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Abstract

Verticillium albo-atrum R. and B. isolates were separated into four strain groups based on morphological type. All isolates were tested for pathogenicity toward tomato, and an attempt was made to relate pathogenicity to morphological type.

Since structural and functional differences in plants and animals are believed to be invariably associated with chemical differences, a survey of the nitrogenous constituents of the isolates was made in an attempt to relate any differences to morphological or pathologic strains.

The alcohol-soluble extract from the mycelium of each isolate was investigated chromatographically for amino acids, amines, amides and peptides. The amino acids found were the same for all isolates. These were aspartic acid, glutamic acid, lysine, histidine, isoleucine, serine, valine, proline, methionine, \( \alpha \) alanine, tyrosine, threonine, glycine and one unidentified neutral compound, A. No amines, amides or peptides were detected.

The amino acids, amines, amides and peptides of the \( \alpha, \beta, \) and \( \gamma \) strains of *Colletotricum lindemuthianum* (Sacc. and Magn.)
Bri. and Cav. were isolated and identified using the techniques of paper chromatography for the purpose of comparison with *Verticillium albo-atrum*. The three Colletotricum strains contained the same amino acids as the Verticillium isolates with the exception of unknown A which was replaced by another unknown, B, common to all three Colletotricum strains indicating a definite species difference. Strain differences apparently do not occur in these two organisms in the groups of compounds studied.

The effect of aeration, age of mycelium, pH of medium and carbon source on the qualitative amino acid content of isolate V3 were studied. These environmental conditions had no qualitative effect on the amino acids of the isolate.

The free nucleotides of the acid-soluble extract of six representative isolates were studied using the technique of anion-exchange chromatography. Nucleotides isolated from all isolates and tentatively identified were guanosine monophosphate, guanosine diphosphate, guanosine triphosphate, cytidine diphosphate, uridine monophosphate, uridine diphosphate, uridine triphosphate and adenosine diphosphate. No other nucleotides were isolated.

The above results indicated that if chemical dif-
ferences do exist between strains, as postulated, they do not occur, in these two organisms, among the nitrogenous compounds studied, but must occur in some other class of compounds not studied in this investigation.
Acknowledgments

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INTRODUCTION

*Verticillium albo-atrum* R. and B., an important fungus pathogen for a large number of truck crops, trees, stone-fruits, and bush fruits, (95) is not a clear-cut entity, but consists of a number of ill-defined strains or races. These strains fall into four main morphological types; type A having sparse mycelium and microsclerotia, type B having fluffy mycelium and no microsclerotia, while type X has abundant mycelium and abundant microsclerotia, (12). These types have been considered separate species by Isaac, (49). Presley (12), however, indicates that these strains are not stable, but undergo mutation resulting in the formation of one type from another which precludes the viewpoint that these types are separate species.

Furthermore, these strain types seem to have little, if any, relationship to pathogenicity types. That is, the ability of a strain to infect a particular species of plant does not seem to be related to its morphological type. Strains can be set up on the basis of their ability to infect or not infect a large variety of plants (95), or even on their relative ability to infect the same species of plant (56). These observations indicate that the strains of *Verticillium albo-atrum* need further clarification.
One possible method of throwing new light on the classification of *Verticillium albo-atrum* isolates is the study of biochemical characteristics. Clarke (27) suggests that the methods for determining the biochemical activities of microorganisms as applied to classification fall into the four following categories:

1. A study of minimal growth requirements.
3. A study of end products of metabolism.
4. Identification of characteristic compounds including enzymes.

This thesis is an attempt to utilize the fourth category. The nitrogenous constituents of a number of isolates of *Verticillium albo-atrum* were studied for the purpose of strain delineation. The techniques of paper chromatography and column partition chromatography using ion-exchange resins was used. An attempt was also made to relate any possible differences in nitrogenous constituents between strains to pathogenicity and to relate any differences to possible effects of cultural and environmental conditions.
HISTORICAL

Verticillium albo-atrum R. and B. is the causal agent of a serious disease of tomato in the Okanagan valley of British Columbia (128). Losses from this disease have been estimated to be up to thirty percent of the crop in some areas. In a comprehensive review article in 1931, Rudolph (95) listed over one hundred and twenty additional species involving thirty-five families and eighteen orders of plants reported to be infected by this organism. In a recent compilation Westcott (119) added an additional fifty-four species to this list. The species of plants in these lists are, with a few exceptions, dicotyledons. Potato, tomato, cotton, pepper, hops, avocado, raspberry, and maple are among the more important hosts throughout the temperate zones of the world. In British Columbia, tomato, sweet cherry, and cantaloupe are the important hosts. In general the disease is referred to as 'wilt' of truck crops and trees, 'black heart' of stone fruits, and 'bluestem' of bush fruits.

According to Rudolph (95) the causal agent of these diseases, Verticillium albo-atrum R. and B. was first isolated by Reinke and Berthold in 1879 from potatoes infected with a disease known at that time as 'Krauselkrankheit.'

The fungus produces typical verticilliate whorls, chains of chlamydospore-like cells, and black cell masses of various sizes and shapes showing no longitudinal walls and which were produced by budding of contiguous mycelial threads which they called 'Dauermycélien' or 'sklerotien.' The conidial are oval, hyaline, non-septate, and borne on the tips of sterigmas arranged in a whorl or series of whorls. Since chlamydospores and microsclerotia may, or may not, be produced considerable confusion has arisen in the classification of this organism.

Klebahn (58) in 1913, isolated a *Verticillium* from potato which he considered to differ from *Verticillium albo-atrum* in that it produced large quantities of black microsclerotia which arose from irregular multilateral septation and budding of a single hypha and not from the anastomosing of several hyphae. He named it *Verticillium Dahliae* Klebs.
According to Isaac (49), Pethybridge (81), in 1910 isolated a Verticillium very similar to *Verticillium albo-atrum* except that it produced spherical, oval, or pear-shaped, darkened cells which he called chlamydospores. He named this organism *Verticillium nigrescens* Pet.

Several other workers (95) have found variants of this organism which they promptly gave specific rank. The *Verticillium angustum* of Wollenweber, the *Verticillium duboys* of Poex and Picado, the *Verticillium tracheiphilum* of Gurzi, and the *Verticillium ovatum* of Berkeley and Jackson fall into this class.

Most of these 'species' have arisen because the drawings of Reinke and Berthold are too diagramatic and the description too sketchy to indicate whether their isolate had microsclerotia or chlamydospores (95).

The majority of workers now consider these organisms to be strains of *Verticillium albo-atrum* although some still consider them to be separate species. The most persistent of these workers is Isaac (49) who separates *Verticillium albo-atrum* into three distinct species: *Verticillium albo-atrum* R. and B. being a resting mycelial form, *Verticillium dahliae* Klebs being a microsclerotial
form, and *Verticillium nigrescens* Pet. being a chlamydosporial form.

To add to the confusion, although Nees von Essenbeck set up the genus *Verticillium* in 1816, Gorda, in 1838, described the same genus and called it *Acrostalagmus*. Hoffman proved that *Acrostalagmus* was a 'nomen nudem' for *Verticillium* in 1854 but some workers refused to accept this ruling (95). Hence, the names *Acrostalagmus alga*, *Acrostalagmus vilmorinii*, and *Acrostalagmus caulophagus* appear in the older literature and are synonyms for *Verticillium albo-atrum* R. and B. Carpenter (19) sums up this situation.

Rudolph (95) suggests that all these 'species' are actually varieties of *Verticillium albo-atrum* and bases his claim on the fact that the morphological and cultural characteristics of this organism may be altered by varying cultural conditions.

Those workers who accept all these varieties as one species are by no means in agreement as to the number of varieties or strains that exist. Bewley (8) reported the isolation of six strains based on their relative pathogenicity to tomato. Presley (87, 88) classified
his isolates into four types on the basis of cultural characteristics. Type A produced microsclerotia with sparse mycelia, type B produced fluffy mycelia with no microsclerotia, type C produced appressed mycelia with no microsclerotia, and type X produced abundant microsclerotia and mycelia. Isaac and Keyworth (50) divided isolates from hops according to their pathogenicity as fluctuating and progressive strains; fluctuating isolates varying in pathogenicity from year to year while progressive isolates became more pathogenic from year to year. They found no consistent morphological difference between cultures. Berkeley (7) divided his isolates into two types according to the character of their resting condition and gave each type specific rank. Those producing chlamydospore-like cells were placed in one group and those producing microsclerotia in another. He did not have a group producing neither as this type did not show up among his isolates. Keyworth (55) divided twenty-seven isolates into Berkeley's two groups but added a third intermediate group from which he concluded the two types were not separate species but variants of *Verticillium albo-atrum*. Isaac (49) divided his isolates into three groups to which he gave species rank. His types were: type A, microsclerotial; type B,
dark resting mycelial: and type C, chlamydosporial. Keyworth and Bennett (56) found that the fluctuating and progressive strains of Isaac and Keyworth (50) resembled *Verticillium albo-atrum* R. and B. and *Verticillium dahliae* Klebs, respectively.

Wagner and Mitchell (116) stated that 'structural and functional differences in plants and animals are invariably associated with chemical differences. Some of these chemical differences may be of a simple type, involving qualitative or quantitative changes in compounds which may be isolated and precisely identified, or they may be at a more complex level, involving proteins or even spatial organization of chemical compounds in cells and parts of the organism.' Since the various varieties or strains of *Verticillium albo-atrum* show obvious structural and functional differences one can assume they will also show chemical differences.

The structural and functional differences in *Verticillium albo-atrum* strains have been shown by Presley (87) to arise by 'saltation' or 'mutation.' A mutation, being a genotypic change will, according to Wagner and Mitchell (116) show a change in metabolism resulting in one
or more of the following chemical consequences:

(1) An increase or decrease in the amount of a single compound or group of compounds.
(2) The disappearance of one or more compounds.
(3) The appearance of one or more compounds.
(4) Inverse, reciprocal changes in the relative concentrations of two or more compounds.

These types of chemical change are not mutually exclusive for any one case of gene change.

Since each genotypic change results in a change in metabolism then the individual progeny from any one culture of a single spore origin may vary within wide limits in regard to performance of any given biochemical activity, despite the fact that for all practical purposes, all may be morphologically indistinguishable. This phenomenon, known as strain specificity (40) is generally revealed only by biochemical analysis.

If the progeny of any one culture may be expected to differ markedly then the differences between morphologically different strains would be expected to be that much greater. Those genotypic changes resulting in a deficiency for some essential metabolite would, of course,
not be found on a minimal medium since this type of mutation would be lethal. Those changes which result in only a partial block in a metabolic pattern not absolutely essential to the growth of the organism would be the types to show up on biochemical analysis (40) (20). Hence, it should be possible to differentiate between fungal strains by means of biochemical analysis of the cellular constituents and by-products of the isolates.

The work of Raistrick et al. (90) (91) on the polychemism of fungi shows that species may be differentiated by this method but they did no investigations on fungal strains. Their studies were mainly on the carbohydrate and organic acid constituents of fungi. Nitrogenous constituents were not studied.

Owing to technical difficulties the content of nitrogenous constituents such as amino acids, amines, amides, and nucleotides were not studied intensively in organic materials until Consden, Gordon, and Martin (30) introduced the technique of paper partition chromatography in 1944. The technique was not applied to plants until 1947 (31). Since then a large number of workers have applied the technique to the elucidation of nitrogenous materials in plants but
few have studied fungi. Prior to the use of paper chromatography and microbiological assay the classical techniques of chemical isolation and analysis were used. These older techniques were used for the isolation of specific compounds and could not cover the whole spectrum of nitrogenous products which it is now possible to isolate from organic materials.

The first isolation of nitrogenous compounds from fungi was done by Alderhalden and Rona (1) from hydrolysed \textit{Aspergillus niger} van Tiegh mycelium. Glycine, alanine, leucine, glutamic acid, and aspartic acid were isolated. Engeland and Kutscher in 1910 (36) isolated agmatine from rye ergot and Kutscher (60) in the same year reported histamine from the same source. Yoshimura (132) isolated histidine and trimethylamine from \textit{Boletus edulis} (Bull) Fr. also in 1910. The same organism was studied in 1912 by Winterstein and Reuter (122) who reported the isolation of alanine, valine, leucine, aspartic acid, glutamic acid, phenylalanine, proline, histidine, and trimethylamine. Kung (59) reported the isolation of putrescine from an Amanita species.

Urea has been reported in a number of fungi by
Vorbrodt in 1919 (115) isolated alanine, leucine, and tyrosine from *Aspergillus niger*.

Butkewitsch in 1922 (14) demonstrated by his research on deamination mechanisms that all amino acids from protein and peptone breakdown do not deaminate at the same rate, some in fact not being deaminated or further attacked at all with the result they accumulate. This observation shows how some of the more unusual amino acids may arise in the free amino acid pool. This observation was confirmed by Waksman and Lomanitz 1925 (117), Chrzaszcz and Ziukow 1931 (24), Chrzaszcz and Pisula 1933 (23), and Chrzaszcz and Zakomorny 1933 (25).

Yukawa (133) isolated tyramine from *Aspergillus oryzae* (Ahlburg) Cohn and demonstrated it was produced from tyrosine. Forst and Weese (38) confirmed Kutscher's (60) observation on the presence of histamine in ergot. Yamada and Ishada (130) isolated putrescine and cadaverine from *Aspergillus oryzae* grown on soybean protein. Takata (111)
isolated betaine, stachydine, six common amino acids and the four main nucleic acid bases from the same organism.

Skinner in 1934 (101) reported tyrosine in a number of fungi. Lim (64) hydrolysed the protein of *Rhizopus japonicus* and obtained histidine, arginine, tyrosine, lysine, tryptophane, and cystine. Keil and Bartman (53) obtained putrescine and phenylethylamine from *Boletus luteus*. Lemoigne and Desveaux (62) obtained hydroxylamine from *Aspergillus niger*. Woolley and Peterson (125) isolated leucine and isoleucine from *Aspergillus sydowi* (Bain and Sart.) Thom and Church. (The following year (126) they reported arginine, histidine, and lysine in the same organism. In another paper published in 1937, (127) they reported the isolation of aspartic acid, glutamic acid, leucine, isoleucine, tyrosine, proline, valine, serine, threonine, and tryptophane for a total of thirteen amino acids from the same organism, a record up to this time. Bohonos et al. (12) confirmed seven of these amino acids from the hydrolysed residue of the same organism. Ovcharov (79) reported thiourea in *Verticillium albo-atrum* and urea (78) the following year in *Botrytis cinerea*, Pers., *Pythium deBaryanum* Hesse, and *Verticillium albo-atrum*. 
His experiments showed that the production of urea and thiourea depended on the nitrogen source. Funk and Fink (41) isolated tyramine and histamine from ergot. Birkinshaw et al. (9) isolated fumarooalanide from *Penicillium resticulosum* Smith. Wieland and Witkop (120) isolated hydroxytryptophane, hydroxyproline, cystine, and alanine from hydrolysed phalloidin, a polypeptide obtained from *Amanita phalloides* (Bull.) Fr. Plattner and Clausson-Kaus (84, 85) isolated lycomarasmin, a tripeptide from *Fusarium lycopersici* Sacc., an example of the many peptides that have been isolated from fungi in the search for antibiotics. Woolley (124) found lycomarasmin to consist of asparagine, glycine, and α-hydroxyalanine. α-hydroxyalanine, a rare unstable compound, is also found in ergotamine (40), an alkaloid produced by the ergot fungus, *Claviceps purpurea* (Fr.) Tull. Stokes and Guinness (109) applied the relatively new technique of microbiological assay to determine the quantities of ten common amino acids in *Penicillium notatum* Westling, *Rhizopus nigricans* Ehr., *Aspergillus niger*, *Saccharomyces cerevisiae* Meyen, *Rhodotorula rubra* (Demme) Lodder and three species of bacteria as affected by cultural and environmental conditions. Sporulation lowered the amino acid content of the mycelium by half. Amount of aeration,
and composition of the medium were found to affect the quantity of amino acids, but did not change the spectrum of amino acids.

Paper partition chromatography, developed in 1944 by Consden, Gordon, and Martin (30) was first used to isolate nitrogenous materials in plants when Dent, Stepka, and Steward (31) used it to detect free amino acids in plant cells. This technique is considerably more sensitive than the conventional chemical techniques, being able to detect innumerable compounds whose very existence in nature had not previously been suspected. Steward, Zacharius, and Pollard (108) record a very large number of nitrogenous compounds found in plant sources by the technique of paper chromatography.

According to Foster (40) there is no reason for not believing that fungi synthesize all known amino acids although the possibility exists that when thorough amino acids analysis of mold mycelium have been done on more of the very large number of fungi known it will be found that some may lack some of the so-called 'recognized' amino acids. Precedence for the absence of amino acids in the protoplasm of biological systems is provided by the absence of some
amino acids in algae low in the phylogenetic series but present in those higher in the series, the absence diminishing up the evolutionary sequence (69, 70). He also points out that these analyses were done before the advent of paper chromatography and therefore, may not be accurate but should be checked by the newer technique. However, Smith and Young (104, 105) using the technique of column chromatography were also able to show amino acid differences between different algal species.

Since 1947 an increasing number of research workers have used the technique of paper chromatography for the detection of nitrogenous constituents of fungi until, at the present time, most of the commonly occurring amino acids in protein have been isolated. (65)

Other more unusual compounds have also been detected. Horowitz (47) detected cystathionine in a Neurospora mutant. Reed (93) found γ-amino butyric acid in baker's yeast. Chamberlain, Cutts, and Rainbow (21) found arylamine was produced by Saccharomyces carlsbergensis Hansen, Saccharomyces cerevisiae, and Brettanomyces species, but not by Schizosaccharomyces octosporus Beijer under conditions of biotin deficiency and excess of methionine. Sheffner, Lloyd
and Grabow (96) isolated glutamine from Saccharomyces cerevisiae under conditions of growth testing the utilization of amide and amino nitrogen. Yokata (131) found alanine was produced by Fusarium solani (Martius) Appel and Wollenw using a variety of nitrogen sources. Thiourea was again reported in Verticillium albo-atrum by Caroselli (18). He indicated this compound was the cause of leaf wilt and was produced by all strains except a white non-matted variant. Glutamine was found by Pyle (89) in Penicillium chrysogenum Thom.

A number of workers have tried to differentiate species and strains of fungi using paper chromatography. Toyohiko et al. (114) found differences between ergot-producing species. Ergots from Elymus mollis Trin, Imperata cylindra, and Sasa nipponica contained aspartic acid, glutamic acid, alanine, histidine, valine, phenylalanine, and leucine as did the ergots of rye wheat and Secale cereale L. stored for twenty-seven years. In addition to the above compounds fresh rye wheat, Secale cereale, and Triticum vulgare Lam. contained tyrosine and glycine. DeVay (33, 34) studied the amino acid compositions of thirty culturally different monosporidial haploid lines of Ustilago zeae (Beckm) Unger
isolated from smut galls on corn and tried to relate differences in free amino acids or in bound amino acids to sex and pathogenicity. Two isolates contained an unknown free amino acid not present in any of the other lines, the unknown in each case being different for each isolate. However, these differences could not be related to sex and pathogenicity.

Hare (44) investigated the amino acid composition of the protein of eight dermatophytic fungi and found them to be qualitatively similar.

Work and Dewey (129) studied the \( \alpha, \epsilon \)-diaminopimelic acid distribution among one hundred and eighteen microorganisms. They found it to be present in all the bacteria except Gram positive cocci, Streptomyces species, and Actinomyces species. Of the algae, fungi, yeasts, viruses, and animal protozoa studied, only blue green algae contained this amino acid. In the course of this study all the free amino acids of each organism were extracted and compared. All the usual amino acids plus a number of unusual compounds were found. Seven spots, which they were unable to identify, were found, some only in one type of organism, others in several. \( \beta \) alanine was found only in the Eubacteriales \( \alpha \) amino
butyric acid was found in three organisms, all unrelated, one of them being the fungus Microsporum gypseum. Microsporum audouini Vuill. and Microsporum canis Bodin did not have this amino acid, showing a definite species difference. Microsporum gypseum Con. also contained glucosamine which was not found in the other two species. \( \gamma \)-amino butyric acid was found in a wide variety of unrelated organisms. Of the fungi studied only Penicillium cyclopium Westling and Penicillium spinulosum Thom contained it. Penicillium notatum, the only other Penicillium studied, did not, indicating another instance of species differences. Of the five Aspergilli studied, four (Aspergillus flavus-oryzae, Aspergillus glaucus LK., Aspergillus oryzae, and Aspergillus ruber Bren.) contained glucosamine. Aspergillus niger did not. Significant variations in concentrations of individual amino acids from different organisms were also observed. From these they concluded that 'each species has a characteristic overall amino acid composition.'

Yokata (131) tested the effect of various nitrogen sources on free amino acid formation by Fusarium solani. Proline was produced only when potassium nitrate was supplied. He was unable to detect any free amino acids in the mycelium.
at all when ammonium chloride was used as the nitrogen source. In all other cases the free amino acid contents of the mycelium were the same.

Simonart and Chow in a series of four papers (97, 98, 99, 100) studied the effect of pH, calcium chloride, carbon source, amino acid used as nitrogen source, and age of mycelium on the free amino acids of Aspergillus oryzae. Each of these changes resulted in qualitative free amino acid differences.

Pittoni and Moret (82, 83) tested the effect of carbon source on the cellular proteins of Oospora lactis (Fres.) Sacc. Aspartic acid, glutamic acid, serine, glycine, threonine, alanine, histidine; lysine, arginine, valine, leucine, isoleucine, tyrosine, and cystine were found in all cases.

Many investigators have reported on the formation of extracellular nitrogen compounds of fungi. Morton (75) using Scopulariopsis brevicaulis (Sacc.) Banier, reports that the free amino nitrogen from the mycelium equals forty-four percent of the total nitrogen of the mycelium while the free amino nitrogen from extracellular sources equals
only ten percent of the total extracellular nitrogen. Most of the extracellular nitrogen was in the form of small peptides indicating the study of extracellular peptides might throw some light on the problem of strain differentiation.

Qualitatively, the amino acids were the same, mycelially and extracellularly, in the early stages of growth but during the later stages a few new amino acids appeared extracellularly.

The above material indicates that a wide variety of nitrogenous compounds occur in fungi and that species (129) and strain (34) differences exist although these differences may be modified by cultural and environmental conditions. As previously indicated this thesis is an attempt to delineate strains of *Verticillium albo-atrum* R. from one another by a survey of their nitrogenous constituents by means of the techniques of paper chromatography and column partition chromatography using ion exchange resins. An attempt will also be made to relate any possible differences to pathogenicity and to relate any differences to possible effects of cultural and environmental conditions.
GENERAL PROCEDURES

I. Organisms and culture methods.

The organisms used in the following studies are listed in Table I.

Table I. Description of isolates of Verticillium albo-atrum and Colletotricum lindemuthianum used in the study of nitrogenous constituents

<table>
<thead>
<tr>
<th>Verticillium Isolates</th>
<th>Presley (a)</th>
<th>Isolated From</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>B</td>
<td>tomato</td>
<td>Summerland, B.C. (b)</td>
</tr>
<tr>
<td>V16</td>
<td>B</td>
<td>potato</td>
<td>&quot;</td>
</tr>
<tr>
<td>V5</td>
<td>B</td>
<td>sweet cherry</td>
<td>&quot;</td>
</tr>
<tr>
<td>SM1</td>
<td>B</td>
<td>soil</td>
<td>Green Timbers, B.C. (c)</td>
</tr>
<tr>
<td>SM67</td>
<td>B</td>
<td>soil</td>
<td>&quot;</td>
</tr>
<tr>
<td>SM72 (1)</td>
<td>B</td>
<td>soil</td>
<td>&quot;</td>
</tr>
<tr>
<td>V14</td>
<td>C</td>
<td>tomato</td>
<td>Summerland, B.C.</td>
</tr>
<tr>
<td>V25</td>
<td>C</td>
<td>apricot</td>
<td>&quot;</td>
</tr>
<tr>
<td>V34</td>
<td>X</td>
<td>tomato</td>
<td>&quot;</td>
</tr>
<tr>
<td>V12</td>
<td>X</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>V32</td>
<td>X</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>V20</td>
<td>X</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>V17</td>
<td>X</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>V3</td>
<td>X</td>
<td>cantaloupe</td>
<td>&quot;</td>
</tr>
<tr>
<td>V9</td>
<td>X</td>
<td>Hop</td>
<td>&quot;</td>
</tr>
<tr>
<td>V14</td>
<td>X</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>V21</td>
<td>X</td>
<td>eggplant</td>
<td>&quot;</td>
</tr>
<tr>
<td>42-I-245A</td>
<td>X</td>
<td>potato</td>
<td>Ottawa, Ont. (d)</td>
</tr>
<tr>
<td>41-B22B</td>
<td>X</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>HNS</td>
<td>X</td>
<td>mint</td>
<td>Oregon State College (e)</td>
</tr>
<tr>
<td>V-H-1-S</td>
<td>X</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>M3X</td>
<td>X</td>
<td>snowberry</td>
<td>&quot;</td>
</tr>
<tr>
<td>V146</td>
<td>A</td>
<td>tomato</td>
<td>Summerland, B.C.</td>
</tr>
<tr>
<td>V149</td>
<td>A</td>
<td>pepper</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Colletotricum Isolates</th>
<th>Isolated From</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Michigan</td>
<td>bean</td>
<td>University of Idaho (f)</td>
</tr>
<tr>
<td>Cornell</td>
<td>bean</td>
<td>&quot;</td>
</tr>
<tr>
<td>Cornell</td>
<td>bean</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

(a)Presley types: A sparse mycelium and microsclerotia;
B fluffy mycelium and no microsclerotia;  C appressed mycelium and no microsclerotia;  X abundant mycelium and abundant microsclerotia.

(b) Summerland isolates supplied by Mr. G. E. Woolliams.
(c) Green Timbers isolates supplied by Mr. G. Wallis.
(d) Ottawa isolates supplied by Miss C. A. Bowerman.
(e) Oregon isolates supplied by Dr. C. E. Horner.
(f) Idaho isolates supplied by Mr. John A. Moisey.

The three Colletotrichum lindemuthianum

(Sacc. and Magn.) Bri. and Cav. were used as a check on the theory that differences in nitrogenous constituents will show strain differences, since these isolates have been shown to be definite strains by pathogenicity tests at Cornell and Michigan. Single spore cultures were made of all isolates of both organisms.

All isolates were grown in 100 ml. portions of Richard's solution which contained, in one litre of solution, 10 gms. potassium nitrate, 5 gms. potassium monobasic phosphate, 2.5 gms. magnesium sulphate, 0.02 gms. ferric chloride, and a carbohydrate source equivalent to 50 gms. sucrose. This medium was chosen because of its readily reproducible character and its absence of organic nitrogen compounds which might interfere with the determination of nitrogenous compounds produced by the isolates. All isolates of Verticillium grew well on this medium. Colletotrichum isolates made adequate, but much slower growth.

All isolates were grown, except where noted, by the shake culture method on a Gump shaker (240 oscillations
per minute) in 500 ml. Florence flasks at 30 °C. After the period of growth the contents were centrifuged at 2000 r.p.m. for 15 minutes and the supernatant discarded. The residue was resuspended in distilled water and centrifuged a further 15 minutes, the supernatant again being discarded. This step was carried out three times and served to remove any traces of extracellular materials associated with the mycelium. Except where noted, the cells are resuspended in distilled water and the cell contents liberated by sonic disruption using a 10 K.C. Raytheon sonic oscillator for 15 minutes. Sufficient 95 percent ethyl alcohol was added to the sonicate to obtain a 70 percent alcohol solution. The material was extracted with the alcohol for twenty-four hours. The mixture was then filtered and the residue washed twice with 70 percent ethanol. The filtrates were combined and concentrated at 50 °C on a Craig flask evaporator. The concentrated solution was then evaporated to dryness in vacuo over anhydrous calcium chloride. The residue was redissolved in 2 c.c. distilled water and stored until use at -10 °C. This material was used for spotting the chromatograms.

II. Chromatography

A. Apparatus.

Two chromatography cabinets were used in this study.
One dimensional descending chromatograms were run in a conventional all glass chromatography cylinder. Two-dimensional chromatograms were run in a wooden cabinet similar to that used by Dent (32).

B. Solvents.

All solvents were of C.P. quality. The phenol was vacuum-distilled and stored in the dark in a brown bottle. All other solvents were used without further purification. For the composition of the general solvents used see Table 11.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Composition</th>
<th>Comment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol: water</td>
<td>100: 20 V./v.</td>
<td>Used with 0.3% NH$_3$ or 1:1 acetic acid: water</td>
<td>Consden 1944</td>
</tr>
<tr>
<td>Butanol: acetic</td>
<td>4:1:5 v./v.</td>
<td>Particularly for 1 dimensional chromatograms employing phenol: water as first solvent.</td>
<td>Slotta 1951</td>
</tr>
<tr>
<td>acid: water</td>
<td>shake 3 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>stand for 2 hrs.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,6 lutidine:</td>
<td>1:1:1</td>
<td>Used as second solvent on two dimensional chromatograms employing phenol: water as first solvent.</td>
<td>Dent (1947)</td>
</tr>
<tr>
<td>collidine:</td>
<td>add 1-2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>water.</td>
<td>diethylamine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isobutyric acid:</td>
<td>4:1</td>
<td>Used as second solvent on two dimensional chromatograms employing Butanol: acetic acid: water as first solvent.</td>
<td>Barry et al. 1951</td>
</tr>
<tr>
<td>water.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Other solvents are described where used.
C. Reagents and special techniques.

1. Ninhydrin: This reagent consists of a 0.2% solution of ninhydrin (triketohydrindene hydrate) in water-saturated butanol (30). If kept in a tightly stoppered brown bottle the reagent will keep for a month. The reagent was sprayed on the dried chromatograms and allowed to dry. The chromatograms was allowed to stand at room temperature in the dark for 24 hours to promote colour development (32). This step avoids the danger of fading of the colour owing to overheating of the oven. However, some chromatograms were heated at 90°C for five to ten minutes in order to detect any ninhydrin-positive compounds which do not undergo the ninhydrin reaction at room temperature.

2. Sakaguchi reaction: The chromatograms were sprayed with a 0.1% solution of naphthol in 1NNaOH. After drying the paper was sprayed with NaClO solution prepared from an equal mixture of ethanol and commercial NaClO (Chlorox). Arginine appeared as a red spot (121).

3. Pauly's Reaction: 1% sulfanilimide was dissolved in 10% v./v. HCL. 5 ml. of this solution was added to 5 ml. of 5% sodium nitrite in a 50 ml. graduate and shaken for one minute. n-butanol was added to the 50 ml. mark, shaken for
one minute, and allowed to stand for four minutes. The butanol layer was decanted and used to spray the chromatogram. The sheet was dried for five minutes in a current of air and sprayed with half-saturated Na₂CO₃ solution. Imidazoles (e.g. Histidine) gave a bright cherry red colour. (11) (13).

4. Hydrogen peroxide reaction: Sulphur amino acids may be detected by using hydrogen peroxide to oxidize them. The resulting difference (Δ) in their movement on the chromatogram is detected with the conventional ninhydrin spray. Thus, methionine, which overlaps valine on conventional chromatograms, is oxidized to methionine sulfoxide and methionine sulphone which appear as separate spots. Similarly, cystine is converted to cysteic acid. (32).

The sample was placed on the chromatogram in the usual manner and dried. An equal volume of 30% H₂O₂ was applied to the spot. Three times this amount of 0.02% ammonium molybdate was added to the spot and dried. The chromatogram was run in the usual manner.

5. Acid-Base technique: (32)- Addition of an acid or a base in the bottom of the cabinet before a run results in a change in pH conditions which affect the movement of acidic and basic compounds. Comparison with a chromatogram run in the
usual manner helps establish basicity or acidity of a compound. Basic or acidic compounds previously obscured by overlapping may be detected in this manner.

6. Peptide detection: Ninhydrin reagent was used for the detection of peptides although this reagent is not as sensitive for peptides as it is for amino acids (86). To test for the presence of peptides the sample was placed in a continuous line across the paper three inches from the top. The one-dimensional chromatogram was run in the conventional manner. Ninhydrin reagent was sprayed along the edges of the chromatogram and the colour allowed to develop. The individual bands thus revealed were cut and eluted with 50% ethanol. The samples were dried in vacuo and hydrolysed twelve hours with concentrated HCl at 80°C. This step was repeated several times to remove the HCl. The residue was taken up with water and used for spotting chromatograms which were run in the conventional manner. The separation of a spot into several components was considered proof of the existence of peptide.

7. Detection of amines: Amines were detected with ninhydrin reagent after removal from the alcohol soluble fraction (10). Cultures of each isolate were grown and the alcohol soluble fraction extracted as described in general
procedures. The extract was taken up in 5 ml. water and added to 6 gms. of a mixture of K₃PO₄:Na₂SO₄ (1:6 w/w.) dissolved in 15 ml. of hot water. The amines were extracted with peroxide-free ether in a continuous liquid-liquid extractor (80) for 24 hours. The receiving flask contained 1 ml. of 4% phosphoric acid. After extraction the ether was removed from the aqueous solution in the receiving flask by heating the solution on a steam bath. The residue was diluted with water, neutralized to pH 7.0 with NaOH and the solution saturated with K₃PO₄: Na₂SO₄ mixture. The amines were extracted from this alkaline solution with n-butanol. The butanol extracts were dried with Na₂SO₄. Gaseous HCl was passed through the butanol solution to neutralize the amines. The butanol was concentrated in vacuo to a convenient volume for paper chromatography.

8. Detection of amides: Amides, such as asparagine and glutamine react with ninhydrin to form a characteristic rusty-brown colour. This colour plus their Rf values against a known standard are sufficient to identify these compounds. Hydrolysis results in the disappearance of these spots and is a further diagnostic test (107).

9. Amino acid standards: These standards contained 0.1M of the amino acid in n-propanol as supplied by Shandon
Company, London, England. The standards were run alongside the unknowns - and served to identify the unknowns.

D. General method of chromatographic investigation.

All Rf values were determined using the rapid determination scale developed by Nettleton et al. (76).

The routine used to investigate the alcohol solublre fraction of each isolate was as follows:

1. A one-dimensional chromatogram was run with butanol: acetic acid: water solvent and developed with ninhydrin. This served as a rough qualitative and quantitative test from which the volume of solution necessary for further tests was determined.

2. Step 1 was repeated using Pauly's reagent (see Reagents 3) as the developer. This test indicated the presence or absence of histidine and other diazo compounds.

3. Step 1 was repeated using Sakaguchi's reagent (see Reagents 2) as the developer. This test indicated the presence or absence of arginine.

4. A one-dimensional chromatogram was run with Butanol: acetic acid: water solvent after oxidation of the unknown spots with H₂O₂ (See Reagents 4). Standards of cysteine, cystine, cysteic acid, methionine, methionine
sulphoxide, methionine sulphone were run alongside these spots. This test served to detect sulphur amino acids.

5. Step 1 was repeated in two parts, using acid in the cabinet or base in the cabinet (See Reagents 5), which served to indicate the presence or absence of acidic and basic compounds. Aspartic acid, glutamic acid, lysine, and hydroxylysine were used as standards for comparison.

6. One dimensional chromatograms were run using butanol: acetic acid: water, phenol: water, and isobutyric acid: water solvents using suitable ninhydrin positive standards for each separated ninhydrin positive unknown spot for comparative purposes. Their behavior in each of the three solvents helped in the identification of the unknown.

7. A test for peptide was run as described in Reagents 6 using butanol: acetic acid: water as the solvent.

8. A test for amines was run as described in Reagents 7 using butanol: acetic acid: water as the solvent.

9. A test for amides was run as described in Reagents 8 using butanol: acetic acid: water as the solvent.

10. Two dimensional chromatograms were run with phenol: water and 2, 6- lutidine: collidine: water solvents which detects nearly all the amino acids when compared with
a 'map of spots' prepared according to the method of Dent (32).

11. Step 10 was repeated using the H₂O treatment as previously described as a further check on sulphur amino acids.

12. Two-dimensional chromatograms were run with butanol: acetic acid: water and isobutyric acid as solvents and compared with a 'map of spots' prepared, using the same solvents as a further check on the identity of the compounds.

A comparison of R.f. values obtained by the methods described above with those reported in the literature was found to be of little value since slight changes in conditions resulted in changes in R.f. values.

All results were checked at least four times before being accepted.
EXPERIMENTAL

Study I. The pathogenicity of Verticillium isolates towards tomato.

A. Methods and Procedures.

All *Verticillium albo-atrum* isolates described in Table I were tested for pathogenicity to tomato to determine if any possible differences in nitrogenous composition could be related to this pathogenicity.

Flats of steam-sterilized soil were sown with seed of the Bonny Best tomato variety. When three weeks old these plants were transplanted to steam-sterilized soil in flats, twenty plants to a flat. Before being transplanted the roots of each plant were inoculated with the mycelium of a *Verticillium albo-atrum* isolate by dipping the roots into a mycelial suspension prepared by growing the isolate in Richard's solution by shake culture as described under General Procedures. Each of the twenty plants comprising one flat were inoculated with the same isolate. Several flats of plants were not inoculated with any isolate, their roots being dipped in distilled water. These flats served as controls.

The plants were allowed to grow for thirty days
at which time they were cut off two inches above the soil level. The roots were then removed from the soil, washed, and cut off one inch below the soil level. This three inch portion was cut into six one-half inch pieces which were placed in a petri plate containing 95% ethanol. The pieces were removed from the alcohol with sterile tweezers and placed in a petri plate containing 0.1% HgCl$_2$ solution for two minutes. Sterile tweezers were used to remove the pieces from the HgCl$_2$, rinse them in sterile water, and place them in another petri plate containing sterile water for five minutes. They were then transferred to another petri plate of sterile water where each half inch piece was cut into several fragments. Five representative pieces were used to inoculate a potato dextrose agar plate which was then incubated for three days at 20°C. The presence of *Verticillium albo-atrum* mycelium on the plate was considered proof that the plant was infected. This procedure was carried out for each plant in the flat and the results tabulated as plants infected per twenty plants inoculated per isolate.

B. Results.

The pathogenicity of each isolate toward tomato is indicated in Table 3.
Table III. Pathogenicity of *Verticillium albo-atrum* isolates to tomato.

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Isolate</th>
<th>Plants infected per 20 plants</th>
<th>Presley group A</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>tomato</td>
</tr>
<tr>
<td>V1</td>
<td>20</td>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V1/4</td>
<td>19</td>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V3/4</td>
<td>20</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V12</td>
<td>20</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V32</td>
<td>20</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V20</td>
<td>18</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V46</td>
<td>20</td>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V17</td>
<td>20</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V3</td>
<td>20</td>
<td>X</td>
<td>cantaloupe</td>
<td></td>
</tr>
<tr>
<td>V28</td>
<td>15</td>
<td>A</td>
<td>hop</td>
<td></td>
</tr>
<tr>
<td>V9</td>
<td>17</td>
<td>X</td>
<td>pepper</td>
<td></td>
</tr>
<tr>
<td>V1/49</td>
<td>20</td>
<td>A</td>
<td>potato</td>
<td></td>
</tr>
<tr>
<td>42-I-245A</td>
<td>20</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41-822B</td>
<td>20</td>
<td>X</td>
<td>mint</td>
<td></td>
</tr>
<tr>
<td>HNS</td>
<td>20</td>
<td>X</td>
<td>snowberry</td>
<td></td>
</tr>
<tr>
<td>V-H-I-H</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 2</th>
<th>Isolate</th>
<th>Plants infected per 20 plants</th>
<th>Presley group</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>sweetcherry</td>
</tr>
<tr>
<td>V5</td>
<td>3</td>
<td>B</td>
<td></td>
<td>apricot</td>
</tr>
<tr>
<td>V25</td>
<td>1</td>
<td>C</td>
<td>hop</td>
<td></td>
</tr>
<tr>
<td>V1/4</td>
<td>8</td>
<td>X</td>
<td>eggplant</td>
<td></td>
</tr>
<tr>
<td>V21</td>
<td>5</td>
<td>X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Isolate | Plants infected per 20 plants | Presley group | Source |
---|---|---|---|
V16 | 0 | B | Potato |
SM7 | 0 | B | soil |
SM67 | 0 | B | " |
SM72 (1) | 0 | B | " |

A Presley group indicates colony type.

- A - sparse mycelium and sparse microsclerotia.
- B - fluffy mycelium and no microsclerotia.
- C - appressed mycelium and no microsclerotia.
- X - abundant mycelium and no microsclerotia.

Relative pathogenicity was determined by arbitrarily setting up three groups. Group 1 is pathogenic to tomato, group 2 slightly pathogenic and group 3 non-pathogenic. The distinction between pathogenic and slightly pathogenic is an artificial one, an isolate falling into either group depending on the number of plants per twenty plants it infected, those infecting eleven to twenty plants being placed in group 1, those infecting one to ten being placed in group 2.

Table 3 clearly shows that there is no apparent relationship between colony type as indicated by Presley group, source of the isolate, and pathogenicity of the isolate. The four so-called strain groups of Presley (87) are not consistent in their pathogenic characteristics. That is, morphological strain types of this organism apparently do not correspond to pathogenic strain types.
Study II. Survey of the ninhydrin-positive compounds of isolate V3.

A. Methods and procedures.

Three flasks of the isolate V3 were harvested and the alcohol soluble extract prepared as described in General Procedures. The routine described in General Method of Chromatographic Investigation was used to separate and identify the ninhydrin-positive compounds in each of the three extracts.

B. Results.

A diagram of a two-dimensional chromatogram of the free amino acids in the mycelial extracts of isolate V3 is shown in Figure 1. The following amino acids were found in the extract: aspartic acid, glutamic acid, lysine, histidine, isoleucine, serine, valine, proline, methionine, alanine, tyrosine, threonine, glycine, and an unknown spot A, which was neutral. No amines, amides or peptides were present. The results had to be obtained on four chromatograms run on four successive days before being accepted.

Study III. Effect of the change of environment on the ninhydrin-positive compounds of isolate V3.

1. Effect of carbon source.

A. Methods and Procedures.

Isolate V3 was grown in Richards' solution as
FIG. 1: TWO DIMENSIONAL AMINO ACID CHROMATOGRAM OF ISOLATE V-3

described under General Procedures. Glucose, mannose, lactose and sucrose were the carbohydrate sources used. Three flasks of isolate V3 were grown with each carbohydrate source and extracted separately as previously described. Each of the resulting extracts (three for each carbon source) was treated according to the routine for the investigation of ninhydrin-positive compounds.

B. Results.

The same amino acids were found to be present in each case, that is, aspartic acid, glutamic acid, lysine, histidine, isoleucine, tyrosine, threonine, glycine and unknown spot A. Since no attempt was made to ensure that the same weight of mycelium was extracted in each case a quantitative comparison cannot be made with any degree of accuracy. However, the isolate grown on lactose did not make more than two thirds the growth of the other cultures, and hence appeared to have somewhat less free amino acids present, particularly valine and isoleucine.

2. Effect of the initial pH of the medium on the nitrogenous constituents of isolate V3.

A. Methods and Procedures.

Twelve flasks of Richards' solution, using sucrose as the carbon source, were prepared and the pH's adjusted with
INHCl and INNaOH so that three flasks were at pH 3, three at pH 5, three at pH 7 and three at pH 9. The flasks were inoculated with isolate V3, incubated and extracted as described in General Procedures.

B. Results.

Aspartic acid, glutamic acid, lysine, histidine, isoleucine, serine, valine, proline, methionine, alanine, tyrosine, threonine, glycine and unknown spot A were present in all cases. A quantitative comparison of the various amino acids was not feasible since much less growth occurred at pH 2 and pH 9 than at pH 5 and pH 7. Final pH's of the medium in each flask are shown in Table 4.

Table 4. Initial and final pH's of the media used for culturing isolate V3.

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>Initial pH</th>
<th>Final pH</th>
<th>Flask No.</th>
<th>Initial pH</th>
<th>Final pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.10</td>
<td>6.00</td>
<td>7</td>
<td>7.20</td>
<td>8.00</td>
</tr>
<tr>
<td>2</td>
<td>3.20</td>
<td>6.00</td>
<td>8</td>
<td>7.05</td>
<td>8.20</td>
</tr>
<tr>
<td>3</td>
<td>3.10</td>
<td>6.10</td>
<td>9</td>
<td>7.10</td>
<td>8.10</td>
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<tr>
<td>4</td>
<td>5.30</td>
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<td>10</td>
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<td>8.40</td>
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<tr>
<td>5</td>
<td>5.20</td>
<td>7.05</td>
<td>11</td>
<td>9.25</td>
<td>8.40</td>
</tr>
<tr>
<td>6</td>
<td>5.20</td>
<td>7.00</td>
<td>12</td>
<td>9.15</td>
<td>8.40</td>
</tr>
</tbody>
</table>

The results shown in Table 4 indicate that under acid and neutral conditions Verticillium albo-atrum excretes a basic substance into the medium.
3. Effect of shake culture vs. standing culture on the nitrogenous constituents of isolate V3.

A. Methods and Procedures.

Six flasks of Richards solution, using sucrose as the carbon source, were prepared, inoculated with isolate V3. Three were grown as shake cultures in the usual manner and the other three were grown as standing cultures. The standing cultures were left standing beside the Gump shaker so that exactly the same temperature conditions prevailed for both types of cultures. The mycelia were harvested and extracted in the usual manner.

B. Results.

No differences were found between the nitrogenous constituents of standing and shake cultures. These constituents were the same as those present in isolate V3 in part 1.

4. Effect of age of mycelium on the nitrogenous constituents of isolate V3.

A. Methods and Procedures.

Eighteen flasks of Richards' solution, using sucrose as the carbon source, were prepared, inoculated with V3, and placed on the Gump shaker in the usual manner. Flasks were removed in such a way that three flasks each of 1 day,
2 day, 4 day, 8 day, and 16 day old pellets were obtained. Each flask was harvested and extracted in the usual manner.

B. Results.

Age of mycelium had no effect on the nitrogenous constituents of isolate V3, the usual fourteen spots being obtained, namely, aspartic acid, glutamic acid, lysine, histidine, isoleucine, serine, valine, proline, methionine, alanine, tyrosine, threonine, glycine, and unknown spot A.

Study IV. Ninhydrin - positive components of monoconidial cultures of a number of isolates of Verticillium albo-atrum R. and B., and Colletotricum lindemuthianum (Sacc. and Magn.) Bri. and Cav.

A. Methods and Procedures.

Twenty-five isolates of Verticillium albo-atrum and three isolates of Colletotricum lindemuthianum as described under "Organisms" were cultured on Richards' solution, using three flasks per isolate.

B. Results.

All Verticillium isolates contained the same nitrogenous constituents as outlined for isolate V3 in previous experiments. The three Colletotricum strains contained all the constituents of the Verticillium isolates and in addition,
all three contained a neutral spot designated unknown B. Figures 1 and 2 show representative two dimensional chromatograms of Verticillium and Colletotrichum isolates.
**Fig. 2**: Two-dimensional amino acid chromatogram of isolate α: *Colletotricum lindemuthianum*

DeVay (34) compared the amino acid composition of a number of isolates of *Ustilago zeae* in order to determine differences between strains or races which could be related to sex and pathogenicity. He found definite qualitative differences between two of the thirty isolates studied but could not relate these differences to sex or pathogenicity. The two distinguishable isolates also differed from each other in that each contained an unknown substance, not present in the other isolates, and which was not present in the other. They did not differ in respect to any known ninhydrin-positive substance. The investigations reported in this thesis showed no qualitative differences at all between the various Verticillium isolates, nor were there any differences between the three Colletotrichum strains. All Colletotrichum strains, however, did possess an unknown (Unknown B) which was not present in the Verticillium isolate showing a definite species difference in the nitrogenous constituents of fungi, a situation previously shown to exist by a number of investigators, as has been discussed adequately in the introduction to this thesis.

The results of this investigation cannot be interpreted to mean that differences of this type do not occur between isolates of *Verticillium albo-atrum* but merely that there
were no differences between the isolates studied. There is no reason to believe that, given enough isolates, one might come up with one which does differ in some respect by possessing a compound not in the other isolates or, conversely, by lacking one of the compounds already shown to be present. However, it is obvious from these studies that even if such differences do exist they will be of little value in the differentiation of the strains of this organism. It is also obvious that such a difference in nitrogenous compounds will have no apparent effect on the pathogenicity of the organism since three groups pathogenic to tomato have been found to have no differences in nitrogenous constituents. These observations do not mean that pathogenicity may not be related to the presence or absence of some chemical component other than nitrogenous materials such as carbohydrate, fatty acids, polyphenols etc., or even to differences in concentration of these components. Further study is needed to clarify this point.

Another area of investigation which might be more profitable in this respect lies in the study of extracellular compounds excreted by the fungus into the medium. Since a large portion of the nitrogenous materials excreted into the medium are peptides (75) a study of these compounds might
throw some light on strain differentiation and its relationship to pathogenicity.

Work and Dewey (129) in a study of one hundred and eighteen species of microorganisms, including bacteria, fungi, actinomycyes, algae and protozoa, showed that each species appears to have a characteristic amino acid composition. In most instances though the differences were quantitative rather than qualitative. These workers considered the general pattern of spots to show significant variations in concentrations of individual amino acids from different organisms. Qualitative differences between species were not consistent. For example with the exception of Aspergillus niger all the five Aspergillus species studied by Work and Dewey (129) contained glucosamine. The four similar species did not differ from one another qualitatively. Hence, the fact that no differences were found among the Verticillium isolates used in this study does not mean that they are necessarily all the same species. Isaac (49) would have us believe they were not. The study of their nitrogenous constituents throws no light on this problem.

As has already been noted, changes in pH, changes in carbon source, age of colony, and type of culture (standing
or shake), did not have any effect on the qualitative aspects of the nitrogenous constituents of *Verticillium albo-atrum* which were studied. Simonart and Chow (97) (98) (99) (100) studied these same aspects of culture growth using *Aspergillus oryzae* as the test organism and their results were exactly opposite to the results of the present study. Carbon sources used were ammonium acetate, ammonium fumarate, ammonium pyruvate, ammonium succinate, ammonium α-keto glutarate and ammonium sulfate plus glucose. Amino acid determinations were made at one and three days using standing liquid cultures. Both age and carbon source changed the qualitative amino acid content of the mycelium. Differences ascribed to influence of age were not due to undetectable concentrations of amino acids in the younger cultures since the younger cultures frequently contained amino acids not present in the older cultures. PH changes, calcium chloride, and the amino acid used as nitrogen source also changed the qualitative amino acid picture. A possible explanation for the uniformity of *Verticillium albo-atrum* nitrogenous constituents as compared to those of *Aspergillus oryzae* during changing environmental conditions may lie in the nature of the two organisms. *Verticillium albo-atrum* is able to infect an extremely heterogenous group of hosts and hence would be expected to be
able to synthesize any necessary metabolite which might be
missing in any one host, whereas Aspergillus oryzae is a
saprophyte, capable of growing only in the presence of specific
nutrients.

A further explanation for the data obtained by
Simonart and Chow (97) (98) (99) (100) may lie in their method
of extraction of nitrogenous constituents. They extracted
the amino acids from the mycelial pads by boiling the homogenized
material in water for five minutes. This method was tried at
the beginning of this study but later abandoned when it was
found impossible to obtain consistent results. Results could
not be duplicated even when lengthy extraction times were used.

The results of this study would seem to indicate that
environmental conditions do not affect the quality of nitrogenous constituents produced, but do affect the quantity. Since
this study involved only one of the twenty-five Verticillium
albo-atrum isolates available it cannot be considered as con-
clusive.

Study V. Quantitative Determination of amino acids of Verti-
cillium albo-atrum isolates.

A. Introduction.

Although a number of investigators (Stokes and Gunness
have shown that quantitative differences in amino acid content exist between species, no one has tried to show these differences between strains of the same species.

B. Organisms.

The organisms used in this study were six isolates representative of the different strain types of *Verticillium albo-atrum* as determined by morphological and pathogenicity studies. Table 5 describes these isolates and their sources.

Table 5. Characteristics of the isolates of *Verticillium albo-atrum* used in the quantitative study of amino acids.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Pathogenicity group A</th>
<th>Presley group B</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI</td>
<td>1</td>
<td>B</td>
<td>tomato</td>
</tr>
<tr>
<td>V5</td>
<td>II</td>
<td>B</td>
<td>sweetcherry</td>
</tr>
<tr>
<td>VI6</td>
<td>III</td>
<td>B</td>
<td>potato</td>
</tr>
<tr>
<td>V14</td>
<td>II</td>
<td>X</td>
<td>hop</td>
</tr>
<tr>
<td>42-L-245A</td>
<td>I</td>
<td>X</td>
<td>potato</td>
</tr>
<tr>
<td>V46</td>
<td>I</td>
<td>A</td>
<td>tomato</td>
</tr>
</tbody>
</table>

A. Pathogenicity groups are based on the number of plants infected per twenty plants inoculated. 1 - 11 - 20 plants infected. II - 1 - 10 plants infected. III - 6 plants infected.

B. Presley group indicates culture type. A - sparse mycelium and sparse microsclerotia. B - fluffy mycelium and no microsclerotia. X - abundant mycelium and abundant microsclerotia.

C. Methods and Procedures.

All isolates were grown in Richards' solution and harvested as described in General Methods. Before extraction,
the isolates were dried by lyophilization. The dried material of each isolate was weighed and stored in a desiccator over CaCl₂ at -4°C until used.

The weighed material was homogenized with distilled water in a Waring blender for five minutes. The homogenized material was washed into a 250 cc beaker with distilled water. Cold 0.6 N perchloric acid was added to the mixture to give a final concentration of 0.4 N perchloric acid and allowed to stand for thirty minutes at 4°C (48). The material was centrifuged at 2000 r.p.m. for fifteen minutes. The solution was decanted into a beaker and the residue extracted for another thirty minutes at 4°C with 0.2 N perchloric acid. After centrifugation, the centrifugates were combined, placed in a beaker in an acetone-ice bath and neutralized with 6N KOH using methyl red as the indicator. The mixture was centrifuged to remove the precipitated potassium perchlorate and the volume of the centrifugate measured. The centrifugate was stored at -10°C until needed.

An aliquot of 0.05 ml. of centrifugate was spotted on Whatman #1 chromatography paper and run as previously indicated for one-dimensional chromatograms using butanol: acetic acid: water as the solvent. Four chromatograms of each
isolate were run. When dry the chromatograms were sprayed very lightly with 0.02% ninhydrin solution in water-saturated n-butanol to locate the spots. The located spots were numbered, cut out, and placed in correspondingly numbered test tubes. The amino acids were eluted from the paper with 50% ethanol. The quantity of chromogen in the spot was determined colorimetrically using a modification of the method of Moore and Stein (73) as follows:— One milliliter of ninhydrin solution was added to the extract still containing the paper and shaken for thirty seconds. Aluminum foil caps were placed on the tubes and the tubes placed in a rack. The racks of tubes were placed in a vigorously boiling water bath for twenty minutes. 5cc distilled water was added to each sample, the tubes wiped dry, and shaken for one minute to mix the contents. After standing for fifteen minutes, the samples were read at 570 m\textsubscript{m} in a Beckman DU spectrophotometer. Blanks were obtained by subjecting a portion of the chromatogram not having spots to the same procedure. The instrument was zeroed with the blanks before reading the samples.

A standard curve was prepared using leucine as the standard. Ten concentrations varying from 0.1 to 1.0 mM per 0.01 cc. were prepared, chromatographed, and determined in the
same manner as the samples. A graph was prepared plotting concentration against optical density. A table was prepared from the graph giving the concentrations corresponding to optical density readings from 0.01 to 1.00 in steps of 0.01 units. The concentrations of the samples were obtained by multiplying the concentration corresponding to the optical density of the sample by five. This figure was used to find mg. amino acid per gram of dried mycelium. All samples and standards were run in quadruplicate.

D. Results and Discussion.

Table 6 summarizes the results of the quantitative determination of amino acids of *Verticillium albo-strum* isolates. Since the samples were run on one-dimensional chromatograms using only one solvent, certain spots contain more than one amino acid. These combinations are indicated in the table.
Table 6. Free amino acid composition in μg/g of six isolates of *Verticillium albo-atrum*.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Amino acids</th>
<th>V1</th>
<th>V5</th>
<th>V16</th>
<th>V14</th>
<th>12-1</th>
<th>245A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>lysine</td>
<td>153.3</td>
<td>127.4</td>
<td>241.8</td>
<td>176.6</td>
<td>262.9</td>
<td>219.0</td>
</tr>
<tr>
<td>2</td>
<td>histidine</td>
<td>93.8</td>
<td>65.6</td>
<td>109.9</td>
<td>79.9</td>
<td>107.5</td>
<td>90.3</td>
</tr>
<tr>
<td>3</td>
<td>aspartic acid</td>
<td>336.3</td>
<td>200.8</td>
<td>369.3</td>
<td>343.9</td>
<td>262.9</td>
<td>629.7</td>
</tr>
<tr>
<td>4</td>
<td>glycine, serine,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unknown A</td>
<td>297.2</td>
<td>42.5</td>
<td>142.9</td>
<td>339.2</td>
<td>286.8</td>
<td>79.4</td>
</tr>
<tr>
<td>5</td>
<td>glutamic acid</td>
<td>242.4</td>
<td>169.9</td>
<td>79.1</td>
<td>241.6</td>
<td>262.9</td>
<td>365.5</td>
</tr>
<tr>
<td>6</td>
<td>threonine, alanine</td>
<td>361.3</td>
<td>142.9</td>
<td>336.4</td>
<td>120.8</td>
<td>95.6</td>
<td>135.5</td>
</tr>
<tr>
<td>7</td>
<td>proline</td>
<td>226.8</td>
<td>239.4</td>
<td>266.0</td>
<td>309.5</td>
<td>95.6</td>
<td>123.2</td>
</tr>
<tr>
<td>8</td>
<td>tyrosine</td>
<td>141.6</td>
<td>179.9</td>
<td>219.8</td>
<td>65.1</td>
<td>157.4</td>
<td>219.0</td>
</tr>
<tr>
<td>9</td>
<td>methionine</td>
<td>164.3</td>
<td>119.7</td>
<td>142.9</td>
<td>55.1</td>
<td>430.3</td>
<td>221.8</td>
</tr>
<tr>
<td>10</td>
<td>valine</td>
<td>60.2</td>
<td>23.2</td>
<td>120.9</td>
<td>148.7</td>
<td>267.7</td>
<td>94.5</td>
</tr>
<tr>
<td>11</td>
<td>isoleucine</td>
<td>46.9</td>
<td>27.8</td>
<td>87.8</td>
<td>127.3</td>
<td>59.7</td>
<td>95.8</td>
</tr>
</tbody>
</table>

A. All figures are averages of four determinations.

A study of table 6 shows that a comparison of quantitative free amino acid composition cannot be used as a basis for strain or species separation. Presley groups (87) which correspond fairly closely to Isaac's (49) species should have a similar amino acid composition if Work and Dewey's (129) belief that each species has a characteristic overall amino acid composition is to hold true. Table 6 shows this not to be the case. For example three isolates of Presley group B; V1, V5,
and V16, fail to correspond quantitatively in several cases. There are 297.2 µg./gm. of the amino acids of spot 4 present in the mycelium of V1 but only 42.5 µg./gm and 142.9 µg./gm in V5 and V6 respectively. Similar discrepancies appear in the amino acids of spots 5, 6, and 10. A similar situation exists in the Presley group X isolates where spots 7, 8, 9, and 11 differ markedly.

If a comparison is made on the basis of pathogenicity strains, a similar situation is found. Group I differs in spots 4, 6 and 10 and group II differs in spots 4, 10 and 11. Further study of the data in Table 6 indicates that no strain or species relationships can be set up on the basis of quantitative amino acid composition. Apparently, with Verticillium albo-atrum at least, strain or species relationship is not dependent on overall amino acid composition.
Study VI. The free nucleotides of Verticillium albo-atrum isolates.

A. Introduction.

Among other nitrogenous constituents of living cells are purines and pyrimidines. These compounds may occur in the free state but are far more often present as glycosides known as nucleosides. The nucleosides may occur free in cells but are found for the most part as their phosphoric esters, the nucleotides, which function as coenzymes or, in a highly polymerized condition, as nucleic acids.

Nucleotides which polymerize to form nucleic acids have all been shown to be phosphate esters of ribonucleosides or deoxyribonucleosides. The former polymerize to form ribonucleic acid (RNA) and the latter deoxyribonucleic acid (DNA). The nucleosides present in RNA are uridine, cytidine, adenosine and guanosine. The nucleosides present in DNA are adenine desoxyribose, guanine desoxyribose, cytosine desoxyribose, and thymidine. 5-methyl cytosine desoxyribose has been shown to be present in the DNA of bacteriophage (2). Each of these nucleosides can form three different monophosphate esters. For example, adenosine forms adenosine 2' phosphate, adenosine 3' phosphate and adenosine 5' phosphate. (29).
A large number of nucleotides do not occur in the cell as nucleic acid building units, but as coenzymes which function as electron carriers in biological oxidations. These nucleotides occur in mono-, di-, and triphosphate forms such as adenosine monophosphate, adenosine diphosphate and adenosine triphosphate, AMP, ADP, and ATP respectively. Other examples of this type of nucleotide are diphosphopyridine nucleotide (DPN or Coenzyme I), triphosphopyridine nucleotide (TPN or Coenzyme II), flavine adenine dinucleotide (FAD), coenzyme A, uridine monophosphate, uridine diphosphate, uridine triphosphate, cytidine monophosphate, cytidine diphosphate, cytidine triphosphate, guanosine monophosphate, guanosine diphosphate and guanosine triphosphate.

Since 1949 a number of nucleotides containing sugars other than ribose or deoxyribose have been found, such as uridine diphosphate glucose (17), uridine diphosphate galactose (61), uridine diphosphate glucuronic acid (46), uridine diphosphate arabinose (45), uridine diphosphate xylose (45), cytidine diphosphate choline (54), guanosine diphosphate mannose (16) and uridine diphosphate N-acetylglucosamine (15). These compounds have been isolated mainly from animal tissues, yeasts and bacteria. However, enzyme studies have shown the coenzymes
to be necessary for a wide variety of biological reactions. They would, from the standpoint of comparative biochemistry, be expected to be present in any cell in which a reaction needing them takes place. In fact, most of the common coenzymes are involved in a number of reactions and are present in all cells. Others are associated with only one known reaction and would be expected to be present only in organisms capable of undergoing the reaction. For example, uridine diphosphate glucose has, so far, been associated only with the inversion of galactose-1-phosphate to glucose-1-phosphate (2) and hence, only organisms capable of carrying out this reaction would be expected to contain uridine diphosphate glucose.

A number of unusual nucleosides have been isolated from a variety of sources. Phosphorylation of these nucleosides would result in the formation of nucleotides. These nucleotides have not been isolated as yet, but there is no reason why an intensive search should not result in their isolation.

Adenine thiomethyl pentoside containing sugar-5-desoxy-5-methylthioribose was isolated by Mandel et al (68) in 1912. Crotonoside (9-β-D ribofuranosylisoguanine) was isolated from the bean Croton tiglium in 1932 (22). Uric acid riboside was isolated from beef blood in 1939 (37). The fungus
Cordyceps militaris Lim. contains an antibiotic, cordycepin, which is a nucleoside containing adenine and a desoxypentose, cordycepose (5). Spongothymidine is a nucleoside isolated from the sponge Cryptothia containing thymine and an unidentified sugar (6). A Neurospora crassa mutant contains the nucleoside orotidine, a glycoside of orotic acid and ribose (7). Vicine, a nucleoside from vetch meal contains D glucose and the pyrimidine divicine (63). Puromycin, an antibiotic from Streptomyces alboniger has a nucleoside-like structure containing a 6-dimethylaminopurine, O-methyl-L-tyrosine, and D-3-amino-3-disoxyribose (118).

The existence of such a wide variety of nucleotides and nucleosides from such a wide variety of sources, coupled with the fact that this field has barely been touched, particularly in the study of fungi, suggests that the study of free nucleotides of Verticillium albo-astrum might be of value in the delineation of strains of this fungus. This section of this thesis is such a study.

B. Methods and Procedures.

The method of Cohn (28) as adapted by Hurlbert et al (48) for the separation of nucleotides from unknown mixtures was used. The acid soluble extracts (48) prepared
in Part II, of this thesis, for quantitative amino acid determination was used without further treatment for column partition chromatography with Dowex 1 ion-exchange resin using the gradient elution method described by Hurlbert et al. (48). The extracts of V1, V5, V16, 42-1-245A, and V46 were used.

i. Preparation of columns (48):
Dowex 1 -X10, 200 to 400 mesh, chloride form had the fines and course particles removed by sedimentation. The resin was then washed in a column with 3M sodium formate until chloride ion was no longer detectable. The resulting formate form of the resin was washed several times with water. The resin was poured into a chromatography column containing a sintered glass disc near the lower end. The resin was poured as a thin aqueous slurry and packed with low air pressure in a number of 2 cm. sections until the resin column was 20 cm. long. A crimped filter paper disc was placed on top of the resin and the column washed with five to ten bed volumes of a mixture of 6M formic acid and 1M sodium formate, then with several bed volumes of 88 percent formic acid. It was then washed with distilled water until the effluent was no longer acidic.

A ten ml. aliquot of the acid soluble extract was
put on the column at a rate of 0.6 ml. per minute and rinsed into the column with one bed volume of distilled water. Care must be taken not to blow the column dry.

ii. Elution.

The apparatus for gradient elution was essentially that of Hurlbert et al. consisting of a two liter reservoir, a 250 ml. round bottom mixing flask with a magnetic stirrer, and Tygon tubing used to join the reservoir to the mixing flask and the mixing flask to the column in an air tight system. The solutions are forced through the system by means of 2 lbs. nitrogen pressure through a tube attached to the top of the reservoir. Details of this system may be found in Hurlbert et al. (48).

Ten ml. distilled water was added to the column to prevent it from being forced dry and the elution started with 250 ml. distilled water in the mixing flasks and 4 M formic acid in the reservoir. The eluate was collected with a Gilson volumetric fraction collector in 3 ml. fractions at a rate of 0.6 ml. per minute. The concentration of the solvent in the column rises smoothly and assymptotically approaches that of the reservoir solution. Successive ranges of eluting power were obtained by changing the solution in the reservoir.
to 4 M formic acid plus 0.2 M ammonium formate at tube 80, to 4 M formic acid plus 0.4 M ammonium formate at tube 160, to 4 M formic acid plus 0.8 M ammonium formate at tube 240, and 4 M formic acid plus 1.0 M ammonium formate at tube 300.

iii. Analysis of fractions.

The optical densities of all fractions were read at 260 m in a Beckman DU spectro-photometer in one cm. quartz cells using water as a blank. This operation served to locate the nucleotide peaks. A suitable dilution of an aliquot from each peak was read in 0.1 N HCl at pH 2 at 230, 245, 260, 275, and 290 m as well as at the wavelengths of maximal and minimal absorption. The solution was made at least pH 11 by the addition of a known volume of 1N NaOH, mixed, and the spectrum readings repeated. Suitable HCl and NaOH blanks were used. These values permit adequate routine spectral characterization of the nucleotides.

Inorganic and total phosphate were determined for each peak using the method of King (57). The standard consisted of 2.1935 grams of potassium dihydrogen phosphate in 500 ml. water. Five ml. of the standard was diluted to 500 ml. with distilled water to obtain 0.1 mg. P/10 ml. A standard curve was constructed using the above methods of determination.
to cover a range of concentrations from 0.1 mg P/10 ml. to 0.8 mg. P/10 ml.

Labile phosphate was determined using the method of Lowry and Lopat (66). A standard curve was prepared in the same way for this method. These standard curves were used for calculating phosphate concentration.

Ribose content of each peak was determined by the orcinol method (3) using pure ribose as the standard. Desoxypentoses are not sensitive to this method.

The relative positions of the peaks when optical density at 260 m is graphed against tube number, the ultraviolet absorption spectra in acid and alkalie, the content of total phosphate and labile phosphate, and the apparent ribose content serve to characterize the individual nucleotides separated.

The identity of the purine and pyrimidine components of the nucleotides was further checked by paper chromatography using the method of Smith and Markham (103). A portion of each peak was hydrolysed with 3 ml. 6NHC1 in an autoclave at 15 pounds pressure for 6 hours. The excess acid was removed by evaporation and the hydrolyzate spotted on Whatman #1
chromatography paper, 1 inch apart, 3 inches from one end, until the spots fluoresced in the dark when illuminated with ultraviolet light at 260 m. Standards of known purines and pyrimidines (2 mg./cc) were run on the same paper. The chromatogram was run using the descending method for one dimensional chromatograms using tert-butanol: HCl: water as the solvent. The solvent contained 700 ml. pure tert-butanol, 132 ml. constant boiling hydrochloric acid, made up to one liter with distilled water. After irrigation the paper was dried for one hour at room temperature and viewed with ultraviolet light. Fluorescent spots were outlined with pencil and compared with the standards.

C. Results and Discussion.

The acid soluble extracts of isolates VI, V5, V16, V14, 42-1-245A, and V46 all contained the same free nucleotides. Figure 3 shows the nucleotide peaks for isolate 42-1-245A as they came out of the anion-exchange column. The peaks were similar for all isolates tested.

Table 7 summarizes the information obtained from spectral, phosphate, and ribose analysis and used to characterize the peaks.
FIG. 3: FORMIC ACID CHROMATOGRAM OF VERTICILLIUM ALBO-ATRUM ISOLATE 42-I-245 ON DOWEX 1-X10 COLUMN 1X15 CM. 250 ML. MIXER

Solvent changes in reservoir were made as follows: A (TUBE 0) = 4.0 M. FORMAL ACID. B (TUBE 80) = 4.0 M. FORMAL ACID + 0.2 M. AMMONIUM FORMATE. C (TUBE 160) = 4.0 M. FORMAL ACID + 0.4 M. AMMONIUM FORMATE. D (TUBE 240) = 4.0 M. FORMAL ACID + 0.8 M. AMMONIUM FORMATE. Fractions = 3 ML. PER TUBE COLLECTED AT A RATE OF 0.6 ML. PER MIN.
Table 7. Analysis of the nucleotide peaks contained in the acid-soluble fraction of isolate 42-245A.

<table>
<thead>
<tr>
<th>Nucleotide Peak</th>
<th>pH</th>
<th>Ultraviolet absorption</th>
<th>Chemical Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wave length</td>
<td>Optical density at given wave length</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Optical density max. in acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Max.</td>
<td>Min.</td>
</tr>
<tr>
<td>1. GMP acid</td>
<td>256</td>
<td>228</td>
<td>1.00</td>
</tr>
<tr>
<td>2. CDP acid</td>
<td>280</td>
<td>214</td>
<td>1.00</td>
</tr>
<tr>
<td>3. UMP acid</td>
<td>261</td>
<td>241</td>
<td>1.00</td>
</tr>
<tr>
<td>4. ADP acid</td>
<td>258</td>
<td>228</td>
<td>1.00</td>
</tr>
<tr>
<td>5. UDP acid</td>
<td>261</td>
<td>231</td>
<td>1.00</td>
</tr>
<tr>
<td>6. GDP acid</td>
<td>256</td>
<td>228</td>
<td>1.00</td>
</tr>
<tr>
<td>7. UTP acid</td>
<td>261</td>
<td>231</td>
<td>1.00</td>
</tr>
<tr>
<td>8. GTP acid</td>
<td>256</td>
<td>228</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Millimolar extinction coefficient (εₘₚ) used in calculations as follows: cytidine 13.7 at 278 mp, uridine 9.9 at 260 mp, guanosine 13.3 at 256 mp, and adenosine 14.1 at 258 mp. Total phosphate, labile phosphate and ribose are expressed \(\mu M/\mu M\) base.
The peaks were thus, tentatively, identified as guanosine monophosphate, cytidine diphosphate, uridine monophosphate, adenosine triphosphate, uridine diphosphate, guanosine diphosphate, uridine triphosphate, and guanosine triphosphate, in order of their appearance from the column (Fig. 3).

Since all these nucleotides were present in the acid-soluble extracts of each isolate, it is apparent that they cannot be used to differentiate strains on a qualitative basis among the isolates tested. There is, however, no basis for assuming that the isolates not tested would all have the same spectrum of free nucleotides. These possible differences would, however, be of little value for strain differentiation since they could not be related to morphological or pathological types.
SUMMARY

1. Twenty-five isolates of *Verticillium albo-atrum* separated into four strain groups on the basis of morphology.

2. These isolates separated into three strain groups on the basis of pathogenicity toward tomato.

3. The morphologic strain groups showed no relationship toward the pathologic strain groups.

4. The alcohol-soluble extracts of these isolates contained no amines, amides or peptides. They all contained the same amino acids, namely, aspartic acid, glutamic acid, lysine, histidine, isoleucine, serine, valine, proline, methionine, alanine, tyrosine, theonine, glycine and an unidentified neutral compound, A.

5. The alcohol-soluble extracts of three *Colletotricum lindemuthianum* strains contained no amines, amides or peptides. They all contained the same amino acids, namely, aspartic acid, glutamic acid, lysine, histidine, isoleucine, serine, valine, proline, methionine, α-alanine, tyrosine, threonine, glycine, and an unidentified neutral compound, B.

6. The isolates of *Verticillium albo-atrum* and *Colletotricum lindemuthianum* differed from each other only in respect to
unknowns A and B which indicates a species difference. No strain differences were apparent.

7. Aeration, pH of medium, age of mycelium and carbon source had no qualitative effect on the amino acid content of isolate V3.

8. Quantitative studies of amino acids of representative isolates showed no correlation between the concentrations of the various amino acids and morphologic or pathologic strain types.

9. Six representative isolates of *Verticillium albo-atrum* all contained eight free nucleotides in their acid-soluble extracts. These were tentatively identified as guanosine monophosphate, cytidine diphosphate, uridine monophosphate, adenosine diphosphate, uridine diphosphate, guanosine diphosphate, uridine triphosphate and guanosine triphosphate. No strain differences were evident.
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