate: 1) variability of ergot isolates in culture and the relationship of cultural characters to host origin and virulence of the isolates; and 2) the effect of selected amino acids, with emphasis on alanine isomers, on growth and sporulation of ergot isolates.

LITERATURE REVIEW

Claviceps purpurea (Fr.) Tul. is a fungus pathogenic to the inflorescences of various cereals and grasses. The fungus possesses a sexual and an asexual stage in its life cycle. These stages have been described by several authors including Barger (1931), Bové (1970), Butler and Jones (1961), Dickson (1947), Heald (1937), Walker (1969), and are summarized as follows: The sexual stage is recognized as a stroma, consisting of a slender, reddish, pale-violet or whitish stalk or stipe surmounted by a reddish, flesh or pale-fawn-colored globular head referred to as the perithecial receptacle or sphaeridium. One to several stromata are produced by the germinating sclerotium after cold treatment. The flask-shaped perithecia are embedded within the head with ostioles protruding to the upper surface of the head. Asci produced in the perithecia are long, curved, hyaline, narrow at both ends and surrounded by paraphyses. Each ascus contains eight slender, filiform, slightly curved, hyaline, and continuous or septate ascospores. The asexual stage is recognized by the formation of honeydew containing conidia and the production of sclerotia on the infected florets.

The following review deals primarily with the asexual stage of the fungus, its nutritional requirements

and its variability.

Honeydew and Conidia

(A) Formation of Honeydew Conidia

Barnett (1972) described the imperfect stage of C. purpurea as a sporodochium consisting of a compact layer of conidiophores bearing conidia terminally; conidia are hyaline, small, ovoid, 1-celled, and produced in a sugary honeydew.

The meaning of the term "sporodochium" is not always clear. It is defined by Ainsworth (1967) as a mass of conidiophores tightly placed together on a stroma or mass of hyphae. On the other hand, Alexopoulos (1962) defines sporodochium as a cushion-shaped stroma covered with conidiophores, a definition which resembles closely that of Ainsworth for an acervulus. According to Barron (1968), if the fructification is flat or saucer-shaped, it is referred to as an acervulus; if cushion-shaped, it is a sporodochium.

The amount of honeydew (as well as the size of the sclerotia) is influenced by the fungus as well as by the host. Smaller sclerotia (kernel size) and less honeydew are produced by the fungus on susceptible cultivars of spring wheat than on cultivars of rye or triticale (Platford and Bernier 1976). Furthermore, several host-isolate combinations within spring and durum wheat were found not to produce honeydew and the isolates were considered to be

low in virulence (Ratanopas 1973).

(B) Chemical Composition of Honeydew

Honeydew of C. purpurea contains large amounts of sugar. According to Kirchhoff (1929) undiluted honeydew contains 2.33 molar sugar solution. The osmotic value of the honeydew has an effect on the size of the conidia. Comparative measurements on identical conidial material in undiluted and diluted honeydew showed spore dimensions of 3.5 to 6 by 2.5 to 3 μ and 6.5 to 7.5 by 4.2 to 4.8 μ respectively (Kirchhoff 1929).

Dry honeydew extract was chromatographically analyzed for its composition by Fuchs and Pöhm (1953). They found seven sugars, namely, glucose, fructose, sucrose, and four unidentified ones. Among the three known sugars, glucose and fructose were present in large amounts, whereas the amount of sucrose was small. The presence of fructose, glucose, and sucrose in the sphacelial stage was confirmed by the findings of Mantle (1965).

In addition to sugars, honeydew was found to contain numerous amino acids which were characterized as asparagine, glutamic acid, glycine, alanine, tyrosine, valine, proline, leucine, and phenylalanine (Fuchs and Pöhm 1953). They acknowledged that honeydew was the product of the infected host. Using chromatographic methods, Gröger (1958) discovered 19 amino acids in the honeydew of four strains of

C. purpurea from rye. The relative amount of each amino acids analysed for each strain showed no appreciable difference. Amino acids present in large amounts were valine, glutamic acid, leucine, alanine, γ-aminobutyric acid. In moderate amounts were serine, glycine, glutamine, threonine, lysine, aspartic acid, and proline. Histidine, arginine, tyrosine, asparagine, phenylalanine, pipecolic acid, and tryptophan were in small amount.

(C) Chemical Composition of Saprophytic Culture

C. purpurea is also capable of producing a variety of amino acids in pure culture. Kirsten et al. (1966) chromatographically analysed the mycelial extracts of C. purpurea, growing in a liquid medium, as well as the culture filtrate, for free amino acids. They found 28 ninhydrin positive substances among which alanine and lysine were present in large amounts. There was no marked difference in composition between free amino acids of the mycelium and the culture fluid.

Grein (1967) observed that conidia of C. purpurea produced on a sucrose-asparagine agar medium, designated as T2 medium (Amici et al. 1967a), tended to stick together, within a liquid drop, and to form globose bodies. The globose bodies were tiny and completely formed after an incubation period of about 15-20 days. By paper chromatographic analysis, Grein (1967) found that mycelium from cultures rich in such globose bodies contained glucose, fructose maltose and

mannitol. The presence of these sugars depended on the carbon sources used in the medium. Glucose was always found in the medium containing either sucrose, maltose or mannitol as a carbon source. Working with two strains of C. purpurea and employing a variety of nutrient media, Vining and Taber (1964) found that the cultures of these two strains always stored trehalose and mannitol in the mycelium. They reported that the accumulation of other carbohydrates depended upon the medium in which the fungus was grown and the strain used.

(D) Mechanism of Honeydew Formation

Mower (1970) proposed a possible mechanism for honeydew production and carbohydrate assimilation by Claviceps spp. infecting sedge and grass flowers. Each species produces a distinct group of sugars and corresponding well-bound β-D-fructosidases and transfructosidases, which effect conversion of sucrose to mono-, di-, and oligosaccharides. Glucose and fructose are found in varying concentration in honeydew during pathogenesis of all species tested except C. gigantea n. sp. which is parasitic on maize.

Mower and Hancock (1973) further demonstrated, by chromatographic and autoradiographic analyses of the infected floral structure, that sucrose is present in the phloem of the rachilla up to the host-parasite interface region. Honeydew in the sphacelial region contains high concentrations of transfer and cleavage products of sucrose.

They also found that the osmotic pressure is 25 fold greater in the honeydew region than in adjacent host tissue. They stated that host sucrose is converted into fungal sugars which may serve not only as nutrients for the sphacelium but as a basis for the movement of carbohydrate from host to parasite. Sucrose is drawn from the host by two processes: 1.) fungal sucrases cause a sucrose deficit in the region of the thallus which allows a net diffusion of sucrose from adjacent host tissues, and 2.) a large number of sugar species derived from sucrose and the evaporation of water from honeydew cause an increase in osmotic pressure which causes a bulk flow of phloem and sap solutions toward the parasite.

Recently, Mower (1974) found that the patterns of sugar components appeared to be independent of the morphological similarities among Claviceps spp. He suggested that the difference in honeydew sugars might be a valuable taxonomic tool in this group of fungi. Sugars varied both quantitatively and qualitatively between species and qualitative differences among sugars were found within a species on several occasions. These differences may be due to strain variation but are more likely the result of mold or bacterial activity in the honeydew. He obtained good separation of honeydew sugars by using a combination of paper chromatographic systems and gel filtration colum chromatography.

Sclerotia

(A) Nature of Sclerotial Formation

Ergot sclerotia are black bodies, formed by the fungus, in place of the kernel of various cereals and grasses. In addition, they can be induced to form on inoculated haulm nodes (Stroll and Brack 1944), seedlings of certain rye varieties (Lewis 1956), and internodes of rye (Garay 1956).

The sclerotia of C. purpurea are usually larger than the kernels of the hosts and extrude beyond the glumes. However, the size of the sclerotia is influenced by the fungus as well as by the host. Smaller sclerotia (kernel size) were found to be produced by the fungus on susceptible cultivars of spring wheat than on cultivars of rye and triticale (Platford and Bernier 1976). Furthermore, sclerotia produced on two of several wheat cultivars tested, were found to be considerably smaller (smaller than kernel size) than sclerotia produced on other wheat cultivars (Platford and Bernier 1970; 1976). The reaction of both cultivars was interpreted to be a resistant reaction. It was recently found that several fungal isolates produced a few small sclerotia or no sclerotia, as well as aborted ovaries and partially infected kernels, particularly on resistant cultivars. Such isolates were considered to be low in virulence (Ratanopas 1973).

In the developing sclerotium the ovary appears as a deformed process at the top of the sclerotium because it

becomes detached from the receptacle. It has been suggested that the fungus merely grows directly on the sap from the rye which bleeds into the hollow formed by the palea of rye flowers. This plant sap serves as nutrient for the fungus throughout its period of development (Ramstad and Gjerstad 1955).

By dissecting growing sclerotia, Kybal (1964) found that the germinating conidia appeared to give rise to sclerotia. Conidia were found to germinate and form long thick sphacelial hyphae: the hyphae then interspersed and grew densely to form the compact sclerotium.

Corbett et al. (1974) studied sclerotial development of two strains of C. purpurea on rye. They reported that within seven days the ovaries were covered with white fungal mycelium impregnated with the honeydew. By the 10^{th} day, the sphacelial mycelium was sporing freely and honeydew was exuding from the florets. After about two weeks, pigmented tissue was seen on the surface of the sphacelium. It was, however, confined to the proximal end of the sphacelium. Growth of the fungus proceded by the proximal addition of new sclerotial tissue. The rate of sclerotial growth is presumed to be governed by the availability of nutrient supply from the host plant. Growth of the parasite ultimately ceased when the host became senescent.

There are indications that fertilization has an influence on ergot infection (Abe and Kono 1957; Campbell

and Tyner 1959; Futrell and Webster 1965; Puranik and Mathre 1971). In a recent study it was found that following fertilization, there was a reduction in the number of sclerotia as well as the amount of honeydew produced and that resistant cultivars expressed these reactions sooner than the susceptible ones (Ratanopas 1973).

(B) Histology of Sclerotia

Several workers have attempted to elucidate the histological structure of ergot sclerotia. Barger (1931) noted and described three layers within the sclerotia of C. purpurea. Outer walls of the outmost layer of cells are thick and brown to black. These walls contain chitin, but no cellulose. Cell contents are composed of oil and protein. The inner part consists of two types of tissue. The central one is composed of small cells having in transverse section the shape of an irregular star, and sometimes showing large, irregular intercellular spaces in the middle part. The peripheral tissues of the inner part are somewhat more elongated and have rounded edges; cell walls are pinkish white.

Ramstad and Gjerstad (1955) revealed that normal ergot sclerotial cells are arranged in a parenchymatous structure. The cells are cubical to spheroidal, relatively thick walled and large. They also found that aberrant sclerotia which occasionally develop have

much smaller rectangular cells, with considerably thinner walls.

Stewart (1957) also found that the sclerotium of C. paspali Stevens and Hall is composed of three layers, namely the surface layer, a transitional layer, and the germ layer. The surface layer which stains green to pink is composed of dead cells, and it acts as a protective layer. The transitional layer stains red with safranin. The germ layer, the vital portion of the sclerotium, stains green. During the germination of the sclerotium, the stipe is initiated in this vital layer.

(C) Chemical Composition of Developing Sclerotia

Recently Corbett et al. (1974) analysed the amino acid content of developing sclerotia and healthy rye seeds in order to obtain information on the growth and metabolism of the fungus under the conditions in which alkaloid synthesis is most regularly achieved. They found that during sclerotial development on rye (cultivar Svalof's Fourex), concentrations of γ-aminobutyric acid and alanine were higher than those of other amino acids at about 7 to 13 days after inoculation which was performed before anthesis. Compared to the healthy developing seed of the same variety, the concentration of alanine was higher than that of other amino acids at day 11 and from day 25 to 39 after the time of inoculation. The concentration of alanine in the free amino acid fraction rose from 2% (w/w of free amino acids) 11 days after

inoculation to a maximum of about 25% between days 20 and 40 (maturity). In healthy developing seeds of rye (cultivar Prolific), triticale (line 6A 190), and durum wheat (cultivar Stewart), alanine retained a high level or increased slightly over the first two weeks after anthesis and declined rapidly thereafter, whereas all the other amino acids remained at generally fixed levels (Dexter and Dronzek 1975).

Nutritional Requirement of C. purpurea in Culture

(A) Early Literature

Classical papers on culturing C. purpurea were reviewed by Bové (1970) and are summarized as follows: Tulasne in 1853 diluted honeydew and germinated conidia in solution. He could obtain mycelium producing secondary conidia. Brefeld in 1881 was the first to grow C. purpurea artificially on bread soaked in a nutrient solution as a substrate. He obtained only mycelium and conidia. Meyer in 1888 tried to immitate nature by using similar chemical substances which were found in rye and its ash constituents as determined by chemical analysis. His liquid medium contained glucose, starch, asparagine, albumin, peptones, ammonium sulfate, magnesium sulfate, potassium chloride, and dipotassium phosphate. This nutrient solution was used to soak bread and cotton for growing the fungus. He could obtain mycelium and conidia, but not sclerotia. In 1902, Engelke grew C. purpurea on a solid medium which consisted of

glucose, ammonium nitrate, monobasic potassium phosphate, magnesium sulfate, and 2% agar. He could induce the formation of tiny knots of packed hyphal threads resembling tiny sclerotia which he called "mikrosclerotia".

(B) Sclerotium-like Structures in Culture

Bonns (1921) grew C. purpurea on various media containing either carrots, string beans, sweet potatoes, rye seeds, rye screenings, rye heads, white corn meal, and yellow corn meal. He added agar to each medium and could obtain only mycelium and conidia. In cultures which were kept a long time, he found compact hyphal threads which resembled pseudoparenchymatic tissue. On both types of corn meal, the dense mycelial growth was a markedly purple color and became heaped-up in vermiculate form resembling sclerotia.

Kirchhoff (1929) described various growth forms of C. purpurea observed in artificial culture. Sclerotia developed from smooth colonies on a two percent nutrient agar containing 0.1 % monopotassium phosphate, 0.025% magnesium sulphate, 10% cane sugar, and 0.1% asparagine. When 1% asparagine was added to the medium, the growth became luxuriant and sclerotial development was more rapid and pronounced. He obtained better results when a small amount of potash lye was added to the medium to produce slight alkalinity of the medium. His sclerotia from culture were

composed of pseudoparenchyma without a cortical layer and differed in external appearance from natural sclerotial bodies. However, he claimed that the microscopic characters of natural and artificial sclerotia were identical. Only the marginal zone (2 to 5 mm broad) of the smooth colony was transformed into purple, later dull black sclerotial tissue. The formation of the artificially grown sclerotia was completed in two months. He believed the fungus possess the capacity to acidify the medium and thereby prevent the development of the smooth colonies from which alone sclerotia subsequently were produced. Large bodies (3 by 5 or 8 by 9 mm) resembling incipient sclerotia were formed on media containing gelatin. He noted that the reaction of the medium was the essential factor in sclerotial formation. For successful sclerotial formation, the composition of the medium should guarantee that there shall be no production of free acid. McCrea (1931) also found pseudosclerotia in old dry agar cultures. These pseudosclerotia consisted of mycelial knots rounded up into pulvinate to hemispherical structures, approximately 2 mm in diameter, embedded in the loose hyphae of mycelial mat. Ergot extract and vitamin E separately added to the agar medium stimulated the growth of the fungus and caused an early development of such pseudosclerotia.

The previous papers reported the formation of scleretium-like structures of C. purpurea on agar media,

but none of them reported the germination of those structures into stromata. It was Schweizer (1941) who first induced germination, into stromata, of a thickened mycelial mat growing on macerated germinating rye seeds which has been cold sterilized by carbon disulfide. He claimed that his sclerotium-like tissues were anatomically similar to those developing under natural conditions. He obtained stromata from a germinating sclerotium by subjecting it to a cold period followed by inoculation with pollen extract. However, he made no histological studies of the stromata obtained to determine whether it was functional.

Michener and Snell (1950) tried to grow C. purpurea on chemically sterilized germinated wheat and rye by the method described by Schweizer (1941). They failed to obtain sterile media even when the amount of chemical and the treatment time were increased above the level recommended by Schweizer. Their media so prepared were heavily contaminated with bacteria and fungi. They also worked with heat sterilized media and found that the growth of the fungus was better on germinated than on ungerminated wheat medium. The growth of the fungus was poor on germinated rye medium. There was no sclerotial formation on these media.

Cummings et al. (1952) attempted to grow C. purpurea in artificial culture with a view to producing sclerotia having an alkaloid content comparable to that obtained from

natural sclerotia produced on rye. However, by sterilization of natural media with carbon disulfide, ethyl chloride, propylene oxide and combinations thereof; they failed to obtain sterile media.

(C) Plant Extract Media

Berman and Youngken (1954) investigated the effect of rye homogenates on the growth of C. purpurea. By comparing homogenates of rye inflorescences at three different growth stages, non-flowering, flowering and fruiting, they found that good growth of the fungus was obtained on all liquid extracts of each of these stages. Maximum growth occurred on extracts prepared from the fruiting stage. On rye mash suspensions, growth occurred only on media prepared from the mature fruiting stage. Thus, this stage contains substances capable of supporting growth of the fungus. No growth occurred when chlorophyll was removed by solvent extraction from rye homogenate and mash suspension. This seemed to indicate that chlorophyll or substances associated with it are essential for Claviceps growth. There were no differences among frozen, dried, or fresh materials used.

Garay (1956) found that extracts of detached parts of rye namely ears, leaves, stems, nodes and roots, either freshly prepared or sterilized by autoclaving or filtration, did not stimulate the germination of ergot conidia. However, extracts

sterilized by autoclaving stimulated fungus growth; a strong effect was obtained in the case of ears. He could obtain growth on rye embryos and isolated wheat roots, but the hyphae grew around the roots and embryo without penetrating into them, even when roots and embryos were injured with a needle. He did not observe any kind of micro-sclerotia or sclerotia.

Guttation fluid of the host has an influence on the growth of C. purpurea in vitro (Lewis 1962a). The amount of fungal growth in the presence of guttation fluid obtained from seedlings of certain cereals, was found to correlate with the degree of susceptibility of the hosts. The fluid of rye (cultivar Rosen) produced the most growth by germinating spores whereas that of barley (cultivar Trail) produced the least. In the same year, Lewis (1962b) investigated the effects of some metabolites on the susceptibility of rye seedling to C. purpurea. He found that DL-α-amino-n-butyric acid, L-arginine HCl, β-alanine, L-glutamic acid, DL- and D-phenylalanine, and succinic acid increased the susceptibility of rye seedlings to C. purpurea. L-histidine, DL-isoleucine, L-leucine, DL-methionine, DL-norvaline, and L-tyrosine had little or no effect on infection.

Potbury and Drysdale (1969), working with rye and barley varieties different from those used by Lewis (1962a), studied the effect of guttation fluid on the growth of C. purpurea. They investigated the effects of guttation

fluid from one variety of rye and four varieties of barley on the growth of the fungus in vitro. Their results were in contradiction to those of Lewis (1962a) in that seedlings of barley varieties proved similar to that of rye in becoming infected, and there was no clear correlation between the effect of the guttation fluids on conidial growth and the susceptibility of seedlings of the cultivars to infection.

Goatley and Lewis (1966) determined the composition of guttation fluid from rye, wheat, and barley seedlings. Glucose was found to be the principle sugar component of the rye and barley fluids with galactose being highest in wheat. Total sugar content was about equal in rye and barley fluids, but lower in wheat. Most of the amino acid content in all three fluids was aspartic acid or asparagine. Barley fluid was far higher than the other two in total amino acids, and wheat the lowest. The majority of inorganic elements (P, K, Na, Ca, Mg, Mn, Fe, Cu, B, Zn, Mo, Al) were found to be highest in barley and lowest in wheat with the exception of Fe, in which rye was highest and barley lowest. For vitamins, barley fluid was highest in choline, p-aminobenzoic acid, and thiamine, while rye was highest in inositol and pyridoxine. Wheat was much lower than the other two in choline and inositol, and uracil was found only in barley.

(D) Utilization of Carbon and Nitrogen Sources

C. purpurea can grow on a wide variety of carbon and nitrogen sources. The fungus was found to be capable of utilizing various sources of carbon, namely, dextrose, levulose, galactose, cane sugar, glycogen and mannitol which induced very good growth at concentrations of 5 to 15% (Kirchhoff 1929). McCrea (1931) also studied the effect of various carbon sources on the growth of C. purpurea on synthetic media. Among four sugars tested, namely, maltose, dextrose, levulose and sucrose, 2-3% maltose proved to be the best. There was not much difference between dextrose and levulose, but sucrose appeared to be a poor carbon source for the growth of this fungus. However, Kirchhoff (1929) had obtained good growth on a media containing 10% cane sugar. According to Michener and Snell (1950), the growth of the fungus was found to be rapid on media supplemented with either glucose, fructose, or sucrose as a source of carbon. Maltose, mannitol, arabinose, dextrin and wheat starch were utilized more slowly. Lactose and raffinose were poorly utilized. Among nitrogen sources studied, asparagine, peptone, casein hydrolysate and yeast extract were good nitrogen sources. Ammonium and nitrate nitrogen were not as well utilized as organic nitrogen.

Effects of various organic and inorganic nitrogen sources on growth of C. purpurea were determined by Sim and Youngken (1951). They found that supplementary feedings

of the liquid synthetic medium with tryptophan, ornithine and indole markedly inhibited mycelial growth. Arginine and ammonium sulfate had no significant effects on growth.

According to Tyler and Schwarting (1952), 2% mannitol and 1% casein hydrolysate incorporated in their basic nutrient solution was the best carbon and nitrogen combination for the maximum production of mycelium.

Working with the nutrition of three strains of C. purpurea in liquid shake culture, Taber and Vining (1957) found that organic nitrogen sources, namely glutamate, asparagine, ammonium succinate and urea were more readily utilized than inorganic nitrogen sources, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and KNO_3. L-asparagine has been widely used as a sole nitrogen source in media for C. purpurea (Amici et al. 1967a; Gjerstad and Ramstad 1955, Grein 1967; Johansson 1964a; Kirchhoff 1929; Mantle and Tonolo 1968; Strnadová and Kybal 1974). Among carbon sources, glucose, fructose, mannose, cellobiose and sucrose were readily utilized, but not xylose, ribose, mannitol, starch, powdered cellulose, carboxy methyl cellulose and methyl cellulose. An exogenous supply of biotin was required for growth of the fungus in the medium containing glucose, ammonium succinate, and minerals. In further studies, Taber and Vining (1960) found that the alkaloid-producing and non-alkaloid-producing strains could utilize succinic acid as a carbon source for growth, but neither of them could utilize L-tryptophan as a carbon or

nitrogen source for growth and this agreed with a previous findings (Sim and Youngken 1951).

Variability of C. purpurea

Variability with regard to pathogenicity, cultural characters and alkaloid production of ergot has been reported. Classification of the variation in pathogenicity and alkaloid production has been attempted (Barger 1931; Campbell 1957; Grein 1967; Kybal and Brejcha 1955; and Ratanopas 1973).

(A) Variation in Pathogenicity

Studies on the pathogenicity of C. purpurea started a long time ago. Barger (1931) reviewed Stäger's work on biological races of Claviceps spp. The specialized races of C. purpurea mentioned by Stäger are summarized as follows: 1) The race attacking rye (Secale cereale L.) also infects wheat (Triticum aestivum L.), barley (Hordeum vulgare L.) meadow fescue (Festuca elatior L.), Bromus sterilis L., and a few Poa spp. 2) The ergot from sweet vernal grass (Anthoxanthum odoratum L.) attacks rye and some other grasses but not barley. 3) Wood brome (Brachypodium sylvaticum (Huds.) Beauv.) and mannagrass (Glyceria fluitans (L.) R. Br. each have an exclusive, single race. 4) The race of perennial rye-grass (Lolium perenne L.), infects meadow brome (Bromus erectus Huds.) and other Lolium spp., but not rye.

Campbell (1957) successfully inoculated 421 isolates of C. purpurea, from 38 different host species, on rye, wheat, and barley growing in the greenhouse. He found that only one of three isolates from Glyceria borealis (Nash) Batch. did not infect these three hosts. However, ergot from rye infected all of the grass species tested, both in the field and in the greenhouse. These results differ from those of Stäger (1903) who failed to secure infection in many hosts in cross inoculation studies between species of the Gramineae. Furthermore, Campbell found that ergot from perennial rye-grass infected rye. This contradicted Stäger's results, but it supported the findings of Békésy (1956) who infected rye with ergot from Lolium perenne L. and L. perenne L. with ergot from rye. Because of the existence of variability in the conidial colonies, Campbell concluded that physiologic races of C. purpurea, as proposed by Stäger, do exist but only in the cultural sense of the term. However, he found no evidence that these races were species specific.

Ratanopas (1973) inoculated the wheat cultivars Kenya Farmer and Carleton, reported by Platford and Bernier (1970) to possess resistance to C. purpurea, as well as two susceptible cultivars, with each of 58 isolates of the fungus collected from various cereals and grasses in Western Canada. He found that disease severity varied with the isolates within, as well as among, the four cultivars. The isolates showed differential interaction with the resistant and

susceptible cultivars indicating that they differ in virulence. Variation in virulence was also observed among monoascosporic isolates originating from the same sclerotium. There was no relationship between virulence and host sources.

(B) Variation and Morphological Characteristics in Culture

Several workers have reported on variation and morphological characteristics of C. purpurea in culture. However, none has attempted a thorough evaluation of the cultural characteristics of the fungus. Furthermore, each author used different terms to describe morphological characteristics.

Michener and Snell (1950) grew C. purpurea in culture aiming to produce ergot alkaloid artificially. They found that cultures from sclerotia known to have a high alkaloid content differed morphologically from each other although their nutritional requirements were similar. In some cases two or more types of growth, appearing within one culture, were separated and treated as separate isolates.

Sozynski et al. (1965) studied the effect of various natural and synthetic media on the morphology and sporulation of C. purpurea. They reported that the composition of the medium has an important effect on the morphological and physiological characters, sporulation, and alkaloid production. Of eight media studied, they found that potato

extract-glucose-wort and mineral media supported good growth and sporulation in both solid and liquid culture. The characteristics of the cultures they described were size of colony, morphological development, edge of the colony, distribution of spores on the colony, spore color, slime production, thickness of the colony, cross section of the colony and dry weight of the fungus.

Grein (1967) reported a very high variability of the cultural characteristics of a number of freshly isolated strains of C. purpurea growing on an agar medium containing asparagine and high amounts of sucrose (T2 medium) used by Amici et al.(1967a). Among 61 strains isolated from sclerotia, he found that 90% showed variability in their cultural characters, and only a few strains were stable and reproducible upon subculturing. He used the following characters to describe the cultures: 1) color of aerial mycelium: white, ivory, beige-violet, and violet; 2) type of aerial mycelium- velvety, cottony and formation of synnemata; 3) type of growth of the whole culture- flat, folded, wrinkled, bulky or clotted; 4) sectoring ability or outgrowth production in giant colonies.

Mantle and Tonolo (1968) described the cultural characters of C. purpurea on a sucrose-asparagine agar medium which differed slightly from the T2 medium used by Grein (1967) and Amici et al. (1967a). They found that the two strains studied produced different morphological characters in

culture. One strain gave rise to raised cushion-like plectenchymatic tissue, having a red-violet color and bearing droplets which contained many conidia. This strain was unstable and had a tendency to change morphologically into a new strain which consistently produced flat white colonies with the presence of conidia and the absence of plectenchymatic tissue. This new isolate was stable. Another strain produced off-white colonies and consisted of a compact white plectenchymatic tissue without producing conidia.

Spalla et al. (1969) reviewed previous work and summarized the morphological characters of C. purpurea cultured on T2 medium as follows: 1) purplish pink giant colonies showing two types of sectors, the first one reddish-violet, bulky with brownish-violet reverse, the second one pinkish-violet with abundant aerial mycelium and violet reverse; 2) flat, whitish colonies with straw-yellow reverse; 3) pinkish colonies, slightly bulky, later violet with light violet reverse; 4) thin, flat, smooth colonies, with a dendritic surface pattern and with straw-yellow reverse. Upon subculturing of some sectors they reported that: 1. isolation from mycelium which had not sporulated yet gave colonies all of the same type; 2. isolation from conidia gave colonies of several types; and 3. the characteristics of colonies obtained either from sectors of the giant colonies or from isolation of

conidia were the same.

Working in solid culture with C. purpurea naturally occurring on Phragmites communis Trin., Mantle (1969) described the cultural characteristics of different isolates into five groups: 1) thick, purple, non-sporulating colony; 2) thin, white, sporulating; 3) thin, purple, non-sporulating tissue surrounding a white sporulating center; 4) thin, light purple, non-sporulating; 5) thin, purple, non-sporulating sector in thin, white, sporulating colony. Isolates from separate fragments of natural sclerotia of one collection produced the same type of growth form characterized as white colonies with abundant sporulation, except one isolate which gave rise to a white sporulating center having thick and compact periphery with a deep red-purple color and no spores. Four sub-isolates were made from the second type of growth form, one from the white sporulating center, and three from different parts of the thick pigmented tissue. He found that in the white isolate, the white area was stable but the pigmented isolates tended to revert to a thin, non-pigmented form during successive subculturing.

(C) Variation in Alkaloid Production

Sclerotia of C. purpurea contain a number of active and inactive alkaloids and other nitrogen-containing compounds. The active alkaloids are ergocryptine, ergocornine, ergocristine, ergotamine, ergosine and ergonovine

(Kingsbury 1964) but the total alkaloid content is variable. Mothes and Silber (1954) described two forms of variability in the alkaloid content of the sclerotia. One form was due to host species. Another form was likely due to strain differences within the fungus, since they obtained large amounts of alkaloids from a black sector and found a total absence of alkaloids in a white sector from the same sclerotium. They concluded that these parti-colored sclerotia arose from multiple infections of two separate strains of the fungus.

On the basis of alkaloid production, Kybal and Brejcha (1955) divided rye ergot into five races which were recognized by a standard content of certain fixed alkaloids in constant proportions. These races were rich in 1) ergotamine; 2) in ergotamine, ergocornine and ergocristine; 3) in ergotamine, ergocornine, ergocristine and ergocryptine; 4) in ergocornine, ergocristine and ergocryptine; and 5) in ergocornine and ergocristine. Ergonovine and ergosine were present in all the sclerotia examined.

Mutation and Heterokaryosis

The sexual organs of C. purpurea was reported by Gäuman and Dodge (1928) found to include antheridia and ascogonia originating from the same thallus; this was taken as evidence of homothallism. Homothallism in this fungus was further demonstrated by McCrea (1931) who crossed several

single ascospore cultures from different locations, and found no evidence of inhibition or acceleration of mycelial growth of the cultures. The hyphae from each source intermingled as they spread over the surface, forming a mat of normal appearance that could not be distinguished from either of the originals. However, she did not report either mutation or heterokaryosis in this fungus.

Following treatment with UV light, Strnadová (1964) isolated an ergot mutant which differed from the original strain in producing, on agar media, a wrinkled colony lacking aerial mycelium. The diameter of the colony was halved and sporulation was only one percent that of the original strain. The mutant when inoculated into rye heads yeilded only a few sclerotia; these had a much lower alkaloid content than the original strain.

Sheu and Paul (1968) induced mutants of C. purpurea by treating with UV light and γ-rays, and found that these mutants produced a high level of tryptamine. The mutants also varied from the wild type parent in growth characteristics, pH during growth and nitrogen utilization.

Amici et al. (1967b) reported heterokaryosis in strains of C. purpurea grown on T2 medium. A heterokaryotic strain produced late sectors on this medium. Mixed cultures of certain strains, isolated from sectors, formed numerous anastomoses on T2 medium. Furthermore, overgrowth of the fungus was formed at the contact zone between the colonies.

Staining the nuclei of hyphae from a culture of the strain that produced sectors, they could demonstrate that each cell usually contains five nuclei. Moreover, heterokaryons produced large quantities of alkaloids, whereas the original individual strains were unable to produce significant amounts. However, they reported that the heterokaryotic condition is rare in culture, but it is frequent in the mycelium comprising sclerotia.

Strnadová (1968) was able to obtain heterokaryotic mycelium of C. purpurea by simultaneous plating of conidia of two different defined auxotrophic mutants of an ergotamine producing strain. This heterokaryotic mycelium was characterized by its ability to grow on a minimal medium and to dissociate into both original auxotrophic mutants during sporulation.

Using the same medium as that described by Amici et al. (1967a), Strnadová and Kybal (1974) confirmed Amici's result in obtaining "late sectors" in cultures of C. purpurea. They found that sclerotial as well as monoconidial cultures could give rise to sectors. From this evidence, they concluded that ergot sclerotia are commonly homokaryotic and that these phenotypically different sectors result from a mutation during growth of the colony. Their conclusion differed from that of Amici et al. (1967b) who believed that the formation of the sectors is attributed to the segregation of nuclei of the parent heterokaryon. Although heterokaryosis

of C. purpurea could be demonstrated in culture, its importance in nature is not yet known.

Cytology of C. purpurea

Jung et al. (1957) studied the cytology of C. purpurea. They were able to demonstrate that only one nucleus of normal size was detected in a conidium stained by Feulgen's method and with gentian violet. Additional information on cytology was presented by Jung and Rochelmeyer (1960) who reported that conidia in young cultures are uninucleate whereas those in older cultures are multinucleate, but develop uninucleate germ tubes. In older mycelium the cells can contain several nuclei. Anastomoses are frequent in the young growing mycelium, but less frequent in older one.

Robbers et al. (1974) worked with a homokaryotic strain of C. purpurea which yielded high amounts of alkaloids in culture. They found that during the growth phase, the hyphal tip cell of such an isolate is multinucleate due to the rapid nuclear division associated with actively growing hyphal tips. As growth slows, the number of nuclei in the hyphal tip decreases. When the rapid phase of mycelial growth has ceased, the hyphal tip cells become mononucleate just as the other cells in the hyphal strand. As the mycelium ages, mononucleate conidia are formed. This homokaryotic strain produced consistently high amounts

of alkaloids, even after many successive transfers in production medium. They stated that heterokaryosis may be a necessary requirement for alkaloid production in some strains of C. purpurea as reported by Amici et al. (1967b).

Role of Amino Acids on Host Reaction and on Growth and Sporulation of Fungi in Culture

Several studies have attempted to relate the amino acid content of plants to their susceptibility and resistance to diseases (van Andel 1966). Increased levels of histidine or lysine, or both, in blueberry fruits coincide with their increase susceptibility to Glomerella cingulata (Ston.) Spaulding and Schrenk (Strech and Cappellini 1965). D and DL isomers of phenylalanine applied through leaf midribs of apple markedly increased the resistance of seven apple varieties to attack by the apple scab fungus Venturia inaequalis (Cke.) Wint. (Kuc et al. 1959). These two compounds incorporated into potato dextrose agar at concentrations equal to those used for infusion, inhibited the growth of several isolates of race 2 of V. inaequalis. Cotton cultivars resistant to Fusarium wilt contained considerably more cysteine in their roots than did susceptible ones (Lakshminaryanan 1955).

Germination and germ tube growth of spores of Fusarium oxysporum Schlecht. f. sp. fabae Yu and Fang were completely inhibited by β-alanine at concentrations above 40% (Abdel-Rehim et al. 1968). Broad bean (Vicia faba L.) seedlings of a susceptible

variety developed severe infection when grown in infected soil but remained healthy when this soil was watered with 60% β-alanine. In addition, applying a 30% β-alanine solution to leaves, one drop twice daily for a period of 10 days before inoculation, prevented infection of seedlings in infested soil. This indicates the translocation of the compound downward from the leaves to the roots. It was also found that root tissues and root exudates of a resistant variety contained β-alanine. Investigating the mechanism of resistance of some varieties of broad bean (Vicia faba) towards infection with F. oxysporum, Hashem (1969) suggested that in resistant seedlings, hydrolysis of globulins in the seeds produced more β-alanine than susceptible variety. β-Alanine was translocated to the roots and passed into the soil, thus preventing infection by the fungus. Although DL-isoleucine inhibited rust development on detached wheat leaves (Samborski and Forsyth 1960), it had no effect on the infection of rye seedlings by C. purpurea (Lewis 1962b). With Fusarium solani f. cucurbitae Snyd. and Hans., race 2, DL-isoleucine induced the formation of perithecia (perfect state) in culture (Toussoun 1962). DL-methionine was effective in controlling Aphanomyces root rot of pea growing in nutrient solution in liquid culture (Papavizas and Davey 1962). However, this amino acid stimulates growth and sexual reproduction of several isolates of Aphanomyces euteiches Drechsler (Papavizas and Davey 1963a) in vitro. From these results, Papavizas and Davey (1963a) pointed out that although

nutritional requirements of plant pathogenic fungi play significant roles in the development of disease syndromes, the physiology of growth of pathogens in vitro may not always be used for predicting the final magnitude of pathogenesis of this fungus. Among non-sulphur amino acids, DL-norvaline and DL-norleucine completely prevented pathogenesis of Aphanomyces root rot of pea in nutrient solutions, whereas DL-valine, DL-leucine, DL-isovaline and DL-isoleucine were partially effective (Papavizas and Davey 1963b).

Several amino acids have an influence on the development of fungi in culture (van Andel 1966). With Venturia inaequalis (Cke.) Wint., Ross (1968) found that DL-(α)-alanine as a nitrogen source supported very good sporulation of certain isolates whereas L-proline, DL-(α)-aminobutyric acid and glycine were good nitrogen sources for another isolate.

β-Alanine can inhibit growth and sporulation of some fungi. With Claviceps microcephala (Wallr.) Tul., β-alanine as a nitrogen source supported poor growth but good sporulation (Singh et al. 1972). This amino acid consistently inhibited the growth of Alternaria solani Kuhn, but it increased growth when high concentrations of proline were present (Lewis 1957). The growth of Neurospora crassa was also inhibited by β-alanine (Herrmann and White 1966).

D-alanine was poor source of nitrogen for growth of Phytophthora infestans (Mont.) de Bary which grew well in the presence

of L-alanine (Newton 1957). Several wood-rotting basidiomycetes failed to utilize D-alanine except for Lenzites trabea Pers. ex Fries, which could utilize D-alanine when applied together with L-alanine (Jennison and Perritt 1960).

Tryptophan is known to play a role in ergot alkaloids synthesis (Kaplan et al. 1969). Teuscher (1964; 1965) has also shown that only mycelium of strains producing alkaloids could actively take up L-tryptophan from the medium.

The amino acid analog DL-p-fluorophenylalanine (FPA) inhibited sporulation of Ceratocystis ulmi (Buis.) and Fusarium oxysporum f. lycopersici (Sacc.) Snyd. and Hans. in liquid culture; sporulation of C. ulmi being more sensitive to FPA than sporulation of F. oxysporum f. lycopersici (Biehn 1973). FPA at the concentration that inhibited 90% of the sporulation of both fungi did not appreciably inhibit their total growth.

Spore germination and mycelial growth of Cladosporium cucumerinum Ell. and Arth., Collectotrichum lagenarium (Pass.) Ell. and Halst., Phytophthora cactorum (Lebert and Cohn) Schroet., Pythium debaryanum Hesse were reduced by FPA (van Andel 1966). The effect of FPA in protecting cucumber seedlings against C. cucumerinum and C. lagenarium, when applied to the roots before inoculation, was reported by an Andel (1962).

RESULTS OF RESEARCH

1. Relationship of Cultural Characters of *Claviceps purpurea* to Host Origin and Virulence.

ABSTRACT

Cultural characteristics varied considerably among 143 isolates of *Claviceps purpurea* (Fr.) Tul. and were classified into 10 groups on the basis of type of growth (raised or flat), surface characteristics (smooth, folded or wrinkled) and color of the colony (white or colored). None of the cultural characteristics appeared to be related to host origin of the isolates or to the virulence of 49 selected isolates. Small, globose conidial masses were observed in cultures of a few isolates and more prominent conidial horns were observed inconsistently in cultures of one isolate. These structures do not appear to have been reported previously. Sectoring occurred with equal frequency in cultures derived from monoascospores as well as those from sclerotia, indicating that sectoring is probably due to mutation in cultures of both types of isolates. However, the variability among 20 monoascosporic isolates from a single sclerotium appeared to be too great to be ascribed to mutation only and suggests that recombination of genetically different nuclei during the sexual stage of the fungus might also be responsible for the wide variation observed in this study among isolates of *C. purpurea*.

INTRODUCTION

Variability within C. purpurea, the causal agent of ergot of cereals and grasses, has been recognized since Stäger in 1903 conducted infectivity tests with fungal isolates from many host sources and reported the existence of several physiologic races (Barger 1931). Campbell (1957) acknowledged the existence of physiologic races, in the cultural sense of the term, but found no evidence that these races are species specific. Virulence was reported to vary considerably among 49 isolates of C. purpurea collected from various cereals and grasses, when they were tested on 2 susceptible and 2 resistant wheat cultivars (Ratanopas 1973). However, virulence was not related to host origin of the isolates.

Isolates of C. purpurea are usually cultured on malt agar and on this medium they show few differences in cultural characters and most isolates produce white, slightly fluffy mycelium (Bernier, unpublished results). Recently, a medium containing asparagine and a high amount of sucrose has been shown to allow ergot isolates to express different cultural characters (Amici et al. 1967a; Grein 1967; Mantle and Tonolo 1968; Spalla et al. 1969). The present studies were initiated to investigate the variability of cultural characters among isolates of C. purpurea from various hosts in the Canadian Prairies and to determine relationships between cultural

characters and host origin and virulence of the isolates.

MATERIALS AND METHODS

One hundred and forty three isolates of C. purpurea collected from cereals and grasses in the Canadian Prairies were utilized in this study. Of these, forty nine had been used in a previous study and knowledge of their virulence was available (Ratanopas 1973). The number and type of isolates tested from each host are presented in Table 1.

Pure cultures were obtained from either sclerotial pieces or single ascospores according to methods described previously (Ratanopas 1973; Appendix 1). Stock cultures were kept on 4% malt agar slants which, after about three weeks growth at room temperature, were stored at 5^{o} C. The malt agar medium consists of 15 g malt extract, 10 g maltose, 40 g agar, and distilled water to make 1000 ml.

The composition of the sucrose-asparagine medium (T2 medium) employed by Amici et al. (1967a) and used throughout this study is as follows (g/l): sucrose, 100; L-asparagine, 10; yeast extract, 0.1; KH_2PO_4, 0.25; $MgSO_4.7H_2O$, 0.25; KCl, 0.12; $Ca(NO_3)_2.4H_2O$, 1; $FeSO_4.7H_2O$, 0.02; $ZnSO_4.7H_2O$, 0.015; agar, 20; tap water to 1000 ml, pH adjusted to 5.2, sterilized by heating at 110^{o} C for 20 minutes.

The experimental cultures were obtained by mass transfers from stock cultures. A small piece of mycelial mat from each isolate was placed in the center of a 60 x 15 mm polystyrene or glass Petri dish containing 12 ml of T2

Table 1. Host origin, type, and number of isolates of *C. purpurea*.

Host	Host code	Type of isolate: Sclerotia	Type of isolate: Single ascospore	Total
Rye (*Secale cereale* L.)	R	23(7)[1/]	12(10)	35(17)
Smooth brome (*Bromus inermis* Leyss.)	B	28(2)	-	28(2)
Durum wheat (*Triticum durum* Desf.)	D	3(1)	21(-)	24(1)
Quackgrass (*Agropyron repens* (L.) Beauv.)	C	20(6)	3(3)	23(9)
Spring wheat (*Triticum aestivum* L.)	M	8(6)	11(-)	19(6)
Triticale (X *Triticosecale* Wittmack)	T	3(3)	2(2)	5(5)
Ryegrass (*Lolium temulentum* L.)	L	3(3)	-	3(3)
Timothy (*Phleum pratense* L.)	P	2(2)	-	2(2)
Oats(*Avena sativa* L.)	O	1(1)	-	1(1)
Reedgrass (*Calamagrostis canadensis* (Michx) Beauv.)	CT	1(1)	-	1(1)
Common reedgrass (*Phragmites communis* Trin.)	PM	1(1)	-	1(1)
Tall fescue (*Festuca arundinacea* Schreb.)	F	1(1)	-	1(1)
Total		94(34)	49(15)	143(49)

1/ The numbers in parenthesis indicate isolates previously tested for pathogenicity (Ratanopas, 1973).

medium. The plates were incubated at 25° C. All isolates were replicated three times.

The cultures were examined periodically until 30 days after inoculation and characteristics were assessed and grouped.

RESULTS

The mycelial growth of all the isolates after about 14 days of incubation was white, without pigmented centers and generally extended to about 2/3 of the plate. Conidia were generally produced over the entire surface of the colonies. Subsequently, the mycelium of some isolates changed to various shades of violet and yellow, particularly at the center, while that of others remained white. The mycelial mat of many isolates continued to thicken while the surface either remained smooth or became wrinkled or folded. After 30 days, the mycelial mat of some isolates had become highly raised and plectenchymatic while that of others remained relatively flat. The isolates thus displayed considerable variation in cultural characters as illustrated in Figs. 1-5.

Sectoring occurred in 10% and 9% of the cultures derived from monoascospores and sclerotia respectively and in only one of three plates of any given isolate (Appendix 2). Sectoring generally occurred early, close to the origin of the colony, and involved the type of growth and/or the color of the colony (Fig. 5: A, C, D and F). Occasionally, sectoring occurred late, at the margin of the colony (Fig. 5B), and some colonies developed both early and late sectors (Fig. 5E).

Figs. 1-3. Cultures of C. purpurea on T2 medium. Fig. 1. Three isolates showing typical flat mycelial growth: with A = smooth, B = folded and C = wrinkled surfaces. Fig. 2. Three isolates showing typical raised mycelial growth: with A = smooth, B = folded and C = wrinkled surfaces. Fig. 3. Side view of cultures showing: A = raised, B = flat mycelial growth.

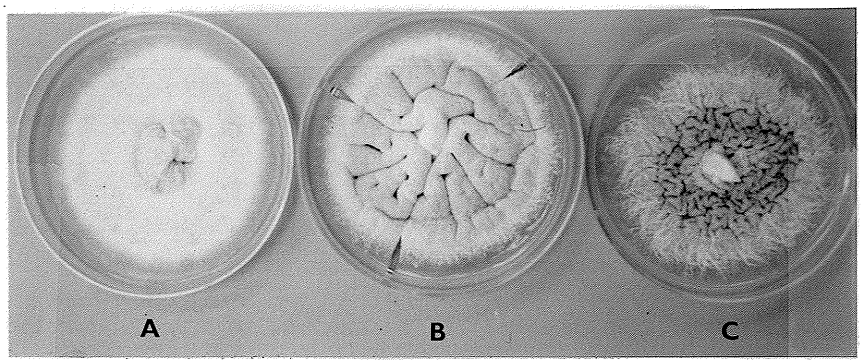


Fig. 1

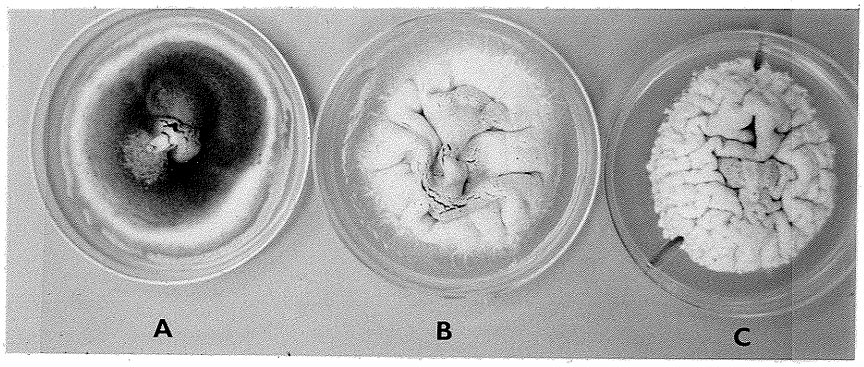


Fig. 2

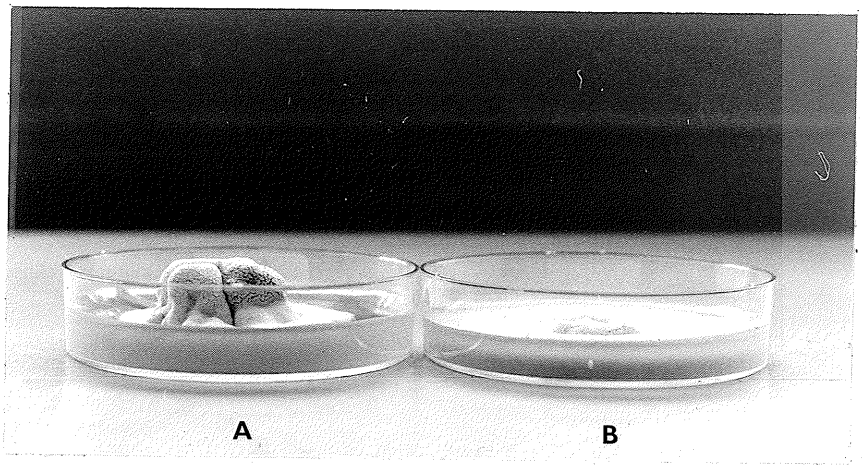


Fig. 3

Figs. 4-5. Cultures of C. purpurea on T2 medium. Fig. 4. Cultural characters of 20 monoascospore isolates from the same sclerotium obtained from Durum wheat. Fig. 5. Sectors formed in cultures of various isolates: A, C, D and F, illustrate early sectors; B, late sector; E, both early and late sectors.

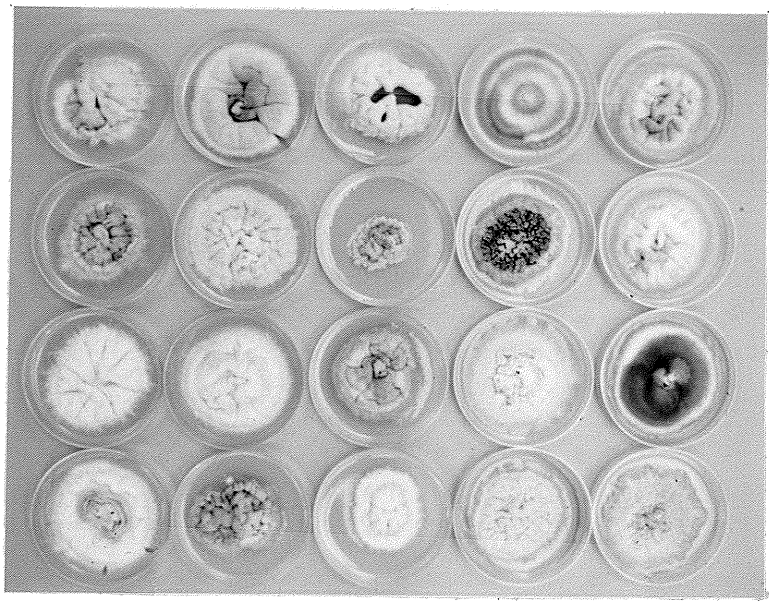


Fig. 4

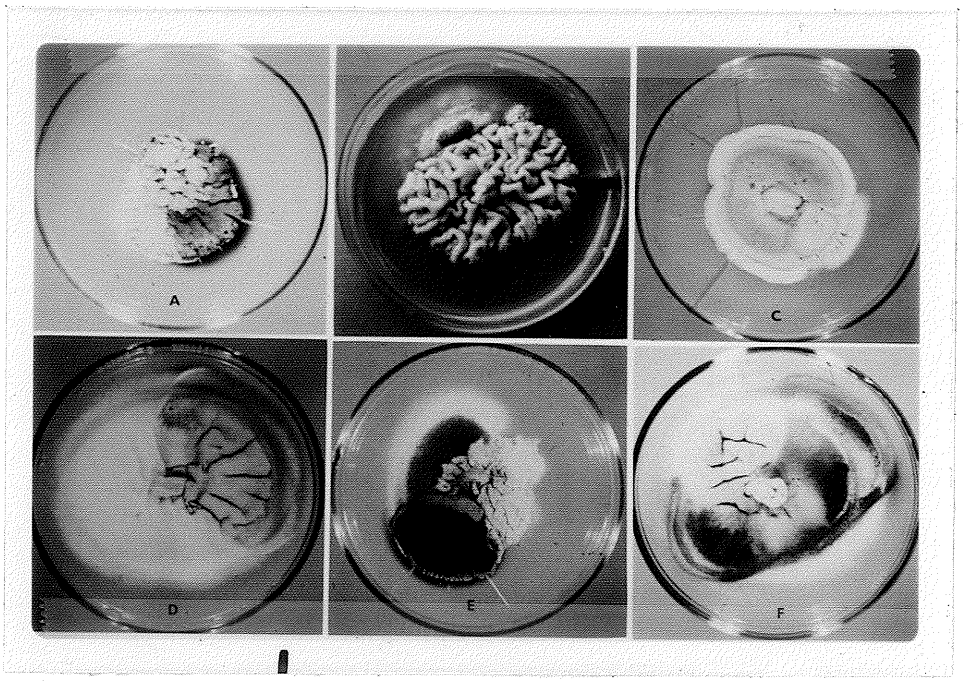
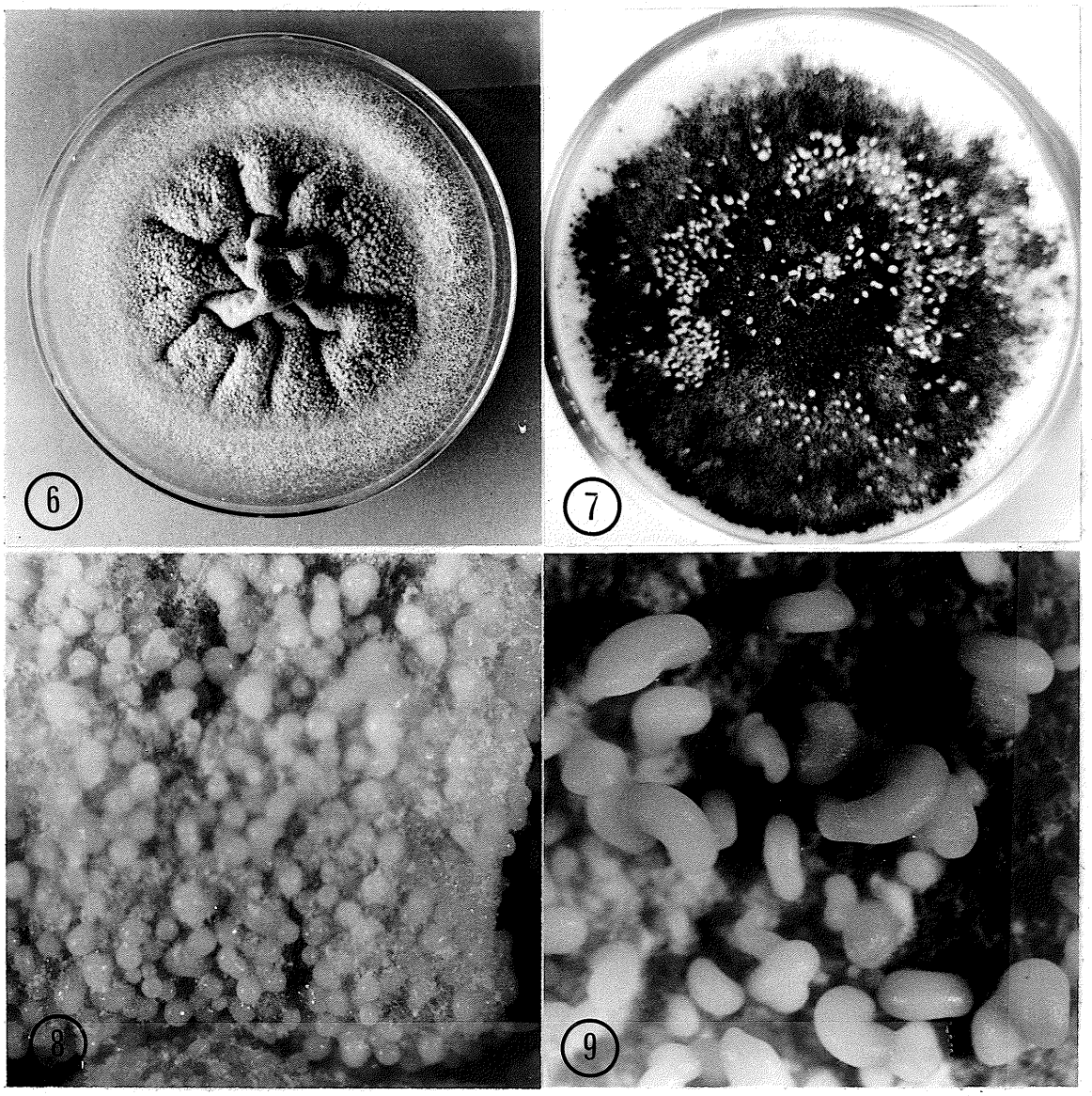


Fig. 5



Figs. 6-9. Conidial structures of C. purpurea on T2 medium. Fig. 6. Small globose structures, 30 days old. Fig. 7. Horn-like structures, 30 days old. Fig. 8. Enlargement of globose structures (x11). Fig. 9. Enlargement of horn-like structures (x11).

Small, globose, yellow structures (Figs. 6 and 8) were observed on the mycelial mat of 9 isolates (Appendix 2). Upon microscopic examination, the structures were found to consist of masses of conidia adhering together. They did not appear to be related to host origin of the isolates since they formed in ergot cultures from rye, durum wheat and spring wheat.

In order to determine relationships between host-source of the isolates and their cultural characteristics, the isolates were placed in one of 10 classes on the basis of type of growth, surface characteristics and color of the colony (Table 2). Minor variations frequently occurred within a plate, and among the three plates of a given isolate for one to several characters; particularly for color, in which case the isolate was classified according to the predominant characters. Isolates showing either violet, yellow and white, or cream pigmentation were grouped and referred to as colored and white respectively.

There were no apparent relationships between any of the cultural characters and the host origin of the 143 isolates studied (Table 2). The cultural characters of the isolates from a given host generally fell into many categories. However, the isolates showed a greater frequency of "flat" vs "raised", "folded" vs "smooth", and "colored" vs "white".

Considerable variation in cultural characters was also observed when cultures from 20 single ascospore isolates

Table 2. Cultural characters of 143 isolates of C. purpurea cultured on T2 agar medium.

Category	Characteristics[1/] Growth	Surface[2/]	Color[3/]	Host Origin[1/] Rye	Smooth brome	Durum wheat	Quack grass	Spring wheat	Triticale	Rye grass	Timothy	Oats	Reed Grass	Common reed grass	Tall fescue
1.	Raised 49(5)	S-2	c-2			1	1								
2.		W-3	c-3		2	1									
3.		F-44(5)	w-15(3)	3	4	2	2(1)	3(1)		1(1)					
4.			c-29(2)	7(2)	5	10	3	4							
5.	Flat 94(44)	S-13(8)	w-10(6)	6(3)			1	1(1)		1(1)					1(1)
6.			c-3(2)	1(1)		1			1(1)						
7.		W-22(6)	w-5(3)	2(2)		1(1)	1	1							
8.			c-17(3)	6(1)	5		3(1)	3(1)							
9.		F-59(30)	w-26(8)	2(1)	6(1)	7	6(1)	2(2)	2(2)					1(1)	
10.			c-33(22)	8(7)	6(1)	1	6(6)	5(1)	2(2)	1(1)	2(2)	1(1)	1(1)		
	Total		143(49)	35(17)	28(2)	24(1)	23(9)	19(6)	5(5)	3(3)	2(2)	1(1)	1(1)	1(1)	1(1)

1/ Values without parentheses are numbers of isolates in each category; values in parentheses are numbers of isolates previously tested for pathogenicity.

2/ Surface: S = smooth, W = wrinkled, F = folded.

3/ Color: c = color, w = white.

obtained from a single sclerotium from Durum wheat were compared (Fig. 4). The isolates were classed into six of the 10 categories recognized in this study; nine were in category 4 (raised, folded, colored), six were in category 9 (flat, folded, white), and one each was in category 1, 2, 3, 6 and 10.

There was no apparent relationship between the cultural characters revealed in this study and the virulence data of the isolates obtained previously (Fig. 10). Isolates in morphological category 10, for example, showed both high and low virulence on each host tested.

Cultures of each isolate were examined microscopically for the presence of conidia 14 and 30 days after inoculation. For the isolates that produced a plectenchymatic type of growth, conidia were generally detected only in young colonies. Only twelve isolates, ten of which were tested for pathogenicity, did not produce conidia on T2 (Appendix 2). The virulence of 7 of these 10 non-sporulating isolates (R-4A, R-6A, R-7A, R-11A, C-7, T-2A and L-9) was relatively high, on at least 2 of the four cultivars tested, whereas the remaining 3 isolates were low in virulence on at least 3 cultivars (Fig. 10). Furthermore, while 3 isolates (P-1, PM-1 and B-2) which were low in virulence on all 4 cultivars produced abundant conidia in culture, many other high sporulating isolates ranged from low to high in virulence indicating that there is no relationship between virulence

Fig. 10. Relationship of morphological category (see Table 2) to virulence of 49 isolates of C. purpurea. Virulence data from Ratanopas 1973. Disease rating: 0 = nonpathogenic, 1 = very low virulence, 2 = low virulence, 3 = moderate virulence, 4 = high virulence, 5 = very high virulence. For host code of isolates, see Table 1.

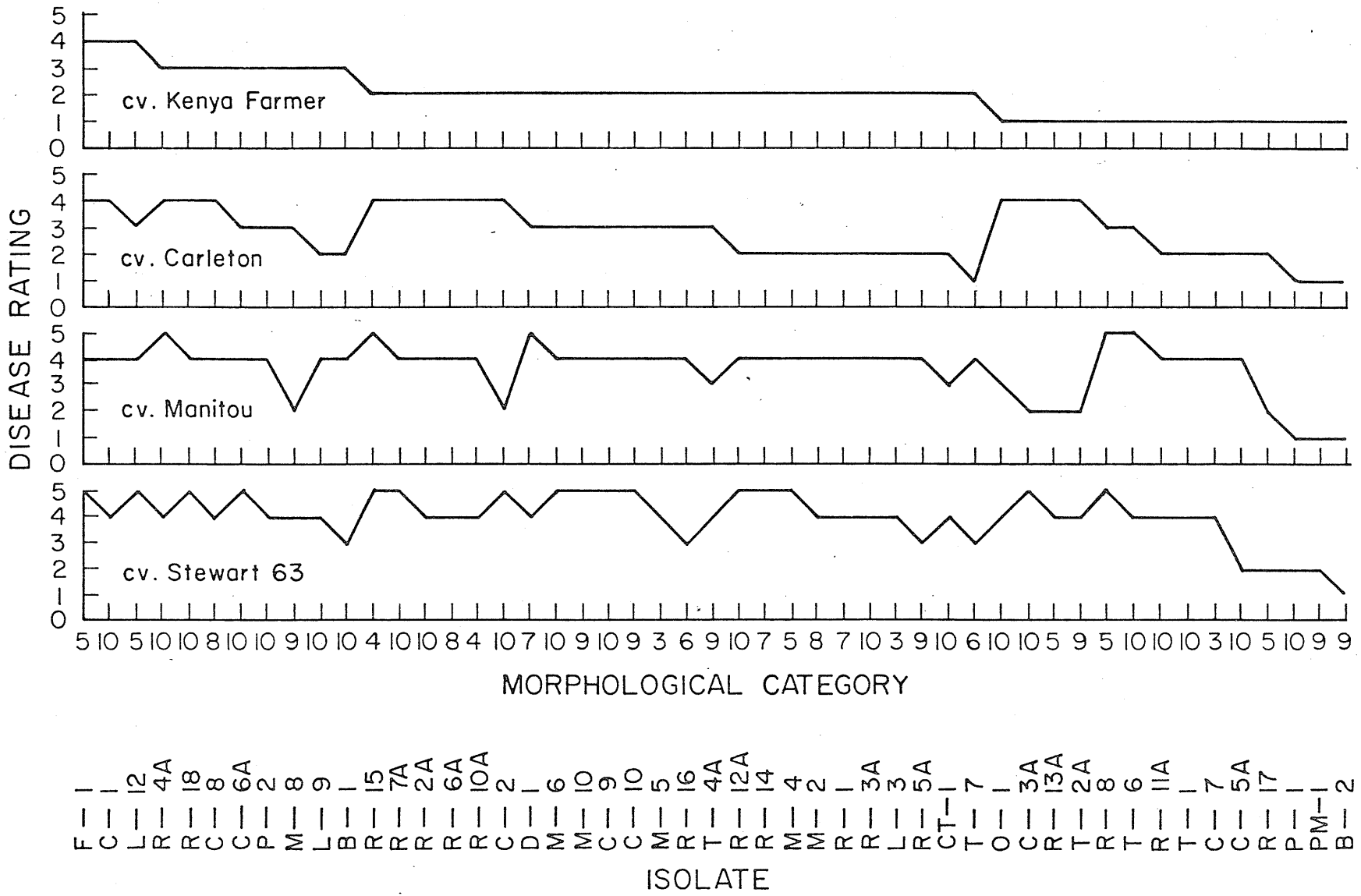

DISEASE RATING
cv. Kenya Farmer
cv. Carleton
cv. Manitou
cv. Stewart 63
5 10 5 10 10 8 10 10 9 10 10 4 10 10 8 4 10 7 10 9 10 9 3 6 9 10 7 5 8 7 10 3 9 10 6 10 10 5 9 5 10 10 10 3 10 5 10 9 9
MORPHOLOGICAL CATEGORY
F — C — L — R — R — C — C — P — M — L — B — R — R — R — R — C — D — M — M — C — C — M — R — T — R — R — M — M — R — R — L — R — CT — T — O — C — R — T — R — T — R — T — C — C — R — P — PM — B —
12 4A 18 8 6A 2 8 9 — 15 7A 2A 6A 10A 2 — 6 10 9 10 5 16 4A 12A 14 4 2 — 3A 3 5A — 7 — 3A 13A 2A 8 6 11A — 7 5A 17 — 1 — 2
ISOLATE

Fig. 10

and ability to sporulate on T2 medium.

To determine whether the sectors would remain stable upon subculturing, mycelium from normal and sectored areas from cultures of each of 13 isolates was transferred to fresh T2 medium. All subcultures were phenotypically identical to the original source of inoculum with the exception of one plate originating from the violet sector of isolate R-2A (Fig. 5A), which differed from the original in producing horn-like structures (Figs. 7 and 9). These structures were composed of masses of conidia adhering together but were larger than the small, globose conidial structures observed previously. However, the horn-like structures failed to form when used as inoculum on T2 medium and did not reappear until the fifth subculture indicating that their formation is probably influenced by other factors.

DISCUSSION

A wide variation in cultural characteristics was observed in this study among the relatively large number of isolates of C. purpurea from the Canadian Prairies. These results are in agreement with those obtained with ergot isolates from Europe (Amici et al. 1967b; Grein 1967). Phenotypic expression depends, among other factors, on the medium, and an asparagine and high sucrose medium clearly favors greater expression of cultural variation of ergot isolates than does more media such as malt extract agar and potato dextrose agar.

On the basis of colony characteristics, the ergot isolates were grouped into 10 cultural types. However, none of the cultural characteristics observed could be related to host origin or virulence of the isolates. Nevertheless, the study did reveal several aspects of C. purpurea which are worthy of discussion, namely the formation of plectenchymatic type of growth, conidial mass and horn formation, and the occurrence of sectoring. The most striking characteristic was the highly raised, plectenchymatic type of growth produced by some isolates as opposed to the flat mycelial type normally observed on malt extract agar. Thickening of the mycelial mat occurred after the production of conidia and appears analogous to the development of sclerotia in host florets following the sphacelial stage. Sclerotial size is influenced by the isolate as well as by the host (Ratanopas 1973;

Platford and Bernier 1976) and it appears worthwhile to determine whether sclerotial size might be related to the degree of mycelial mat thickening in culture. Furthermore, alkaloid production has been shown to be correlated with thick, plectenchymatic fungal growth in either surface or submerged cultures (Mantle and Tonolo 1968) and the physiological function of this type of growth would also warrant further investigation.

The formation of globose conidial masses and conidial horns by a few ergot isolates does not appear to have been reported previously. Grein (1967), however, found upon microscopic examination of young mycelial mats that conidia adhere together in small masses. Perhaps in cultures of some isolates these microscopic structures continue to develop until they form visible conidial masses. If so, conidial horns might simply be large, extended conidial masses. The morphology of these unusual structures as well as the factors affecting their formation were the subject of a separate investigation, the results of which will be reported elsewhere (Thesis section 2).

The 10% frequency of sectoring observed in this study among sclerotial isolates was considerably less than that reported by Spalla et al. (1969) and Strnadová and Kybal (1974), who found, respectively, that 78% and 34% of ergot colonies formed evident sectors on T2 medium. This discrepancy might be due to the fact that in this study,

the isolates had been subcultured several times by mass transfers on malt extract agar prior to their use, whereas in the European studies, colonies were derived directly from sclerotia plated on T2 medium. Spalla et al. (1969) attributed the formation of sectors to the segregation of nuclei in the mycelium and claimed that sclerotia formed by C. purpurea on host plants are heterokaryotic. On the other hand, Strnadová and Kybal (1974) observed sectors in cultures derived from individual conidia and attributed sectoring to mutation during growth of the colony and concluded that ergot sclerotia are commonly homokaryotic. The fact that in this study, sectoring did occur in cultures of monoascospore isolates and that the frequency was about equal to that of cultures derived from sclerotia, indicates that sectoring is due to mutation.

The observations on sectoring in this study do not allow any conclusion regarding the genetic nature of ergot sclerotia. However, the variability of 20 monoascosporic isolates from a single sclerotium appears too great to be ascribed to mutation alone and might be more properly ascribed to the presence of two or more genetically different nuclei in the mycelium of the parent sclerotium. As theorized by Spalla et al. (1969), the heterokaryotic nature of sclerotia might arise after infection of florets by high numbers of conidia or ascospores, followed by anastomoses and exchange of nuclei between hyphae originating

from genetically different spores. Thus, variation among monoascosporic isolates might be due to recombination of genetically different nuclei during the sexual stage of the fungus. The wide variation of C. purpurea observed in this and other studies (Grein 1967; Spalla et al. 1969; Ratanopas 1973) tends to support this theory and suggests a fruitful area for further investigation.

2. Development, Morphology and Histology of Conidial Horns Produced by Claviceps purpurea in Culture and Factors Affecting their Formation.

ABSTRACT

Cultures of a monoascosporic isolate (R-37C) of Claviceps purpurea (Fr.) Tul. on media containing asparagine and a high concentration of sucrose (T2 medium) developed either conidial horns, small globose conidial masses or a thick plectenchymatic mycelial mat. Globose conidial masses and conidial horns appeared to originate from microscopic conidial masses. Twenty-day old horns disintegrated readily in water whereas older horns did not. In mature horns the conidia lost their oval shape and became rounded and compacted.

Extracts prepared by suspending young conidial horns in water for 15 minutes contained fructose, glucose, sucrose and one unidentified sugar suggesting that sugars might be involved in the adhesion of conidia in the horns. However, no conclusions could be made regarding the similarity of the sugars in conidial horn extracts and honeydew.

Conidial structures occurred more consistently and abundantly on media adjusted to pH 7 and prepared with either tap water or deionized water containing mineral salts similar to that in tap water. Thick plentenchymatic mycelial mats appeared to resemble tissue of ergot sclerotia. Small,

black, hemispherical structures were occasionally observed among conidial horns and were also composed of plectenchymatic tissue.

INTRODUCTION

Claviceps purpurea (Fr.) Tul. is an ascomycetous fungus causing ergot of cereals and grasses. The fungus infects the ovaries and within 5-6 days produces honeydew and conidia, followed by the development of frequently prominent purplish-black sclerotia. The sexual stage of the fungus occurs in the stromata of germinating sclerotia yielding ascospores. The amount of honeydew produced and the size of the sclerotia are determined by the host as well as by the fungus (Ratanopas 1973; Platford and Bernier 1976). The fungus can be grown readily in culture producing abundant mycelium and conidia, but not sclerotia. Mycelial growth is white and nondescript on common media such as malt agar and potato dextrose agar. However,on media containing asparagine and a high concentration of sucrose, ergot isolates display wide variation in growth types (Thesis section 1; Amici et al. 1967a).

The formation of honeydew conidia occurs in sporodochia consisting of a compact layer of conidiophores bearing conidia terminally (Barnett 1972). Conidial production in culture was reported to occur on short conidiophores, arranged in a rosette-like structure (Grein 1967). Alkaloid production by C. purpurea in liquid culture has been extensively investigated (Bové 1970). However, sclerotial development in culture has not been achieved although the formation of sclerotium-like structures has been reported

(Bonns 1921; Kirchhoff 1929; McCrea 1930; Schweizer 1941).

During studies on the variability of C. purpurea in culture, one fungal isolate was found to produce prominent, horn-like structures composed of conidia (Thesis section 1). Conidial horns did not form consistently and do not appear to have been reported previously.

This communication reports on: 1) the development, morphology and histology of conidial horns; 2) factors affecting their formation; and 3) the presence of sugars in conidial horns.

MATERIALS AND METHODS

Origin of Isolate Producing Conidial Horns

A single monoconidial isolate of C. purpurea (R-37C), previously found to produce conidial horns in culture (Thesis section 1), was used throughout this study. This isolate was the only isolate among 14 monoconidial isolates of R-2AH which produced conidial horns on sucrose-asparagine medium (T2 medium). Isolate R-2AH originated from the violet sector of isolate R-2A cultured on T2 medium. Isolate R-2A itself did not produce conidial horns and originated as a single ascospore isolate from a rye sclerotium collected at Clear Water Bay, Ontario in 1968.

Preparation of Stock Cultures and Inoculum

Stock cultures of isolate R-37C were maintained on a defined glucose-asparagine agar medium (T medium) and prepared about every three months using conidial horns produced on T2 medium as inoculum. Cultures in 6 cm pyrex Petri dishes were incubated 20 days at 25° C in the dark and then stored at 5° C.

To prepare inoculum, conidia from stock cultures were transferred to 5 ml aliquots of sterile distilled water in test tubes, and shaken vigorously to dislodge spores. The spore suspensions were filtered through sterile cheese cloth to remove the mycelium and adjusted to a concentration of

10^6 conidia/ml.

The experimental cultures were prepared by applying 10 µl of spore suspension to a sterile 6 mm Whatman No. 1 filter paper disc. This was then placed in the center of a 6 cm pyrex Petri dish containing 12 ml of the test medium. Five replications per treatment were employed unless otherwise indicated. The cultures were incubated at 25^{o} C and observations made periodically.

Culture Media

Stock culture medium - The medium was a glucose-asparagine defined medium (T medium) modified from Taber and Vining (1957) and contained the following constituents: glucose, 15 g; L-asparagine, 2.36 g; K_2HPO_4, 100 mg; KH_2PO_4, 700 mg; $MgSO_4.7H_2O$, 201 mg; $CaCl_2.2H_2O$, 5.95 mg; $FeSO_4.7H_2O$, 5 mg; $ZnSO_4.7H_2O$, 4.4 mg; $MnSO_4.H_2O$, 2.75 mg; NaCl, 2.25 mg; $CuSO_4.5H_2O$, 0.27 mg; $(NH_4)_6Mo_7O_{24}.4H_2O$, 1.82 mg; agar, 20 g; glass distilled water to 1000 ml, pH adjusted to 5.2 before autoclaving.

Standard medium - The medium is a sucrose-asparagine medium (T2 medium) which was employed by Amici et al. (1967a). It consists of the following ingredients (g/l): sucrose, 100; L-asparagine, 10; yeast extract, 0.1; KH_2PO_4, 0.25; $MgSO_4.7H_2O$, 0.25; KCl, 0.12; $Ca(NO_3)_2.4H_2O$, 1; $FeSO_4.7H_2O$, 0.02; $ZnSO_4.7H_2O$, 0.015; agar, 20; tap water to 1000 ml, pH adjusted to 5.2 before autoclaving.

Basal medium - To compare the effects of mineral salt formulation on the formation of conidial horns the major ingredients of T2 medium in deionized water were used as a basal medium. Two salt mixtures developed for maximum alkaloid production by C. paspali Stevens and Hall were compared to T2 medium. The mixture created by Mary et al. (1965), designated as TR medium contained the following (mg/l): $AlCl_3.6H_2O$, 0.054; $Ca(NO_3)_2.4H_2O$, 300; $CuSO_4.5H_2O$, 7.45; $FeSO_4.7H_2O$, 53.3; H_3BO_3, 2.5; KCl, 219; KI, 0.75; $MnSO_4.4H_2O$, 9; $NaNO_3$, 120; Na_2SO_4, 200; $ZnSO_4.7H_2O$, 5.3. That developed by Mizrahi and Miller (1970), designated as TM medium contained the following (mg/l): $AlCl_3.6H_2O$, 5; $CaCl_2$, 100; $Ca(NO_3)_2.4H_2O$, 500; $CoSO_4.7H_2O$, 1; $FeSO_4.7H_2O$, 1; KH_2PO_4, 2000; $MgSO_4.7H_2O$, 300; $NaNO_3$, 100; $ZnSO_4.7H_2O$, 10. The pH was adjusted as required with HCl or NaOH prior to autoclaving at 110^{o} C for 20 minutes.

Measurement of Fungal Structures

The size of fungal structures was determined by measuring 25 samples of each structure with the aid of a micrometer and dissecting microscope, and the results expressed as the mean and standard error.

Factors Affecting Formation of Conidial Horns

The effects of pH, purity of water, agar concentration and mineral salts were investigated. Details of the method are given in the result section.

Infectivity Test

Inoculum was prepared by transferring young conidial horns to 5 ml of sterile distilled water and shaking vigorously to dislodge the conidia. The conidial suspension was adjusted to 10^4 conidia/ml for inoculation. Infectivity was assessed on the four wheat cultivars, Kenya Farmer, Carleton, Manitou and Stewart 63 in the greenhouse. Five heads were inoculated for each cultivar at 10 florets/head using a hypodermic needle and syringe (Platford and Bernier 1970). Host reaction was evaluated as described by Platford and Bernier (1970; Appendix 3).

Histology

Samples of fungal structures were fixed in a solution of Formalin-Propiono-Alcohol (FPA) 5:5:90 by volume and embedded in paraffin blocks by the butyl alcohol method. The material was then sectioned with a rotary microtome and stained with Conant's quadruple stain (Johansen 1940).

Determination of Extracellular Sugars in Conidial Horns

The presence of sugars in conidial horns was determined by Benedict's test (Harrow et al. 1967; Appendix 4), followed by thin-layer chromatography. The presence of sugars in ergot honeydew was also determined for comparison.

Samples were prepared from young, 20 day old conidial horns and from 15 day old honeydew produced on Hercules

durum wheat by HD-21 isolate. Conidial horns were removed by using a fine inoculating needle under a dissecting microscope taking care not to include any mycelium or medium. They were suspended in distilled water in the amount of 1 g/10 ml. Honeydew was diluted with distilled water in the amount of 1 g/120 ml. After 15 minutes, the spores from each suspension were removed by low-speed centrifugation, and the clear liquid was passed through a sterile membrane filter with 0.02 nm pore size. The filtrate was stored at 5^{o} C until used.

Cellulose powder MN 300 was used to prepare the plates (Schweiger 1962). Fifteen grams of cellulose was homogenized in a blender with 90 ml distilled water for 60 seconds. The suspension was applied onto five glass plates, 20 x 20 cm, with the Desaga spreader adjusted to 250µ in thickness for the layer.

Reference standard sugars were prepared as follows: 0.01 g/10 ml distilled water for L(+) arabinose, fructose, D(+)mannose, D(+)galactose, and glucose; 0.02 g/10 ml distilled water for sucrose and maltose. Five microliters of each sugar solution and of the filtrate from honeydew and 10 microliters of the filtrate from conidial horn extract were used to spot the plate.

Samples and standard sugars were applied as spots to the base line, at 2 cm from the edge of the plate, with the aid of a micropipette. After drying, the plates were

developed by ascending chromatography using a mixture of distilled water with freshly distilled ethyl acetate and pyridine in a ratio of 25:100:35 (Raadsveld and Klomp 1971). Each chromatographic run was made three times, and dried with warm air between runs. The running distance from the origin to the solvent front was 15 cm. After the third run, the sugar spots were visualized by spraying the plates with the following reagents: 1. silver nitrate-sodium hydroxide followed by diluted sodium thiosulfate solution (Randerath 1966; Stahl 1969); 2. p-anisidine phosphate in ethanol (Mukherjee and Srivastava 1952); 3. p-aminobenzoic acid reagent (Ersser and Andrew 1971); 4. aniline-phosphoric acid (Raadsveld and Klomp 1971); 5. benzidine-trichloroacetic acid (Stahl 1969). The preparation of these spray reagents is outlined in Appendix 5.

The Rg values were used to determine the movement of the sugars on the thin layer plates. They were calculated from the formula:

$$Rg = \frac{\text{Distance substance travels from origin}}{\text{Distance glucose travels from origin}} \times 100$$

RESULTS

Development and Morphology of Conidial Horns

The selected isolate was tested initially on three lots of T2 medium. Conidial horns (Figs. 11, 13) formed in cultures on only one lot of the medium. In cultures of the other two lots only small globular and slightly raised conidial masses (Fig. 15) were produced on the mycelial mat. In cultures that did not form either the globular conidial masses or conidial horns, the mycelial mat thickened and became plectenchymatic (Fig. 17). When mature, the mycelial mat tended to split in the center of the colony exposing the thick layer of plectenchyma (Fig. 17).

The conidial horns became visible 10-15 days after inoculation and were fully developed by about 20 days. Conidial horns consisted of a straw-yellow sticky conidial masses. From microscopic observation of several young cultures, it appeared that the structures began as very small straw-yellow masses, scattered over the entire surface of the colony , consisting of many conidia (Fig. 14). These structures appeared to develop into globular conidial masses or conidial horns. Conidial horns are straight when short but slightly curved at the tip when long (Figs. 11, 13). Conidial horns of different ages were transferred, using a fine needle, to a drop of water on a glass slide for microscopic examination. Young conidial horns readily disintegrated in water (Fig. 16). Mature

horns, on the other hand, were firm and did not collapse in the presence of water (Fig. 13). Mature conidial horns were 1506 ± 114μ in height and 659 ± 36μ in diameter.

Small, black structures occasionally formed in cultures that developed conidial horns (Figs. 11, 12). Such structures had not been observed previously in cultures of this isolate or of other isolates (Thesis section 1). These structures were found to be soft, hemispherical, embedded in the mycelial mat and measured on the average 779±76μ in height and 1054±72μ in diameter. Samples of the above three structures were collected and preserved in a solution of FPA 5:5:90 for histological examination later.

Histology of Fungal Structures

Longitudinal sections of mature conidial horns revealed that they appear to form from closely interwoven sporogenous hyphae located under fluffy aerial mycelium (Figs. 18, 19). During growth, the conidial masses appear to push through the aerial mycelial mat and gradually become horn-like structures. Conidiophores are very difficult to observe from the sections, and they are probably short and closely interwoven (Fig. 19). The conidia lose their oval shape and become rounded and compacted (Fig. 20). Examination of cross-sections of the small black, hemispherical structures indicate that they are composed of plectenchymatic tissue (Fig. 21). Sections of thickened mycelial mat also resembled plectenchymatic tissue

(Fig. 22). However, the cells of both tissues are larger and not as uniform in size as cells from sclerotial tissue (Fig. 23). The plectenchymatic tissues of these two structures are very similar to that of the cross section of an ergot sclerotium (Fig. 23).

Infectivity of Conidia From Conidial Horns

The results of the infectivity test indicate that conidia from young horns are infectious: 5, 0, 16 and 22 sclerotia were produced on 50 inoculated florets of the cultivars Kenya Farmer, Carleton, Manitou and Stewart 63 respectively.

Factors Affecting the Formation of Conidial Horns

Effect of pH - To determine the effect of pH on the formation of conidial horns, T2 medium was adjusted to five pH levels ranging from 5.2 to 8. The experiment was repeated three times using a separate lot of medium each time. Five Petri dishes were inoculated for each experiment.

The results differed in each experiment except at pH 8 where mycelial mats were consistently produced. At pH 7, conidial horns were formed in all three experiments although relatively few horns occurred in one experiment. At pH 5.2, conidial horns were formed in one experiment only. There were no conidial horns at pH 5.5 and 6, however, at both pH values, small conidial masses were formed in two experiments, whereas only mycelial mats were produced in the other

Figs. 11-17. Cultural, morphological and histological characteristics of a conidial horn producing isolate of C. purpurea (R-37C). Fig. 11. Conidial horns and small black globose structures produced on T2 agar medium (x6). Fig. 12. Longitudinal section of a small black hemispherical structure (x20). Fig. 13. Longitudinal section of a mature conidal horn (x20). Fig. 14. A microscopic conidial mass (x100), likely the origin of a conidial horn or a small globose conidial mass. Fig. 15. Small globose conidial masses (x15). Fig. 16. Oval shaped conidia of a disintegrated young conidial horn mounted in water (x400). Fig. 17. Plectenchymatic mycelial mat of isolate R-37C on T2 agar medium.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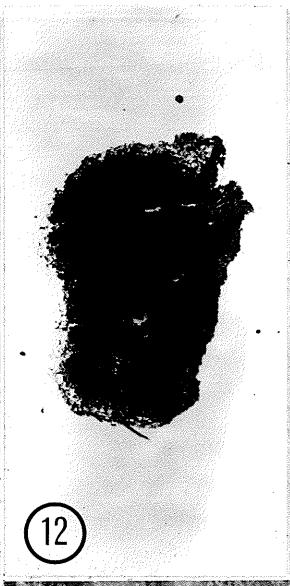
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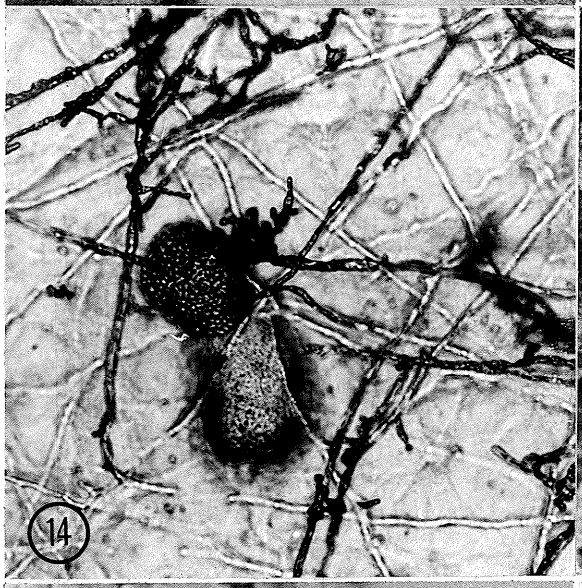
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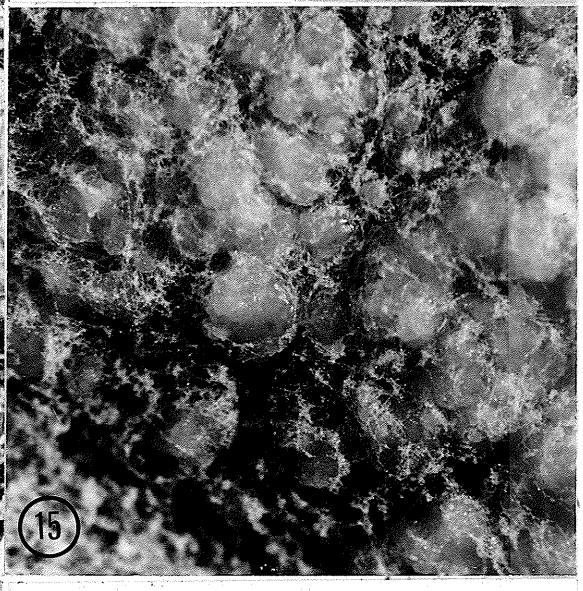
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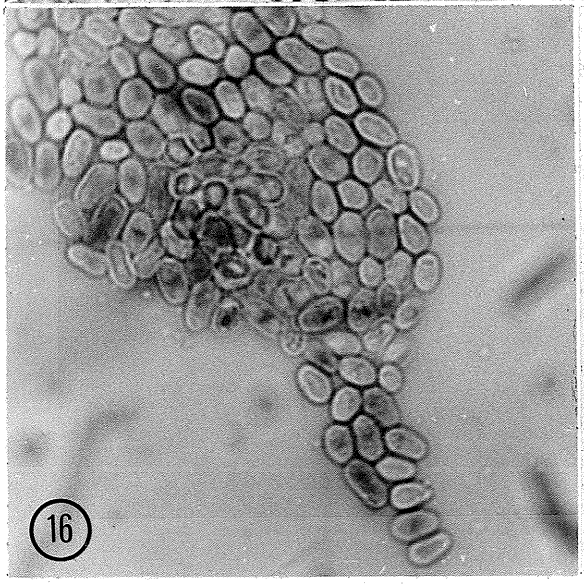
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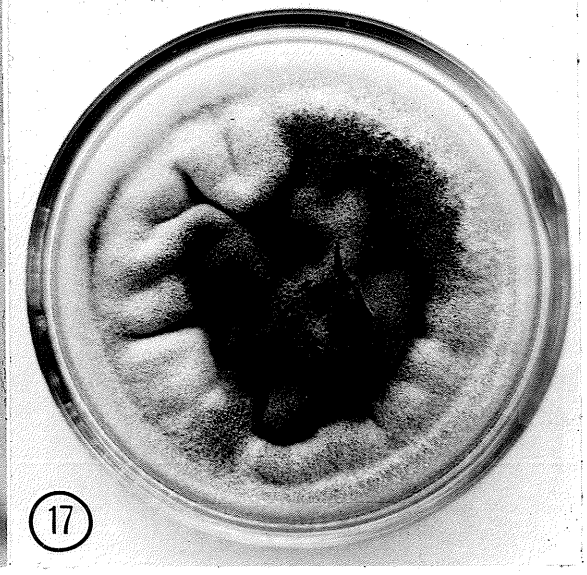
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Figs. 18-23. Histological characteristics of isolate R-37C of C. purpurea. Fig. 18. Longitudinal section of conidial horn (x20) showing the horns attached to the sporogenous hyphae on the T2 agar medium.
Fig. 19. Section at the base of a conidial horn showing sporogenous hyphae (x400). Fig. 20. Longitudinal section of a mature conidial horn (x400). Fig. 21. Cross section of small black hemispherical structure (x400). Fig. 22. Cross section of plectenchymatic mycelial mat (x400). Fig. 23. Cross section of the central portion of an ergot sclerotium (x400).


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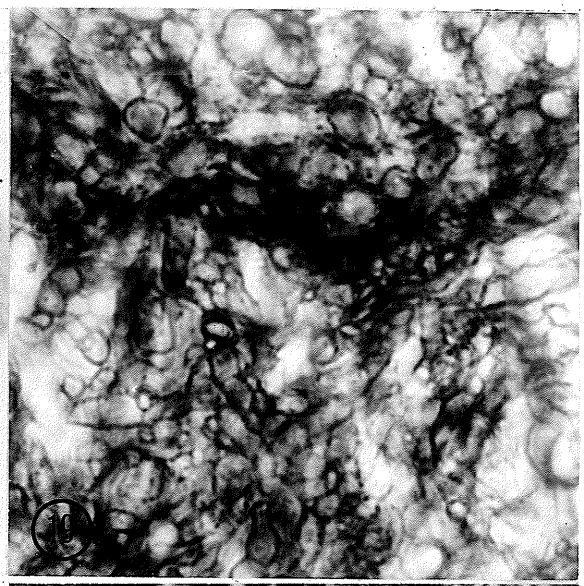
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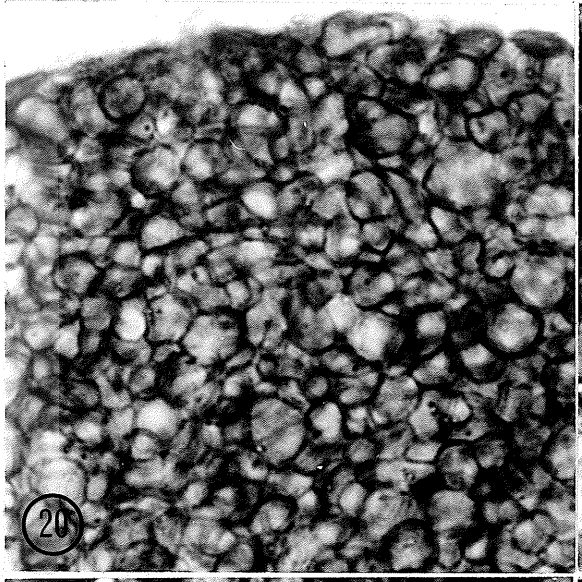
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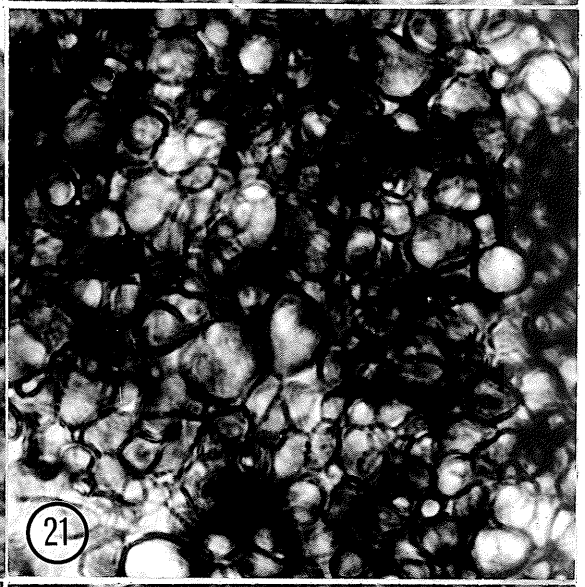
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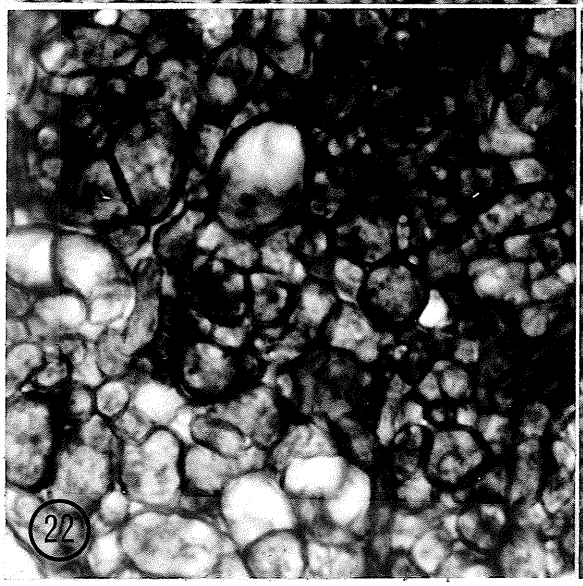
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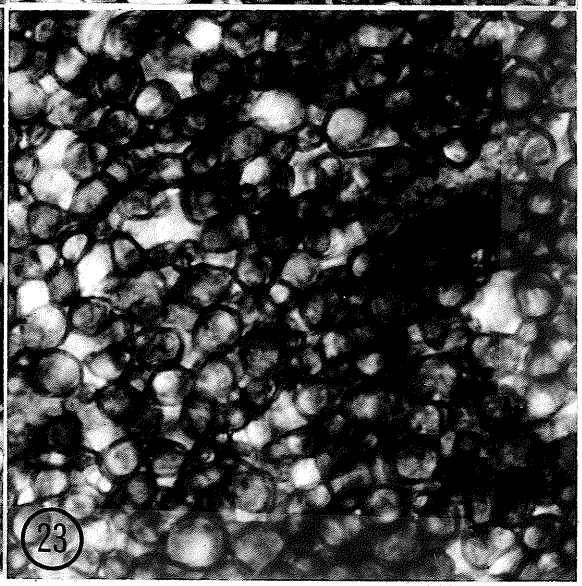
23

experiment.

Effect of water purity - T2 medium prepared with either tap water or glass-distilled water, was compared at pH 5.2 and 7 for its ability to support the formation of conidial horns. Four experiments were conducted using a different lot of medium each time.

Conidial horns developed only on T2 medium prepared with tap water and were considerably more abundant on media adjusted to pH 7 than at pH 5.2 (Table 3). The formation of conidial horns varied with each lot of T2 medium tested. No conidial structures formed on media made with glass distilled water at any pH.

Effect of agar concentration - Agar concentrations of 2, 3, 4, 5, and 6% were compared in two separate experiments with T2 medium at pH 7. Agar concentration had no apparent effect on the formation of conidial horns. A few conidial horns developed at all agar concentrations in one experiment whereas in the other, small conidial masses developed at all agar concentrations.

Effect of water purity, agar concentration, and pH - The results of four experiments are summarized in Table 4. Conidial horns were produced consistently and abundantly only on T2 medium prepared with tap water and 2% agar at pH 7. T2 prepared with tap water and 4% agar at pH 7 supported conidial horn formation in three experiments, whereas that at pH 5.2 gave rise to a few horns in

Table 3. Effect of water purity and pH on the development of conidial horns in T2 medium.

Experiment	Growth characteristics[1/]			
	Tap water		glass distilled water	
	pH5.2	pH7	pH5.2	pH7
1	H	HHH	M	M
2	C	H	M	M
3	M	C	M	M
4	M	HH	M	M

1/ C = small conidial masses.
M = mycelial mat only.
H = predominantly conidial horns (H, few; HH, many; HHH, very abundant).

Table 4. The effect of pH, water purity and agar concentration on the development of conidial horns.

Treatment	Growth characteristics[1/] Experiment 1	2	3	4
pH 5.2				
T2-tap water, 2% agar	M	H	H	C
T2-tap water, 4% agar	M	C	H	M
T2-glass distilled water, 2% agar	M	M	C	M
T2-glass distilled water, 4% agar	M	M	C	M
PH 7				
T2-tap water, 2% agar	HH	HH	HH	HH
T2-tap water, 4% agar	H	H	H	C
T2-glass distilled water, 2% agar	M	C	M	M
T2-glass distilled water, 4% agar	C	C	H	M

1/ C = small conidial masses.
M = mycelial mat.
H = predominantly conidial horns (H, few; HH, many; HHH, very abundant).

one experiment only. Medium T2 prepared with glass distilled water and 4% agar at pH 7 supported a few horns in one experiment. This had not been found in previous tests.

Effect of tap water sampled at different times - Although T2 medium prepared with tap water and adjusted to pH 7 generally supported the formation of many horns, their formation still remained erratic. To determine if the variability might be due to differences in the quality of the water, tap water drawn at monthly intervals was compared in T2 medium at pH 7. Conidial horns formed on media prepared with water from all sampling dates but one. However, the number of horns produced varied with the sampling date as follows: April, 1974, no conidial horns; May, 1974, very abundant horns; September and October, 1974, few horns; November and December, 1974, many horns; January, 1975, very abundant horns. This indicated that water quality might be responsible, in part, for the inconsistencies observed previously. To verify whether water quality might be a factor, water samples drawn on December, 1974 and January, 1975 were analysed for several cations. Tap water analysis for December and January, respectively, revealed the presence and the amount of the following cations (ppm): K, 1.19 and 1.3; Ca, 14.5 and 14.1; Mg, 8.92 and 8.90; Fe, 0.02 and 0.02; Cu, 0.04 and 0.16; and Zn, 0.00 and 0.04.

Effect of mineral salts - In view of the above results, a third salt mixture was formulated on the basis

of the analysis of the tap water sample taken in January, 1975 and compared with TR and TM media in 3 separate experiments. The concentration of the cations was adjusted to the values determined in analysis of tap water using the following salts (mg/l): $Ca(NO_3)_2 \cdot 4H_2O$, 83.0; $CuSO_4 \cdot 5H_2O$, 0.62; $FeSO_4 \cdot 7H_2O$, 0.09; KH_2PO_4, 4.5; $MgSO_4 \cdot 7H_2O$, 90.2; $ZnSO_4 \cdot 7H_2O$, 0.18. These salts were combined with salts from T2 medium, added to the basal medium and designated as TD medium. T2 medium prepared with tap water drawn in January, 1975 and with deionized water served as controls. All media were adjusted to pH 7.

Of the three test media, only TD medium consistently supported the formation of conidial horns (Table 5). However, in two experiments, fewer conidial horns were produced than on T2 medium made with tap water. T2 prepared with deionized distilled water appears to be superior to TR and TM media in that conidial masses were produced in two experiments and conidial horns in the other, whereas only mycelial mats without conidia were produced on the later two media.

The Presence of Sugars in the Conidial Horns

Thin layer chromatographic analysis indicated the presence of four sugars in the extracts of conidial horns. The Rg-value and the color reactions of each sugar to several reagents are given in Table 6. Three of the four sugars could be detected by four of the five reagents used and by

Table 5. Formation of conidial horns on various salts media.

Media (pH 7)	Growth characteristics[1/] Experiment		
	1	2	3
T2-tap water[2/]	HHH	HHH	HHH
T2-deionized distilled water	H	C	C
TD	HH	HHH	H
TR	M	M	M
TM	M	M	M

1/ C = small conidial masses; M = mycelial mat only; H = predominantly conidial horns (H, few; HH, many; HHH, very abundant).

2/ Same lot of T2 medium, prepared with tap water drawn in January, 1975, was used in all three experiments.

TD = Basal medium + mineral salts comparable to tap water.

TR = Basal medium + Mary's salts.

TM = Basal medium + Mizrahi's salts.

Table 6. Average Rg values and color reactions for various sugars in extracts of conidial horn and ergot honeydew.

Sugar	Rg value[1/]	Color reaction with detecting agent[2/]				
		A	B	C	D	E
Reference sugars:						
Fructose	120	brown	brown	lemon yellow	brown	brown
Glucose	100	brown	brown	brown	brown	brown
Sucrose	73	-	brown	brownish yellow	brown	-
Maltose	61	brown	brown	brown	brown	brown
Conidial horns:						
Sugar 1	119	brown	brown	lemon yellow	brown	brown
Sugar 2	100	brown	brown	brown	brown	brown
Sugar 3	73	-	-	brownish yellow	-	-
Sugar 4	59	brown	pink brown	brown	brown	brown
Honeydew:						
Sugar 1	120	brown	brown	lemon yellow	brown	brown
Sugar 2	100	brown	brown	brown	brown	brown

1/ Average Rg values were determined from 10 chromatograms detected with p-anisidine.

2/ A= silver nitrate, B = p-aminobenzoic acid, C = p-anisidine, D = aniline phosphoric acid, E = benzidine trichloroacetic acid.

p-anisidine only. Rg-values of sugars detected with all detecting agents employed were similar. Since p-anisidine could detect more sugars, the average Rg-value of sugars from 10 chromatograms sprayed with this reagent are presented in Table 6. All four sugars separated well with the solvents used. On the basis of Rg-values and color reactions, three sugars were identified as fructose, glucose, sucrose and one having Rg-value of 59 remained unidentified. However, the spot of this unidentified sugar appeared as pink-brown on the chromatogram sprayed with p-aminobenzoic acid indicating it might be isomaltose as reported by Ersser and Andrew (1971). Only two sugars, fructose and glucose, could be detected from the honeydew extract tested.

DISCUSSION

The results of the present study indicate that the ability of isolate R-37C to form conidial horns can be maintained and improved by culturing on T2 medium adjusted to pH 7 and prepared with either tap water or deionized water containing mineral salts similar to those in tap water. Microscopic examination of young cultures revealed the presence of very small conidial masses which appeared to give rise to either globose conidial masses or conidial horns. The microscopic conidial masses appeared to be similar to the "globose bodies of conidial masses" reported by Grein (1976). However, Grein did not indicate having seen conidial masses visible to the naked eye. Conidial horns appeared to develop from interwoven sporogenous hyphae at the base of the horns. Although conidiophores are difficult to observe, the sporogenous hyphae are likely short conidiophores. Conidia and honeydew of C. purpurea are formed on the host in sporodochia consisting of a compact layer of conidiophores bearing conidia terminally (Barnett 1972). Thus, conidial horns appear to be sporodochia of this fungus in pure culture.

In addition to pH, the production of conidial structures was greatly influenced by the mineral salt content of the media. T2 medium prepared with tap water proved superior to that with distilled water; however, the

requirement of the isolate for salts was only partly satisfied by a formulation based on cations found in tap water. This suggests that trace elements or some other factors present in tap water might be involved. The fact that two salts formulations (TR and TM) containing additional cations, did not support the production of conidial horns, indicates that the fungus does not require complex salts for the formation of conidial structures.

It is evident from the data in Table 3, 4 and 5, that the production of conidial structures is influenced by factors other than pH and mineral salts, which could not be determined in these studies.

It is apparent from the results of these studies that factors unfavorable for the production of conidial structures i.e. high or low pH, and the use of glass-distilled water, favored the development of a thick mycelial mat observed frequently by several workers (Thesis section 1; Amici, et al. 1967a; Johansson 1964b) and recently shown to be associated with the production of alkaloids in culture (Mantle 1969). Histologically, the thick mycelial mat was found to be composed of plectenchymatic tissue and to resemble the tissue of sclerotia. These results are in agreement with the finding of several workers (Bonns 1922; Johansson 1964b; Mantle and Tonolo 1968; Mantle 1969). The small, black, globose structures occasionally observed among the conidial horns were also composed of plectenchymatic tissue

and might be small sectors of thickened mycelial mat.

Four sugars were found in the water extract of young conidial horns; they were fructose, glucose, sucrose and one unidentified sugar. Although only fructose and glucose were detected in the honeydew extract tested in this study, these, as well as sucrose, have been reported as the major sugars in honeydew (Fuchs and Pöhm 1953; Mantle 1965). However, sucrose was found in very small amounts only (Fuchs and Pöhm 1953) and failure to detect it in this study might be due to its presence in quantities too low to be detected. It is also possible it is not produced by the isolate tested, since the sugars present in honeydew have been shown to vary qualitatively within a species of Claviceps (Mower 1974). The mycelial mat of C. purpurea was also reported to contain glucose, fructose, trehalose, mannose and mannitol (Grein 1967; Vining and Taber 1964).

Since extracts were prepared by suspending young conidial horns in water for 15 minutes it would appear that the sugars are present in the horns extracellularly and thus might be involved in conidial adhesion. However, further work is required to determine if the sugary material of the conidial horns is identical to honeydew produced by this fungus on cereal hosts.

The low incidence of conidial structure formation among isolates of C. purpurea observed previously (Thesis section 1) might be attributed to the use of culture media

unsuitable for their development. A better appraisal of the ability of ergot isolates to produce conidial structures would be attained by testing ergot isolates on the medium defined in this study.

3. Influence of Selected Amino Acids on Growth and Conidial Production of *Claviceps purpurea* in vitro.

ABSTRACT

L-asparagine, L-glutamine and to a lesser extent L-proline were good sources of nitrogen for mycelial growth of five isolates of *Claviceps purpurea* (Fr.) Tul., but supported appreciable conidial production in only two isolates (F-2C and PM-2C). All five isolates grew well on L-tryptophan but only one isolate produced conidia. DL-methionine, DL-valine, DL-alanine and to a lesser extent DL-isoleucine were poor sources of nitrogen for both growth and sporulation of all isolates. Mycelial growth of all the isolates on asparagine was good even at 50 ppm nitrogen but two isolates only (F-2C and PM-2C) produced conidia at levels of nitrogen lower than 500 ppm. β-Alanine and D-alanine were relatively poor sources of nitrogen for growth and sporulation of all five isolates whereas L-alanine was about as good as L-asparagine. In the presence of high levels of L-asparagine, sporulation of several isolates was enhanced by β-alanine and DL-alanine but reduced by D-alanine. At low levels of L-asparagine, β- and D-alanine were highly inhibitory to both growth and sporulation of isolates F-2C and PM-2C. The amino acid analog DL-p-fluorophenylalanine (FPA) was inhibitory to both growth and sporulation of isolates F-2C and PM-2C. The degree of inhibition depended on the concentration of L-asparagine and FPA in the medium.

INTRODUCTION

Ergot caused by the fungus Claviceps purpurea (Fr.) Tul. is recognized on florets of cereals and grasses, by honeydew containing conidia and by purplish-black sclerotia.

The amount of honeydew and the size of the sclerotia are influenced by the fungus as well as by the host. Smaller sclerotia (kernel size) and less honeydew are produced by the fungus on cultivars of spring wheat than on cultivars of rye or triticale (Platford and Bernier 1976). Furthermore, the production of small amounts of honeydew and small sclerotia (smaller than the size of the kernel) in two wheat cultivars, was considered as an expression of host resistance (Platford and Bernier 1970; 1976). Ergot isolates found to produce small amounts of honeydew and small sclerotia on wheat cultivars resistant or susceptible, were considered to be low in virulence (Ratanopas 1973).

Since reduced sporulation is an expression of resistance in the host as well as of low virulence in the pathogen it appeared that knowledge of the nutritional requirements for growth and sporulation in culture might ultimately lead to a better understanding of the phenomenon of reduced sporulation in the host. Although growth and alkaloid production of C. purpurea in liquid culture have been widely investigated (Bové 1970), the nutritional requirements for growth and sporulation on solid media have been largely neglected. Organic sources of nitrogen were

reported to be more readily utilized than inorganic sources with glutamate and asparagine being of equal value in supporting fungal growth (Taber and Vining 1957). Asparagine seems to have been included in most media used in attempting to study the production of alkaloids in liquid culture (Amici et al. 1967a; Gjerstad and Ramstad 1955; Grein 1967; Johansson 1964a; Kirchhoff 1929; Mantle and Tonolo 1968; Strnadová and Kybal 1974). However, the utilization of glutamine for growth and sporulation bears reexamination since it has been reported to be the predominate amino acid in cereal seeds (MacMaster et al. 1971). The recent finding that alanine also occurs in high concentration in developing rye, triticale and wheat seeds (Corbett et al. 1974; Dexter and Dronzek 1975) coupled with the fact that honeydew production is reduced when florets are infected at or after anthesis (Ratanopas 1973) indicate that this amino acid could have an influence on sporulation.

Other naturally occurring plant amino acids have been related to the susceptibility or resistance of plants to diseases (Abdel-Rehim et al. 1968; Kuc et al. 1959; Lakshminaryanan 1955; Strech and Cappellini 1965; van Andel 1966). With ergot, Lewis (1962b) found that DL-α-amino-n-butyric acid, L-arginine HCl, β-alanine, L-glutamic acid, DL- and D-phenylalanine increased the susceptibility of rye seedlings to C. purpurea.

Several amino acids have been reported to have an influence on the development of fungi in culture (van Andel

1966). With Venturia inaequalis (Cke.) Wint., Ross (1968) reported that DL-(α)-alanine, as a nitrogen source, supported good sporulation of certain isolates whereas L-proline, DL(α)-aminobutyric acid and glycine were good nitrogen sources for sporulation of another isolate. DL-p-fluorophenyl-alanine was reported to inhibit sporulation of Ceratocystis ulmi and of Fusarium oxysporum f. lycopersici in liquid culture (Biehn 1973). β-Alanine inhibited the growth of Claviceps microcephala (Wallr.) Tul. (Singh et al. 1972), of Alternaria solani Kuhn (Lewis 1957), and of Neurospora crassa (Herrmann and White 1966).

This paper reports on the influence of selected amino acids on growth and sporulation of five isolates of C. purpurea, which were either high or low in virulence.

MATERIALS AND METHODS

Isolates

Five monoconidial isolates of C. purpurea were selected on the basis of their pathogenicity on the spring wheat cultivar Manitou. Isolates C-25C, R-37C, F-2C were high in virulence whereas PM-2C and R-38C were low in virulence, when rated according to the disease severity scale used previously (Ratanopas 1973; Appendix 6). Isolate R-37C was also selected because of its ability to produce conidial horns (Thesis section 2). The isolates initially were maintained on T2 medium. Later, they were maintained on the synthetic SD2 medium which supports abundant sporulation of each isolate. After incubation for 20 days at 25° C, the cultures were kept at 5° C and used as inoculum within three months.

Media

The standard sucrose-asparagine medium (T2 medium) used previously (Thesis section 1 and 2) was used in the first experiment. Subsequently, a synthetic medium containing sucrose and asparagine (SD2 medium) was developed and utilized. SD2 medium consisted of agar, 20 g; sucrose, 100 g; L-asparagine, 10 g (2118 ppm nitrogen); K_2HPO_4, 100 mg; KH_2PO_4, 700 mg; $MgSO_4.7H_2O$, 4.4 mg; $MnSO_4.H_2O$, 2.75 mg; NaCl, 2.55 mg; $CuSO_4.5H_2O$, 0.27 mg; $(NH_4)_6Mo_7O_{24}.4H_2O$, 1.82 mg;

glass distilled water to 1000 ml. The medium was adjusted to pH 5.2, and autoclaved at 110° C for 20 minutes.

Initially, T2 medium without asparagine was used as a basal medium and is referred to as T2 basal medium. Subsequently, the "standard basal medium" was SD2 medium without asparagine.

Preparation of Amino Acids

Each amino acid ('Baker Analyzed' reagent) was dissolved in glass distilled water and used at a concentration of 500 ppm nitrogen, unless otherwise indicated. These solutions, after adjusting to pH 5.2, were sterilized by filtration through sterile membrane filter (Metricel GA-8) with 0.2μ pore size and added to the basal medium.

Preparation of Inoculum and Method of Inoculation

Conidial suspensions were prepared by transferring a small piece of agar from the stock cultures to 5 ml sterile distilled water in a test tube and shaking vigorously. The conidial suspension was passed through two layers of sterile cheese cloth to remove the mycelial debris and the concentration adjusted to 10^6 conidia/ml.

Disposable sterile plastic Petri dishes (15x60 mm) containing 12 ml of media were inoculated by applying 10 μl of the inoculum onto a sterile 6 mm filter paper disc (Whatman No. 1) which was then placed into the center of each dish. Three plates were used for each treatment, and

each experiment was conducted once. The inoculated plates were incubated at 25° C.

Measurement of Fungal Growth

Fungal growth was assessed by measuring the diameters of the colony 15 days after the first visible mycelial growth. The amount of sporulation was determined by randomly cutting four 7 mm discs containing mycelium and conidia from each Petri dish. These four discs were transferred to a screw-cap vial (3 drams, 19x65 mm), and inactivated by suspending in 5 ml of 1% $CuSO_4$ solution. The vial was then shaken vigorously to dislodge the spores. Five hemacytometer grids, each 1 mm^2, were counted for each replication and the average number of spores expressed as number of conidia/cm^2 of mycelial growth. The significance of the difference between the means was determined by Duncan's multiple range test.

RESULTS

Effect of Selected Amino Acids on Growth and Sporulation

In an initial experiment, four amino acids at 500 ppm nitrogen were compared to asparagine at 2118 ppm nitrogen, as nitrogen sources for growth and conidial production of five isolates of C. purpurea. Each amino acid was added to T2 medium prepared without nitrogen.

Mycelial growth and conidial production varied among the five isolates on the high asparagine medium as well as on the other sources of nitrogen: mycelial growth of isolates PM-2C and R-38C was generally less, whereas conidial production of isolates F-2C and PM-2C was generally greater than that of the other isolates (Table 7). Reducing the concentration of asparagine to 500 ppm significantly reduced conidial production of all but one isolate (F-2C) but had no appreciable effect on the growth of any isolate with the exception of PM-2C. Mycelial growth of isolates R-37C, PM-2C and R-38C was less on glutamine than on asparagine at 500 ppm. However, conidial production of all the isolates was similar in these two amino acids, with the exception of PM-2C on glutamine which was equal to that of asparagine at 2118 ppm nitrogen. Although mycelial growth of isolates PM-2C and R-38C was slightly less on proline than on asparagine (at 500 ppm), proline was an excellent source of nitrogen for sporulation of these two isolates. Two isolates only

Table 7. Effect of selected amino acids as sole nitrogen sources on the growth and sporulation of five isolates of C. purpurea in T2 basal medium.

Amino acid	Isolate									
	C-25C		R-37C		F-2C		PM-2C		R-38C	
	C[1/]	G[1/]	C	G	C	G	C	G	C	G
	x10^4	cm	x10^4	cm	x10^4	cm	x10^4	cm	x10^4	cm
2118 ppm nitrogen										
L-asparagine	2,597a[2/]	4.7a	333a	4.8a	27,234a	4.8a	18,065a	3.7a	286a	4.4a
500 ppm nitrogen										
L-asparagine	195b	4.5ab	0b	4.7a	21,597ab	4.8a	13,364b	3.1b	130b	4.4a
L-glutamine	0b	4.7a	0b	4.3b	19,650b	4.7ab	14,558ab	2.4d	130b	3.6b
L-proline	417b	4.5ab	0b	3.7c	9,637c	4.6b	15,117ab	2.6c	169ab	3.5b
DL-alanine	0b	4.4b	0b	3.8c	0d	4.0c	1,818c	2.5cd	92b	3.0c

1/ C = number of conidia per cm^2 of mycelial growth, G = diameter of mycelial growth in cm; values of C and G are average of 3 plates.

2/ a-d Duncan's multiple range test; values within each column followed by the same letter are not significantly different (p = 0.05).

(PM-2C and R-38C) produced conidia in a medium containing DL-alanine even though mycelial growth of all isolates was fairly good.

In another experiment, seven amino acids were compared at a concentration of 500 ppm nitrogen in the standard, defined basal medium. L-asparagine, L-glutamine and to a lesser extent L-proline, were all good sources of nitrogen for mycelial growth of all the isolates, however they supported appreciable conidial production in only two isolates (Table 8). On the other hand mycelial growth of all the isolates was fairly good on L-tryptophan, but none sporulated on this amino acid. DL-methionine, DL-valine and to a lesser extent DL-isoleucine, were poor sources of nitrogen for both growth and sporulation of all the isolates.

Effect of Decreasing Concentration of L-asparagine on Growth and Sporulation

Mycelial growth of all the isolates was relatively good even at 50 ppm nitrogen (Table 9). However, only two isolates (F-2C and PM-2C) produced conidia at levels of nitrogen lower than 500 ppm. Sporulation of these two isolates decreased with decreasing nitrogen concentration and only PM-2C produced conidia at 50 ppm nitrogen.

Effect of Alanine Isomers

β-Alanine and D-alanine were relatively poor sources of nitrogen for growth and sporulation of all five ergot

Table 8. Effect of selected amino acids as sole nitrogen sources on the growth and sporulation of five isolates of C. purpurea.

Amino acid (500 ppm nitrogen)	Isolate									
	C-25C		R-37C		F-2C		PM-2C		R-38C	
	C 1/	G 1/	C	G	C	G	C	G	C	G
	x10^4	cm	x10^4	cm	x10^4	cm	x10^4	cm	x10^4	cm
L-asparagine	0b 2/	4.5a	0	4.7a	8,507b	4.7a	37,662a	2.3a	0	4.3a
L-glutamine	28a	4.8a	0	4.7a	26,970a	4.5a	21,991b	2.2a	0	4.5a
DL-methionine	0b	2.6cd	0	2.4cd	0d	2.4cd	0d	1.9ab	0	2.3de
L-tryptophan	0b	3.9b	0	3.5b	0d	3.2b	0d	2.3a	0	3.4bc
DL-valine	0b	2.1d	0	2.1d	0d	1.8d	0d	1.5b	0	1.7e
DL-isoleucine	0b	2.8c	0	2.9c	133d	2.9bc	234d	1.5b	0	2.7cd
L-proline	0b	3.0c	0	4.3a	3,413c	4.2a	8,693c	2.6a	0	4.0ab

1/ C = number of conidia per cm^2 of mycelial growth, G = diameter of mycelial growth in cm; values of C and G are average of 3 plates.

2/ a-e Duncan's multiple range test; values within each column followed by the same letter are not significantly different (p = 0.05).

Table 9. Effect of decreasing concentration of L-asparagine on the growth and sporulation of five isolates of C. purpurea.

L-asparagine (ppm nitrogen)	Isolate									
	C-25C		R-37C		F-2C		PM-2C		R-38C	
	C[1/]	G[1/]	C	G	C	G	C	G	C	G
	x10^4	cm	x10^4	cm	x10^4	cm	x10^4	cm	x10^4	cm
2118	1,392a[2/]	5.0a	19,026a	5.0a	30,584a	4.9a	25,498a	3.0a	12a	4.5a
500	0b	4.6d	0b	5.0a	4,545b	4.8a	18,766ab	2.6b	0b	4.3c
300	0b	4.7c	0b	5.0a	3,571b	4.9a	11,797bc	2.5b	0b	4.3c
100	0b	5.0a	0b	4.7b	22c	4.6b	1,591c	2.4b	0b	4.4b
50	0b	4.8b	0b	4.9a	0c	4.3c	555c	2.0c	0b	4.2d

1/ C = number of conidia per cm^2 of mycelial growth, G = diameter of mycelial growth in cm; values of C and G are average of 3 plates.

2/ a-d Duncan's multiple range test; values within each column followed by the same letter are not significantly different (p = 0.05).

isolates whereas L-alanine was about as good as L-asparagine (Table 10). The racemic mixture of this amino acid supported fairly good mycelial growth of all isolates but conidia were produced by isolate PM-2C only. The mycelial growth of all isolates on β-alanine, although considerably restricted, was very thick and tissue-like.

To determine whether the four alanine isomers were either inhibitory to growth and/or sporulation of some ergot isolates or poor sources of nitrogen, they were tested again in the presence of L-asparagine at the concentration used in SD-2 medium. β-Alanine significantly reduced the amount of mycelial growth of all the isolates except F-2C (Table 11). Mycelial growth of all the isolates on the other alanine isomers was equal to that in SD-2 medium except for PM-2C: here growth was significantly reduced by all the isomers. Conidia were produced by all isolates in every treatment, however, certain isolate-treatment combinations enhanced or reduced sporulation while others had no effect. Thus, DL-alanine enhanced sporulation of isolates C-25C and PM-2C and β-alanine that of R-38C whereas D-alanine reduced sporulation of isolates C-25C and R-37C only and β-alanine that of isolates R-37C and PM-2C.

β-Alanine and D-alanine which reduced sporulation, and DL-alanine which enhanced sporulation, of several ergot isolates in the previous experiment, were tested again to determine whether their effects would be greater at lower

Table 10. Effect of alanine isomers as sole nitrogen source on the growth and sporulation of five isolates of C. purpurea.

Amino acid (500 ppm nitrogen)	Isolate									
	C-25C		R-37C		F-2C		PM-2C		R-38C	
	C[1/]	G[1/]	C	G	C	G	C	G	C	G
	x10^4	cm	x10^4	cm	x10^4	cm	x10^4	cm	x10^4	cm
L-asparagine	77b[2/]	5.0a	127a	4.5b	11,688b	4.8a	39,719a	2.6a	20a	4.8a
β-alanine	0b	0.7d	0b	0.8c	0c	0.8d	368b	1.1b	0c	0.7c
D-alanine	0b	0e	0b	0d	0c	0e	0b	1.2b	0c	0d
L-alanine	231a	4.7b	23b	5.0a	35,931a	4.4b	41,559a	2.5a	13b	4.4a
DL-alanine	0b	4.6c	0b	4.5b	0c	4.0c	13,528b	2.6a	0c	3.8b

1/ C = number of conidia per cm^2 of mycelial growth, G = diameter of mycelial growth in cm; values of C and G are average of 3 plates.

2/ a-e Duncan's multiple range test; values within each column followed by the same letter are not significantly different (p = 0.05).

Table 11. Effect of alanine isomers on the growth and sporulation of five isolates of C. purpurea in SD 2 medium.

Treatment[2/]	Isolate									
	C-25C		R-37C		F-2C		PM-2C		R-38C	
	C[1/]	G[1/]	C	G	C	G	C	G	C	G
	x10[4]	cm	x10[4]	cm	x10[4]	cm	x10[4]	cm	x10[4]	cm
SD2 medium	14,177b[3/]	5.0a	71,299a	5.0a	44,178a	5.0	42,749b	4.1a	948b	5.0a
β-alanine	15,736b	4.0b	24,610c	3.7b	27,576ab	5.0	9,134c	1.7d	24,632a	2.4b
D-alanine	7,705c	4.9a	177d	5.0a	37,771ab	5.0	25,389bc	2.8c	1,106b	4.8a
L-alanine	6,364c	5.0a	40,065b	5.0a	17,035b	5.0	67,381a	3.5b	579b	5.0a
DL-alanine	22,316a	5.0a	59,848a	5.0a	40,325ab	5.0	67,121a	3.7b	3,515b	5.0a

1/ C = number of conidia per cm^2 of mycelial growth, G = diameter of mycelial growth in cm; values of C and G are average of 3 plates.

2/ 500 ppm nitrogen of each alanine isomer was used.

3/ a-d Duncan's multiple range test; values within each column followed by the same letter are not significantly different (p = 0.05).

levels of total nitrogen. Each alanine isomer was combined with L-asparagine in several ratios not exceeding 400 ppm total nitrogen and compared with L-asparagine at 300 ppm. Only isolates F-2C and PM-2C, which had previously been shown to sporulate well in medium containing 300 ppm nitrogen as L-asparagine (Table 9) were used in this experiment.

Mycelial growth and conidial production were good for both ergot isolates in L-asparagine as the sole nitrogen source (Table 12). The two isolates differed considerably in their response to the alanine isomers: conidial production of F-2C was significantly reduced from that of L-asparagine alone by all three isomers at all mixtures tested, whereas that of PM-2C was unaffected in 1-3 mixtures of each isomer. Conidial production of F-2C was more sensitive to the isomers, particularly to D-alanine and DL-alanine, than mycelial growth. All mixtures with D-alanine significantly reduced mycelial growth of both isolates, whereas all mixtures with DL-alanine were without affect on growth of either isolate. Mixtures with β-alanine, on the other hand, reduced mycelial growth of F-2C in all but one mixture (100:200) and of PM-2C in only one mixture (200:200). Mycelial growth was severely restricted and sporulation completely inhibited for both isolates in β- and D-alanine used alone whereas sporulation, but not mycelial growth, of both isolates was significantly reduced in DL-alanine used alone.

Table 12. Effect of combining alanine isomers with L-asparagine at low levels of total nitrogen on the growth and sporulation of two isolates of C. purpurea.

Treatment (ppm nitrogen)	Isolate			
	F-2C		PM-2C	
	C[1/]	G[1/]	C	G
	x10^4	cm	x10^4	cm
L-asparagine (300)	10,325a[2/]	4.5a	14,567ab	2.1ab
β-alanine : L-asparagine				
50:50	27c	3.5bcd	8,052bc	1.6abcde
100:100	542c	3.4cde	12,814ab	1.6abcde
100:200	2,732b	3.9abc	12,900ab	1.7abcde
200:200	95c	2.8ef	18,615a	1.5bcde
300:0	0c	0.8g	0d	1.0ef
D-alanine:L-asparagine				
50:50	0c	2.7f	52d	1.3cdef
100:100	0c	2.7f	394d	1.2def
100:200	0c	3.5bcd	19,567a	1.3cdef
200:200	0c	3.2def	10,823b	1.2def
300:0	0c	0.8g	0d	0.7f
DL-alanine:L-asparagine				
50:50	0c	4.1ab	498d	1.8abcd
100:100	0c	4.1ab	3,095cd	2.0abc
100:200	0c	4.4a	12,922ab	2.0abc
200:200	0c	4.3a	19,978a	2.1ab
300:0	0c	3.9abc	3,333cd	2.3a

1/ C = number of conidia per cm^2 of mycelial growth, G = diameter of mycelial growth in cm; values of C and G are average of 3 plates.

2/ a-g Duncan's multiple range test; values within each column followed by the same letter are not significantly different (p = 0.05).

Effect of DL-p-Fluorophenylalanine (FPA)

The effect of FPA on mycelial growth and conidial production of isolates F-2C and PM-2C was assessed by adding this analog in concentrations ranging from 5-200 ppm, to basal medium containing L-asparagine at 2118 and 300 ppm nitrogen.

The effects of FPA on mycelial growth and conidial production of both isolates were more pronounced in media with a low level than with a high level of L-asparagine (Table 13). At a low level of L-asparagine, conidial production of both isolates was significantly reduced at an FPA concentration of 5 ppm, whereas at a high level of L-asparagine conidial production was reduced at 10 and 50 ppm FPA for isolate PM-2 and F-2C respectively. Reductions in mycelial growth were concomitant with the above reductions in sporulation with the exception of isolate F-2C at 300 ppm nitrogen for which a reduction in mycelial growth required a higher concentration of FPA. Mycelial growth and conidial production of both isolates subsequently decreased with increasing concentration of FPA at both levels of L-asparagine.

Inhibition of sporulation as a percent of the control was calculated for each treatment-isolate combination (Appendix 7) in order to obtain the effective dose of FPA required for a 90% reduction in sporulation (ED_{90}). The ED_{90} of FPA for both isolates was 100 and 10 ppm at high and low levels of L-asparagine respectively.

Table 13. Effect of DL-p-fluorophenylalanine (FPA) on mycelial growth and conidial production of two isolates of C. purpurea in basal medium containing two levels of L-asparagine as a nitrogen source.

FPA (ppm)	L-asparagine							
	2118 ppm nitrogen				300 ppm nitrogen			
	F-2C		PM-2C		F-2C		PM-2C	
	C[1/]	G[1/]	C	G	C	G	C	G
	x10^4	cm	x10^4	cm	x10^4	cm	x10^4	cm
0	99,697a[2/]	4.8a	74,589a	2.6a	7,143a	4.4a	27,922a	2.1a
5	90,022a	4.6a	63,290ab	2.3ab	1,754b	4.1ab	23,333b	1.8b
10	83,615a	4.7a	54,611b	1.9bc	519bc	3.7b	2,173c	1.3bc
50	27,316b	3.5b	35,303c	1.5c	260c	1.2c	0c	0.8c
100	3,983c	2.6c	2,857d	0.9d	130c	1.0c	0c	0.7c
200	3,225c	1.4d	0d	0.7d	0c	0.7c	0c	0.7c

1/ C = number of conidia per cm^2 of mycelial growth, G = diameter of mycelial growth in cm; values of C and G are average of 3 plates.

2/ a-d Duncan's multiple range test; values within each column followed by the same letter are not significantly different (p = 0.05).

DISCUSSION

These studies show that the utilization of amino acids as nitrogen sources for mycelial growth and production of conidia, varies among isolates of C. purpurea. Furthermore, amino acids suitable for growth were not necessarily good for sporulation and others, such as β- and D-alanine, which appeared to be poor sources of nitrogen for growth and sporulation were shown to be highly inhibitory to both.

Pronounced differences in sporulation were observed among isolates on media containing high levels of L-asparagine (Table 7). The two abundantly sporulating isolates also produced the most spores at lower levels of nitrogen (Table 9) whereas the other isolates produced few or no conidia at concentrations of 500 ppm nitrogen and lower indicating that sporulation requires high levels of nitrogen in these isolates. There did not appear to be any relationship between virulence of the isolates on a cultivar of spring wheat and nitrogen requirements for sporulation. However, the total free amino acids of developing endosperms have been reported to differ significantly during the first two weeks after anthesis, and to be less in durum wheat than in either triticale or rye and less in triticale than in rye (Dexter and Dronzek 1975). Thus, the nitrogen requirement of isolates for sporulation in culture should receive greater attention in future studies, in view of its potential relationship to performance

of the isolates on the above cereal hosts.

L-glutamine did not appear to be a better source of nitrogen than L-asparagine for growth or sporulation of ergot isolates when tested in either complete or defined media. These results are in agreement with the findings of Taber and Vining (1957). L-proline and L-alanine were found to be relatively good sources of nitrogen for growth of most isolates; however, L-alanine was also a good source of nitrogen for sporulation of all the isolates whereas L-proline was a good source for two isolates only. The free amino acid fractions of rye, triticale and durum wheat were reported to contain high amounts of L-alanine and L-proline as well as of L-asparagine-aspartic acid and L-glutamine-glumatic acid (Dexter and Dronzek 1975). In view of the differential response of ergot isolates demonstrated in this study it would seem worthwhile to further compare the growth and sporulation of isolates of known virulence (Ratanopas 1973) on media consisting of various combinations and concentrations of the above amino acids.

Results of the comparative utilization of alanine isomers as sole nitrogen sources or in combination with high and low levels of L-asparagine suggest that β-and D-alanine are not only poor sources of nitrogen for growth and sporulation, but highly inhibitory to both, particularly at low levels of L-asparagine (Table 12). However, the isolates differ in their response to the isomers and the

production of conidia in particular, was inhibited to a greater extent in isolate F-2C than in PM-2C.

The results with β-alanine are not in agreement with the findings of Singh et al. (1972) who reported that this amino acid supported poor growth but good sporulation of C. microcephala (Wallr.) Tul. However, Singh et al. apparently used a single isolate in their tests and if the variability within C. microcephala is as great as that of C. purpurea, perhaps other isolates would respond differently.

Sporulation of several isolates was enhanced significantly by β-alanine and DL-alanine in the presence of high levels of L-asparagine (Table 11). The performance of β-alanine in this regard would seem to provide an explanation for the increased susceptibility to C. purpurea of rye seedlings treated with this amino acid (Lewis 1962b). These results also support the conclusions of van Andel (1966) who stated that stimulating and inhibitory effects of amino acids on growth of plant pathogenic fungi are dependent on the concentration of the amino acid concerned and the composition of the medium, particularly in regard to the presence of other amino acids, trace elements and vitamins. Van Andel also points out that strains of the same species can respond differently to a given amino acid and the results of this study indicate that C. purpurea resembles fungi such as Phytophthora infestans and Helminthosporium gramineum in this regard.

DL-p-fluorophenylalanine which was reported to be highly inhibitory to sporulation but not to growth of Ceratocystis ulmi and of Fusarium oxysporum f. lycopersici (Biehn 1973) was inhibitory to both sporulation and growth of the two ergot isolates tested. The systemic activity of FPA in cucumber seedlings against Cladosporium cucumerinum and Collectotrichum lagenarium (van Andel 1962) as well as that of β-alanine against Fusarium oxysporum f. sp. fabae (Abdel-Rehim et al. 1968) indicates that these two amino acids, as well as D-alanine, should be tested further for chemotherapeutic and antisporulant activity against ergot.

GENERAL DISCUSSION

Variability within C. purpurea has been reported with regard to pathogenicity (Barger 1931; Campbell 1957; Ratanopas 1973), alkaloid production (Kybal and Brejcha 1955) and cultural characteristics (Amici et al. 1967b; Grein 1967; Mantle and Tonolo 1968; Strnadová and Kybal 1974). Recently, virulence was also reported to vary considerably among isolates of C. purpurea, but was not related to host origin of the isolates (Ratanopas 1973). The first part of the study was performed to investigate the variability of cultural characters among isolates of C. purpurea from various hosts in the Canadian Prairies and to determine relationships between cultural characters and host origin and virulence of the isolate.

The cultural characteristics of the 143 ergot isolates studied were found to vary considerably. This finding is in agreement with that of Amici et al. (1967b) and Grein (1967) with ergot isolates from Europe. Unfortunately, there appeared to be no relationship between cultural characteristics and host origin. Furthermore, identification of virulent isolates by specific cultural characteristics was not possible. The method of floret inoculation used to establish the virulence of isolates is tedious (Platford and Bernier 1976; Ratanopas 1973) and a preliminary screening on the basis of cultural characters would

have been useful in reducing the number of isolates to be tested on hosts. Thus, although cultural characteristics were found to be as variable as other attributes of C. purpurea which have been studied, and can be grouped into readily recognizable categories, there seems to be no reason to do so.

Variability in cultural characteristics was also demonstrated among monoascosporic isolates from a single sclerotium. Such variability appeared to be too great to be due to mutation alone and might be due to recombination of genetically different nuclei during the sexual stage of the fungus as suggested by Amici et al. (1967b).

The formation of globose conidial masses by a few ergot isolates and of conidial horns by one isolate does not appear to have been reported previously and the development of these interesting structures as well as the factors which affect their formation were investigated further.

Both types of conidial structures appeared to originate from microscopic conidial masses which under suitable condition, continue to enlarge. On the basis of similarities between the formation of honeydew and conidia on the host and conidial horns in culture, it is suggested that conidial horns might be sporodochia of the ergot fungus in culture.

Studies of the factors affecting the formation of conidial horns by isolate R-37C indicate that the ability

to form these structures can be maintained and improved by culturing on T2 medium adjusted to pH 7 and prepared with either tap water or deionized water containing mineral salts similar to those in tap water. These results provide an explanation for the low incidence of conidial structures observed in section 1 and suggest that a more meaningful assessment would be obtained by culturing ergot isolates under conditions defined in section 2.

The highly raised pletenchymatic type of growth produced by some isolates in these studies and also observed by other investigators (Amici et al. 1967a; Johansson 1964b; Mantle and Tonolo 1968; Mantle 1969) is most striking. Thickening of the mycelial mat occurred after the production of conidia and appears analogous to the development of sclerotia in host florets following the sphacelial stage.

Histologically, the thick mycelial mat was found to be composed of plectenchymatic tissue and to resemble tissue of sclerotia. These results are in agreement with the finding of several workers (Bonns 1922; Johansson 1964b; Mantle and Tonolo 1968; Mantle 1969). It became apparent during the course of these studies that factors unfavorable for the production of conidial structures i.e. high or low pH, and use of glass distilled water, favored the development of a thick mycelial mat in isolate R-37C.

Although the results from the first study indicate that only some isolates produce thick mycelial mats perhaps

other isolates would do so under more favorable conditions. The histological similarities between the thick mycelial mat and the sclerotial tissue, the fact that the former was recently shown to be associated with alkaloid production in culture (Mantle and Tonolo 1968; Mantle 1969) and the fact that similar tissues have been reported to produce stromata (Schweizer 1941) suggests that the function and germinating capacity of plectenchymatic mycelial mats should be further investigated.

Three sugars reported to be present in honeydew (Fuchs and Pöhm 1953; Mantle 1965) were found in water extracts of conidial horns. Since extracts were prepared by suspending young conidial horns in water for 15 minutes it would appear that the sugars are present in the horns extracellularly and might be involved in the adhesion of conidia. However, sucrose was not found in the honeydew extract tested in this study either because the isolate used does not produce this sugar or because it is present in amounts too low to be detected. Therefore, further work is required to determine if the sugary material of the conidial horns is identical to honeydew.

Recent studies have indicated that the amount of honeydew and the size of the sclerotia in C. purpurea are influenced by the fungus as well as by the host (Platford and Bernier 1970; 1976; Ratanopas 1973). These differences in host-parasite relationships might be due to qualitative or quantitative differences in the nutrients supplied by

the hosts or to differences in nutrient requirement among isolates of the fungus. Thus, in this study, several amino acids were compared as nitrogen sources on the assumption that better knowledge of the nutritional requirements for growth and production of conidia in culture might ultimately provide a better understanding of the phenomenon of reduced sporulation in the host.

Utilization of amino acids varied appreciably among the isolates tested in this study and amino acids suitable for growth were not necessarily good for production of conidia. Also, three of the five isolates tested required high levels of nitrogen for sporulation. Nevertheless, there appeared to be no relationship between the virulence of the isolates and nitrogen requirements for sporulation. However, the total free amino acids of developing endosperms have been reported to differ significantly during the first two weeks after anthesis, and to be less in durum wheat than in either triticale or rye and less in triticale than in rye (Dexter and Dronzek 1975). Thus, the nitrogen requirement of isolates for sporulation in culture should receive greater attention in future studies.

The differential response of ergot isolates demonstrated in this study, particularly to L-proline and β-alanine, suggests that growth and sporulation of isolates of known virulence (Ratanopas 1973) should be tested further on media consisting of various combinations and concentrations

of the amino acids reported by Dexter and Dronzek (1975) to predominate in the free amino acid fraction of rye, triticale and durum wheat i.e. L-alanine, L-proline, L-asparagine-aspartic acid and L-glutamine-glutamic acid. Furthermore, such tests should be conducted on media adjusted to pH favorable to the production of conidial structures or plectenchymatic growth as demonstrated in section 2.

Knowledge derived from the present studies suggests that it might be worthwhile to analyse cereal hosts with varying degrees of resistance to ergot, for total free amino acids including β-alanine, which was not included in analyses conducted by Dexter and Dronzek (1975).

The resistance to ergot found to date (Platford and Bernier 1970) is not likely to be very effective in male sterile lines used to produce hybrid wheat, since many florets would be infected and produce either small sclerotia or aborted ovaries and no kernels. Therefore, other ways of restricting ergot infection should not be neglected. The results obtained in this study with DL-p-fluorophenylalanine, β-alanine and D-alanine suggests that these amino acids should be tested further for chemotherapeutic and anti-sporulant activity against ergot.

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Appendix 1. Method for obtaining pure isolate of Claviceps purpurea in culture.

1. Sclerotial isolates:

Peeled sclerotia were soaked for approximately one minute in a 2% sodium hypochlorite aqueous solution. This was followed by three washings in sterile distilled water. Each sclerotium was cut aseptically into several pieces and placed onto 4% malt agar plates that had been acidified with one drop of 2% lactic acid. After germination, mycelium was transferred to 4% malt agar slant held at 24° C. These sclerotial cultures were stored at 3° C for further use.

2. Single ascospore isolates:

Unsterilized sclerotia were placed on moist sterile vermiculite in covered plastic containers and stored at 3° C for approximately six weeks. For germination they were held at 24° C until the stromata were fully developed (approximately 4 weeks). Ascospores were collected by the technique of securing one stromatized sclerotia to the interior bottom of an aerobic culture dish (95 x 62). It was inverted for a time interval over a 4% malt agar plate so that the ascospore population on the plate was invariably sparse. This facilitated a single ascospore transfer to an agar slant of the same medium. Germination was permitted at room temperature (24° C). Following germination, mycelium was

Appendix 1. (Continued)

transferred onto 4% malt agar slants and maintained at 3^{o} C as a stock culture of a single ascospore isolate.

Appendix 2. Cultural characteristic of individual isolates used in the study.

Isolate[1/]	Characteristics			Plecten-chyma	Globose conidial mass	Conidial formation	Sectoring
	Growth	Surface	Color				
Rye (Secale cereale L.)							
R-1*	Flat	wrinkled	white	+	-	+	+
R-2A*	Flat	folded	colored	+	-	+	+
R-3A*	Flat	folded	colored	-	-	+	-
R-4A*	Flat	folded	colored	+	-	-	-
R-5A*	Flat	folded	white	+	-	-	+
R-6A*	Flat	wrinkled	colored	+	-	-	-
R-7A*	Flat	folded	colored	+	-	-	-
R-8*	Flat	smooth	white	+	-	+	-
R-10A*	Raised	folded	colored	+	-	+	-
R-11A*	Flat	folded	colored	+	-	-	+
R-12A*	Flat	folded	colored	-	+	+	-
R-13A*	Flat	smooth	white	-	-	+	-
F-14*	Flat	wrinkled	white	+	-	+	-
R-15*	Raised	folded	colored	+	-	+	-
R-16*	Flat	smooth	colored	-	-	+	-
R-17*	Flat	smooth	white	+	-	-	-
R-18*	Flat	folded	colored	-	-	+	-
R-19	Flat	folded	white	+	-	+	-
R-20A	Raised	folded	white	+	-	+	-
R-21	Raised	folded	colored	+	-	-	-
R-22	Flat	smooth	white	+	-	+	-
R-23A	Flat	smooth	white	-	-	+	-
R-24	Flat	wrinkled	colored	+	-	+	-
R-25	Raised	folded	white	-	-	+	-

Appendix 2. (Continued)

Isolate	Characteristics			Plecten-chyma	Globose conidial mass	Conidial formation	Sectoring
	Growth	Surface	Color				
R-26	Flat	folded	colored	+	-	+	-
R-27	Raised	folded	colored	+	-	+	-
R-28	Flat	smooth	white	-	+	+	-
R-29	Flat	wrinkled	colored	+	-	+	-
R-30	Raised	folded	colored	+	-	+	-
R-31	Flat	wrinkled	colored	-	-	+	-
R-32	Flat	wrinkled	colored	+	-	+	-
R-33	Flat	wrinkled	colored	-	-	+	-
R-34	Raised	folded	colored	-	-	+	-
R-35	Raised	folded	white	+	-	+	-
R-36	Raised	folded	colored	-	-	+	-
Smooth brome (*Bromus inermis* Leyss.)							
B-1*	Flat	folded	colored	+	-	-	-
B-2*	Flat	folded	white	+	-	+	+
B-8	Raised	folded	colored	-	-	+	-
B-9	Raised	folded	white	+	-	+	-
B-10	Flat	folded	white	-	-	+	-
B-11	Flat	folded	white	-	-	+	-
B-12	Flat	folded	white	+	-	+	-
B-13	Flat	folded	white	+	-	+	-
B-14	Raised	folded	colored	-	-	+	-
B-15	Raised	folded	colored	-	-	+	-
B-16	Raised	folded	colored	-	-	+	-
B-17	Raised	folded	colored	-	-	+	-
B-18	Flat	folded	colored	-	-	+	-

Appendix 2. (Continued)

Isolate	Characteristics			Plecten-chyma	Globose conidial mass	Conidial formation	Sectoring
	Growth	Surface	Color				
B-19	Flat	folded	colored	-	-	+	-
B-20	Raised	folded	white	+	-	+	-
B-21	Raised	folded	white	-	-	+	-
B-22	Flat	folded	colored	-	-	+	-
B-23	Flat	folded	colored	-	-	+	-
B-24	Flat	folded	colored	-	-	+	-
B-25	Flat	folded	white	-	-	+	-
B-26	Flat	wrinkled	colored	-	-	+	-
B-27	Flat	wrinkled	colored	-	-	+	-
B-28	Raised	wrinkled	colored	-	-	+	-
B-29	Raised	wrinkled	colored	-	-	+	+
B-30	Raised	folded	white	+	-	+	-
B-31	Flat	wrinkled	colored	-	-	+	-
B-32	Flat	wrinkled	colored	-	-	+	-
B-33	Flat	wrinkled	colored	-	-	+	-
Durum wheat (*Triticum durum* Desf.)							
D-1*	Flat	wrinkled	white	+	-	+	-
D-2A	Flat	folded	colored	+	-	+	-
D-3A	Flat	smooth	colored	+	-	+	-
D-4A	Raised	folded	white	+	-	+	-
D-5A	Raised	folded	colored	+	-	+	-
D-6A	Raised	folded	colored	-	+	+	-
D-7A	Raised	folded	colored	-	-	+	-
D-8A	Raised	wrinkled	colored	+	-	+	-
D-9A	Raised	folded	colored	-	+	+	+
D-10A	Flat	folded	white	-	-	+	-

Appendix 2. (Continued)

Isolate	Characteristics			Plecten-chyma	Globose conidial mass	Conidial formation	Sectoring
	Growth	Surface	Color				
D-11A	Raised	folded	colored	+	-	+	+
D-12A	Raised	smooth	colored	+	-	-	-
D-13A	Flat	folded	white	+	-	+	-
D-14A	Raised	folded	colored	+	-	+	-
D-15A	Raised	folded	colored	+	-	+	-
D-16A	Flat	folded	white	+	-	+	-
D-17A	Flat	folded	white	+	-	+	-
D-18A	Flat	folded	white	-	-	+	-
D-19A	Flat	folded	white	-	-	+	-
D-20A	Raised	folded	colored	-	+	+	-
D-21A	Raised	folded	colored	+	-	+	-
D-22A	Raised	folded	colored	-	-	+	-
D-23	Flat	folded	white	-	-	+	-
D-24	Raised	folded	white	+	-	+	-
Quackgrass (*Agropyron repens* (L.) Beauv.)							
C-1*	Flat	folded	colored	+	-	+	-
C-2*	Flat	folded	colored	+	-	+	+
C-3A*	Flat	folded	colored	-	-	+	-
C-5A*	Flat	folded	colored	-	-	+	-
C-6A*	Flat	folded	colored	-	-	+	-
C-7*	Raised	folded	white	+	-	-	+
C-8*	Flat	wrinkled	colored	+	-	+	-
C-9*	Flat	folded	colored	+	-	+	+
C-10*	Flat	folded	white	+	-	+	+
C-11	Flat	wrinkled	colored	-	-	+	-

Appendix 2. (Continued)

Isolate	Characteristics			Plecten-chyma	Globose conidial mass	Conidial formation	Sectoring
	Growth	Surface	Color				
C-12	Raised	folded	colored	+	-	+	-
C-13	Raised	smooth	colored	+	-	+	-
C-14	Raised	folded	colored	-	-	+	-
C-15	Flat	folded	white	+	-	+	-
C-16	Flat	wrinkled	colored	-	-	+	-
C-17	Flat	folded	white	-	-	+	-
C-18	Flat	wrinkled	white	+	-	+	-
C-19	Flat	folded	white	-	-	+	-
C-20	Raised	folded	white	+	-	+	-
C-21	Flat	smooth	white	-	-	+	-
C-22	Flat	folded	white	-	+	+	-
C-23	Raised	folded	colored	-	-	+	-
C-24	Flat	folded	white	+	-	+	-
Spring wheat (*Triticum aestivum* L.)							
M-2*	Flat	wrinkled	colored	+	-	+	-
M-4*	Flat	smooth	white	-	-	+	-
M-5*	Raised	folded	white	+	-	+	-
M-6*	Flat	folded	colored	+	-	+	-
M-7	Flat	folded	colored	-	-	+	-
M-8*	Flat	folded	white	+	-	+	-
M-10*	Flat	folded	white	+	-	+	-
M-14	Raised	folded	white	-	-	+	-
M-15A	Raised	folded	colored	-	-	+	-
M-16A	Raised	folded	colored	+	-	+	-
M-17A	Flat	wrinkled	colored	+	-	+	-

Appendix 2. (Continued)

Isolate	Characteristics			Plecten-chyma	Globose conidial mass	Conidial formation	Sectoring
	Growth	Surface	Color				
M-18A	Raised	folded	colored	-	-	+	-
M-19A	Flat	wrinkled	colored	+	-	+	-
M-20A	Flat	wrinkled	white	-	-	+	-
M-21A	Raised	folded	colored	-	+	+	-
M-22A	Flat	folded	colored	+	-	+	-
M-23A	Flat	folded	colored	-	+	+	-
M-24A	Flat	folded	colored	-	+	+	-
M-25A	Raised	folded	white	+	-	+	-
Triticale (X Triticosecale Wittmack)							
T-1*	Flat	folded	colored	+	-	+	+
T-2A*	Flat	folded	white	+	-	-	-
T-4A*	Flat	folded	white	-	-	+	-
T-6*	Flat	folded	colored	-	-	+	-
T-7*	Flat	smooth	colored	-	-	+	-
Ryegrass (Lolium temulentum L.)							
L-3*	Raised	folded	white	+	-	+	-
L-9*	Flat	folded	colored	+	-	-	-
L-12*	Flat	smooth	white	-	-	+	-
Timothy (Phleum pratense L.)							
P-1*	Flat	folded	colored	+	-	+	-
P-2*	Flat	folded	colored	+	-	+	-
Oat (Avena sativa L.)							
O-1*	Flat	folded	colored	+	-	+	-

Appendix 2. (Continued)

Isolate	Characteristics			Plecten-chyma	Globose conidial mass	Conidial formation	Sectoring
	Growth	Surface	Color				
Reedgrass (*Calamagrostis canadensis* (Michx) Beauv.)							
CT-1*	Flat	folded	colored	+	-	+	-
Common reedgrass (*Phragmites communis* Trin.)							
PM-1*	Flat	folded	white	-	-	+	-
Tall fescue (*Festuca arundinacea* Schreb.)							
F-1*	Flat	smooth	white	-	-	+	-

1/ "A" indicates monoascosporic isolates; * indicates the isolate previously tested for pathogenicity.

Appendix 3. Evaluation of host reaction produced by *C. purpurea*.

Visual rating of sclerotia size measured twenty-one days after inoculation.

1. Sclerotia smaller than normal kernel.
2. Sclerotia approximately the size of normal kernel.
3. Sclerotia larger than kernel, extending beyond the glumes.

Visual rating of honeydew production measured ten days after inoculation.

1. No visible honeydew.
2. Honeydew confined within glumes.
3. Honeydew exuding from infected florets in small drops.
4. Honeydew exuding from infected florets in large drops and running down the head.

Appendix 4. Benedict's test for the presence of reducing sugar (Harrow et al. 1967).

Benedict's reagent is prepared by dissolving 173 g of crystalline sodium citrate and 100 g of anhydrous sodium carbonate in about 800 ml of water. Stir thoroughly and filter. Add to the filtered solution 17.3 g of copper sulfate dissolved in 100 ml of water. Make up to 1 liter with distilled water.

Add 8 drops of each of the sample solutions to 5 ml of Benedict's qualitative reagent, and shake each tube. Place all the tubes at the same time into a boiling water bath and continue boiling for three minutes. Allow to cool and observe.

Appendix 5. Preparation of visualising reagents for chromatograms.

1. Silver nitrate-sodium hydroxide (Randerath 1966; Stahl 1969).

Spraying reagent 1: 1 ml saturated aqueous silver nitrate solution is diluted to 200 ml with acetone and 5-10 ml water then added until the precipitate has dissolved. Spraying reagent 2: 0.5 N solution of sodium hydroxide in aqueous ethanol which is prepared by dissolving 20 g sodium hydroxide in a minimum amount of water and the solution diluted to 1 l with 95% ethanol. Spraying reagent 3: 5% aqueous sodium thiosulfate solution which provided permanent zone location without heating. Spraying is carried out with 1 and allowed to dry, followed by 2. After the maximum color of spots appears the plate is sprayed with reagent 3.

2. p-Aminobenzoic acid reagent (Ersser and Andrew 1971).

A stock solution is prepared by dissolving 2 g p-aminobenzoic acid in a mixture of glacial acetic acid (36 ml), water (40 ml) and 85% orthophosphoric acid (1.6 ml). The stock solution (2 parts) is diluted with acetone (3 parts) for use. After spraying, the plate is heated in an oven at 95-100^{o} C for 10 minutes.

Appendix 5. (Continued)

3. p-Anisidine phosphate in ethanol (Mukherjee and Srivastava 1952).

This reagent is prepared by dissolving 0.5 g p-anisidine in 2 ml 85% orthophosphoric acid and diluting the solution with ethanol to 50 ml. The precipitated p-anisidine phosphate is filtered and the filtrate used as the spray reagent. After spraying, the plate is heated in an oven at 95-100° C for 5 minutes.

4. Aniline-phosphoric acid (Raadsveld and Klomp 1971).

It is prepared by dissolving 1.3 ml orthophosphoric acid (85%) and 1.3 ml aniline in 100 ml 70% ethanol. The reagent is stored in the dark in the refrigerator. After spraying, the plate is dried for 15 minutes at a temperature of 100° C.

5. Benzidine-trichloroacetic acid (Stahl 1969).

Benzidine in the amount of 0.5 g is dissolved in 10 ml acetic acid. Ten ml 40% aqueous trichloroacetic acid is added. The mixture is diluted to 100 ml with ethanol. After spraying, the plate is heated to 110° C for 10 minutes.

Appendix 6. Rating system for disease caused by Claviceps purpurea (Ratanopas 1973).

Varietal reaction	Infection			
	Rating	Type	Frequency of Sclerotia	Amount of Honeydew
Immune (I)	0	No infection; seed in all florets.	-	1
Very resistant (VR)	1	Abortive reaction (AR) mainly; no sclerotia.	-	1
Resistant (R)	2	AR and sclerotia of size 1 & 2 only.	Not greater than 40%	1-2
Moderately resistant (MR)	3	Sclerotia mainly size 1 & 2; 1 to 3 sclerotia of size 3.	Not greater than 60%	1-2
Moderately susceptible (MS)	4	Sclerotia of size 2 & 3.	Not greater than 80%	3
Susceptible (S)	5	Sclerotia mainly size 3.	Greater than 80%	4

Appendix 7. Percent inhibition of p-fluorophenyalanine (FPA) on mycelial growth and conidial production of two isolates of C. purpurea in basal medium containing two levels of L-asparagine as a nitrogen source.

Concentration of FPA (ppm)	% Inhibition of							
	2118 ppm nitrogen				300 ppm nitrogen			
	F-2C		PM-2C		F-2C		PM-2C	
	C[1/]	G[1/]	C	G	C	G	C	G
5	10	4	15	12	75	7	16	14
10	16	2	27	27	92	16	92	38
50	73	27	53	42	96	73	100	62
100	96	46	96	65	98	77	100	67
200	97	71	100	73	100	84	100	67

1/ C = conidial production; G = mycelial growth.