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PYCNIDIA PRODUCTION BY *PLENODOMUS MELILOTI* MARK.-LET. IN LIGHT AND DARKNESS, AND RELATED STUDIES ON THE HOST METABOLISM AT LOW TEMPERATURES

by

© SAEED IQBAL ZAFAR

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "PYCNIDIA PRODUCTION BY *PLENODOMUS MELILOTI* MARK.-LET. IN LIGHT AND DARKNESS, AND RELATED STUDIES ON THE HOST METABOLISM AT LOW TEMPERATURES" submitted by Saeed Iqbal Zafar in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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TO MY PARENTS

ABSTRACT

Mycelial colonies of *Plenodomus meliloti* Mark.-Let., incitant of brown root rot of legumes, were of similar size whether cultured in the light or in darkness on synthetic agar medium. However, the aerial mycelial growth was less when the fungus was cultured in the light as compared to when it was grown in darkness. When various inorganic oxidants and other organic compounds were incorporated in the synthetic medium, few pycnidia were induced in the presence of some of them, while the others did not have any effect on pycnidial production. It was observed that whenever the mycelial growth was reduced or temporarily arrested by mechanical or chemical means, pycnidia were induced in the dark. In the presence of 'active' horseradish peroxidase, beef liver catalase, and polyphenol oxidase enzymes, mycelial growth, in comparison to controls, was accelerated and numerous pycnidia were produced in cultures incubated in light or darkness. When horseradish peroxidase and beef liver catalase were denatured by autoclaving, cultures were similar to controls. *Aspergillus niger* catalase inhibited mycelial growth and pycnidia production. The inhibitory factor was found in the protohaematin fraction of this enzyme.

The mycelial extracts from cultures grown in light and darkness had different protein patterns and peroxidase isozyme complement, when isozymes and proteins were separated using disc gel electrophoresis.

Quantitative and qualitative analyses of proteins, peroxidase, polyphenol oxidase, soluble amino acids, protein amino acids, and soluble sugars present in roots of sweet clover variety Arctic - resistant to *P. meliloti*, and Denta - susceptible to *P. meliloti*, were carried out on samples harvested between October, 1968 and June, 1969.

The catalase activity was higher in the resistant variety as compared to the susceptibile variety; whereas, the peroxidase levels in the susceptible variety were only slightly higher than in the resistant variety. Association of environmental conditions, quantitative and qualitative differences of various oxidizing enzymes, proteins, amino acids, carbohydrates, and growth substances, and pathogenicity is discussed.

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INTRODUCTION

The phenomenon of reproduction as influenced by light has intrigued many research workers for a long time. A useful, though non-critical review of the vast literature that has accumulated through the years on the subject of photobiology in fungi has been compiled by Marsh *et al.* (1959). A more detailed and a critical review on this aspect of fungal physiology was prepared by Carlile (1965). However, the metabolic changes underlying fungal photoresponses have not yet been elucidated. Various theories involving photoreceptor sites as the controlling factors in photoresponses of fungi have been put forward. However, the interpretation of the involvement of these receptors is quite difficult, and at present must remain speculative until supporting biochemical studies have been performed. The usually considered photoreceptors are flavins (Lukens, 1963; Leach and Trione, 1966; Leach, 1968), and carotenoids (Zalokar, 1955). Lukens (1963) considered such a flavin receptor to be existing as a flavoprotein or possibly a flavin nucleotide. Light has been reported to activate or destroy oxidative enzyme systems (Carlile, 1965; Mitchell and Anderson, 1965; MacMillan and Brandt, 1966; Bjorn, 1967). What effect these changes have on the metabolic processes of fungi has not been worked out, although there are indications that carotenoid biosynthesis is affected (Zalokar, 1955; Carlile, 1965). In *Blastocladiella emersoni* a new electron-transfer path is initiated, when the cultures are grown under illumination (Cantino and Horenstein, 1956; Cantino and Horenstein, 1959). Turian and Cantino (1959) showed that the effect of light is involved in the production or destruction of certain metabolites, to which the cell wall is permeable.

Induction of pycnidia by light has been noted for *Plenodomus meliloti* (Wu, 1965), but as noted for a large number of other fungi, the mode of action of light is not clear.

P. meliloti, an imperfect fungus which causes the brown root rot of legumes, has only one spore form produced in pycnidia. The range of hosts and distribution was reviewed earlier by Netolitzky (1965), and Wu (1965). *P. meliloti* attacks the roots of legume plants during the latter part of the winter, producing dark coloured, necrotic lesions in which pycnidia are usually formed. Occasionally pycnidia have also been found on the crowns of host plants (Netolitzky, 1965). Infection and invasion of host tissue by *P. meliloti* are considered to occur while plants are dormant in the winter, since very little infection and very few pycnidia are formed when soil temperatures are high (Sanford, 1933; Cormack, 1934; McDonald, 1955; Netolitzky, 1965). During the early spring, when the soil temperature is just above freezing, numerous mature pycnidia embedded in the diseased portions of roots may be observed (Netolitzky, 1965). When cultured on standard potato-dextrose agar and malt-yeast-dextrose agar media, isolates of *P. meliloti* produced pycnidia abundantly in light but very few, if any, in darkness (Wu, 1965). Light has been found to be necessary for pycnidial production by *P. fuscomaculans* (Coons, 1916), for *Physalospora obtusa* (Fulkerson, 1955), and for *Macrophomina phaseoli* (Ashworth, 1959). However, in all of these cases, unlike *P. meliloti*, the pycnidia in nature are produced on the above ground parts where light could have a direct role in their initiation. On the basis of his results, Wu (1965) concluded that the process(es) which leads to pycnidial production in cultures receives some form of stimulus from the lower part of the

visible spectrum; whereas, this process(es) occurs naturally when the fungus is associated with roots in the dark.

The effects of light on other fungi with regard to reproduction, morphology, pigmentation, and phototropic phenomena has been reviewed by Marsh *et al.* (1959). More recent studies by Carlile (1965), and Knox-Davies (1965) also show that fructification can be influenced by light.

When *P. fuscomaculans* was cultured on filter papers and in darkness, numerous pycnidia were produced (Coons, 1916). Kilpatrick (1966) also showed that sporulation of many fungi, including *Plenodomus* sp., can be induced on non-sterile filter papers in a moist chamber. Coons (1916) was also able to induce sporulation in the dark by reducing the quantity of various nutrients on which the fungus was grown. He concluded that this kind of induced sporulation was due to a lack of enough food material. Under such conditions of depletion of readily available nutrients, some stimulus becomes operative which starts the utilization, by oxidation, of reserve nutrients, and leads to the phenomenon of reproduction. Inadequate nutrient supply and mechanical treatment were found to induce sclerotia in *Sclerotium rolfsii* (Henis *et al.*, 1965; Chet and Henis, 1968). Chet and Henis (1968) postulated that iodoacetic acid, chelating agents, and potassium iodate induced sclerotial formation in *S. rolfsii* by modifying a sulfhydryl-containing copper-linked protein entity which acts as a repressor of sclerotial formation. Whenever vegetative growth was suppressed, the effectiveness or levels of this repressor would be reduced due to a greater accumulation of chelating agents and other oxidizing compounds. A support to the hypothesis given by these workers can be provided from the earlier observations of Coons (1916) who found that various oxidizing com-

pounds were able to replace the effect(s) of light by reducing the amounts of vegetative mycelium and increasing pycnidia production in the dark.

Irradiation of culture media to visible, and uv-light has been known to inhibit the growth of many bacteria and fungi. Leonard and March (1928) reported that the growth of *Bacillus subtilis* was reduced on media exposed to sunlight for 270 hr. Fields *et al.* (1961) showed that growth of *Aspergillus oryzae* was retarded when grown in a sucrose-nitrate broth previously exposed to high energy radiation. Weinhold and Hendrix (1962) found that inhibition of *Rhizoctonia solani* occurred when it was grown on potato dextrose agar previously exposed to light. A later study by Weinhold and Hendrix (1963) provided evidence which indicated that the inhibition may be due to the formation of peroxides in the medium. Frey and Pollard (1966) also found that inhibition of growth in bacteria by ionization radiation was also due to the production of hydrogen peroxide. They concluded that the hydrogen peroxide inhibited the synthetic processes of the cell.

Pigmentation was always found present in cultures of *P. fuscomaculans* (Coons, 1916) and *P. meliloti* (Wu, 1965) when cultured in light, and absent when cultured in the dark. Carley *et al.* (1967) were able to inhibit pigmentation in *Aspergillus niger* by the addition of dimethyl sulfoxide in the medium. However, the pigment inhibition did not affect the growth rate or sporulation, therefore, pigmentation was considered by these authors to be a superfluous characteristic relative to the life cycle of *A. niger*. The significance of pigmentation in pycnidia production by the growing cultures of *P. meliloti* is not known. However, it was observed that the outer surface of immature pycnidia was light green and that the green pigmentation

increased in intensity as the pycnidia matured (Wu, 1965). He also found that the greatest number of pycnidia and pigmentation occurred in the cultures incubated in blue light. No attempt has yet been made to determine the nature of this green pigment in *P. meliloti*.

In wheat roots Bjorn (1965) found that blue light was necessary for glycolic acid oxidase formation. This enzyme is capable of producing hydrogen peroxide. In the presence of suitable catalysts hydrogen peroxide readily reacts with sulfhydryl groups (Randall, 1946; Barron and Dickman, 1949; Barron and Flood, 1950; Little and O'Brien, 1966). This reaction may result in the inactivation of the sulfhydryl enzymes. Variations in sulfhydryl content and in the activities of sulfhydryl enzymes are considered to play a major role in cell development (Brachet, 1961; Libbert and Richter, 1963; Libbert and Zatschler, 1963). The inactivation of these -SH containing enzymes by hydrogen peroxide is probably similar to the modification of a hypothetical -SH containing Cu-linked protein present in *Sclerotium rolfsii* (Chet and Henis, 1968). Such a modification is essential for sclerotial formation in this fungus and can be achieved by using oxidants and chelating agents (Chet and Henis, 1968). It is quite likely that when *P. meliloti* cultures are incubated in the light, hydrogen peroxide and possibly other peroxides are produced in greater amounts, this would modify certain unknown compounds in a manner(s) similar to the one proposed by the above workers. The modified compound will then act as a repressor of vegetative growth and shift the metabolism towards pycnidia production in the same way as it may be involved in the sclerotial production by *S. rolfsii*.

Bjorn (1967) showed that the activities of the enzymes involved in the production of hydrogen peroxide and those that destroy it are

affected by light. The activity of catalase was lost during an exposure to sunlight (Mitchell and Anderson, 1965). Peroxidase specific activity decreased in light-grown first-leaves but increased in light grown coleoptiles of wheat (Parish, 1969). He also observed that synthesis of all peroxidase isozymes increased in coleoptiles of light-grown wheat seedlings. It may be suggested that changes in the levels of these enzymes would determine growth responses.

In addition to the role these enzymes may have in controlling growth, they have also been implicated in the disease resistance phenomenon. Much attention during recent years has been given to the possible role of oxidative enzymes in the development of certain diseases of higher plants particularly in disease resistance (Oku, 1960; Farkas and Kiraly, 1962; Kuc, 1963; Uritani, 1963; Weber and Stahmann, 1964; Staples and Stahmann, 1964; Clare *et al.*, 1966; Farkas and Stahmann, 1966; Weber and Stahmann, 1966; Stahmann *et al.*, 1968). Most of these studies have dealt with the qualitative changes in proteins, and isozyme patterns of peroxidase, polyphenol oxidase, and catalase, the levels of their activities, and the products of their interaction due to a resistant or susceptible host-parasite interaction. Clare *et al.* (1966) found that peroxidase and polyphenol oxidase activities were higher in sweet potato tissue inoculated with pathogenic isolates of *Ceratocystis fimbriata* than they were in uninfected tissue. Weber and Stahmann (1964) inoculated sweet potato with pathogenic and non-pathogenic isolates of *C. fimbriata* and analyzed extracts from tissue sections cut at known distances from a plane of infection. The chromatographic and electrophoretic results showed that similar changes in several proteins and enzymes occurred with both isolates. Stahmann *et al.* (1966) later on found that resistance of sweet potato tissue to *C. fimbriata* can

be induced by treatment with ethylene. Ethylene-induced resistance in healthy tissue also accompanied increased polyphenol oxidase and peroxidase activities. It was suggested that ethylene provides localized metabolic changes leading to necrotic and hypersensitive reactions in plants following infection. Gahagan *et al.* (1968) also showed that ethylene increased peroxidase activity of nine out of ten varieties of sweet potato root discs. They found a relationship between low peroxidase activity and the susceptibility of sweet potato varieties and vice versa. Umaerus (1959) and Kedar (1959) reported that high peroxidase activity as a tool in the selection of potato varieties resistant to late blight fungus *Phytophthora infestans* is useful only with restrictions. However, the whole concept of higher peroxidase activity and resistance has been severely criticized by Henniger (1963), and Henniger and Bartel (1963). This correlation was found not to be applicable in resistance to late blight casued by *P. infestans* on potatoes. During a field test, consisting of 50 potato varieties having different degrees of resistance to the late blight pathogen, they found only a slight correlation between the peroxidase activity and resistance. Some varieties with a high degree of resistance had only a slight peroxidase activity. Due to the presence of high peroxidase activity in plants the rate of mycelial growth of the pathogen was not found to be inhibited. Walker (1963) also found that when cabbage leaves were infected with cucumber mosaic virus the peroxidase activity in susceptible plants was two times more than the resistant varieties.

Data presented by Macko *et al.* (1968) reveal that susceptible wheat variety Little Club always had slightly higher peroxidase activity as compared to resistant Khapli. However, when these wheat varieties were inoculated with *Puccinia graminis* var. *tritici* an increase in the peroxidase

activity in both varieties was noted, although the increases in Khapli were much higher. On the other hand Lobenstein and Linsey (1966) found a close relationship between symptom severity and peroxidase activity in virus-infected tobacco leaves. Peroxidase activities were greater with stronger symptom development. Highest peroxidase activity occurred in those hosts which on virus infection showed necrosis and lowest in those which did not show any symptoms. The activation of enzyme activity in those plants which show severe symptoms could have resulted due to their liberation from inactive forms. The activation process is probably associated with damage to sub-cellular particles e.g. ribosomes, mitochondria (Farkas *et al.*, 1964).

Although the oxidative enzymes of host plants and pathogens have been studied intensively with regard to their role in pathogenicity, some effects of peroxidase, catalase, and polyphenol oxidase on spore germination and mycelial growth have only recently been reported (Macko *et al.*, 1968).

Whether or not mechanisms of disease resistance or susceptibility can be convincingly explained on the basis of the levels of and/or the degree of induction of peroxidase activity due to the host-parasite relationship is not very certain. As can be noted from the different views of different workers such explanations for disease resistance involving living cells are controversial.

Not only are there changes in the activities of oxidative enzymes, but protein and isozyme electrophoretic patterns also undergo changes following host-parasite interaction. Staples and Stahmann (1964) reported a change in one protein band and isozymes of several enzymes, when bean leaves were infected by *Uromyces phaseoli*. When healthy tobacco and

tobacco leaves infected by *Colletotrichum destructivum* were compared, one and possibly more proteins which exhibited peroxidase activity were observed in infected plants (Yu and Hampton, 1964). Qualitative changes in protein and isozyme patterns using gel electrophoresis have been demonstrated in a number of other host-parasite combinations as well (Tomiyama and Stahmann, 1964; Farkas and Stahmann, 1966; Johnson *et al.*, 1966; Lovrekovich *et al.*, 1968). The nature and role of these changes is not known at the present time and major questions concerning their origin and the true nature of these changes have arisen.

Recently Stahmann *et al.* (1968) formulated a hypothesis in an attempt to elucidate the role of enzymes and their changes in host-parasite relationships. The main point in their hypothesis is the production of hybrid enzymes from the interaction of isozymes of host and the pathogen (probably these show up as new isozymes after infection). These hybrid enzymes are capable of participation in the metabolism of both host and the pathogen if the resulting interaction is a compatible combination, but will be out of phase for the metabolic requirements of the parasite in an incompatible interaction.

Quantitative and qualitative changes in proteins, peroxidase, polyphenol oxidase, and catalase have also been related to winter-hardiness of many plant species. Increases in soluble protein contents during hardening were observed in the bark of the black locust tree by Siminovitch and Briggs (1949). This was later confirmed by various researchers working with different plant materials (Bula and Smith, 1954; Bula *et al.*, 1956; Coleman *et al.*, 1966; Gerloff *et al.*, 1967). Coleman *et al.* (1966) observed a zone of highly charged and/or low molecular weight protein contents in the cold-hardened than in the non-hardened root material of

Medicago sativa. Gerloff *et al.* (1967), using amido Schwartz, showed quantitative changes in two protein bands in extracts from alfalfa roots which increased during the hardening period. However, they concluded that these analyses showed no shift large enough to account for the large inherent differences in the hardening capacity that exists between winter-hardy and non-hardy varieties. Craker *et al.* (1969) working with apple bark and arborvitae foliage also noted that qualitative protein changes, as evidenced by the appearance and disappearance of specific protein bands, occurred at times when changes in hardiness were taking place. Quantitative increases in peroxidase and catalase and qualitative changes in peroxidase were found to be related to winter-hardiness of alfalfa varieties (Gerloff *et al.*, 1967). The increases were more rapid in winter-hardy than in non-hardy varieties. They also observed two new peroxidase isozymes during the hardening period. A direct relationship with soil temperature and activities of peroxidase and polyphenol oxidase were noticed by Korovin and Barskaya (1962). They observed that with a lowered soil temperature there were increased peroxidase, and decreased polyphenol oxidase activities in the roots of corn, potato, and wheat. However, no relationship between root zone temperature and catalase activity was found in wheat and potato roots. Active metabolic changes in roots in response to low soil temperature and other ecological factors support Kursanov's hypothesis (1960) that roots are the most active plant organs from the metabolic standpoint, and in which the enzymatic systems react vigorously, rapidly, and sequentially.

The apparent relationship between water-soluble proteins and cold-hardiness prompted Wilding *et al.* (1960) to investigate changes in the amino acid composition of plants during cold-hardening and dehardening.

A 20 per cent increase in the free amino acids from August to November was noted in the roots of the most hardy alfalfa variety. Changes in the non-hardy variety were little. Results obtained by Zech and Pauli (1962) indicated that most of the changes in total free amino nitrogen associated with hardiness probably was caused by an increase in an amide nitrogen rather than by increases in nitrogen in ammonia or free amino acid form. However, the association of amides with changes in cold-hardiness was found to be inconsistent. Later, Pauli and Zech (1964) reported increases in total amino acid recovered, alanine, arginine, aspartic acid, glutamic acid, and histidine followed closely the increasing trends of cold-hardiness.

The seasonal variations in carbohydrates, consisting largely of starch, sucrose, and to a lesser extent some reducing monosaccharides, had a direct bearing on hardiness development in black locust tree bark (Siminovitch *et al.*, 1953). In early summer the starch and sucrose levels rose, and by October they were at their maximum amounts. Starch showed a decreasing trend by October, without any increase in the soluble sugars. The soluble sugars underwent a slight decrease during winter, but as soon as there was an increase in temperature during the spring, a quantitative conversion of sucrose to starch was noted. Such seasonal conversions were later on confirmed by Levitt (1956), and Kramer and Kozlowski (1960). Heinrichs *et al.* (1960) studied the relationship between carbohydrate content in the roots of alfalfa varieties to evaluate winter-hardiness in alfalfa. They found that lower hexosan contents indicated greater winter-hardiness, while pentosans did not show any relationship to hardiness. For this reason they concluded that it is best to use only hexosans rather than total soluble carbohydrates as an indication of hardiness qualities

in alfalfa.

Ullrich and Heber (1957), and Heber (1959) explained the role of sugars in winter-hardiness of plants. Their data indicated that sugars retard the denaturation of prcteins. This protective effect of sugars was considered to be the prevention of, by freezing, the denaturation or coagulation of proteins. Sugars may solvate the proteins in the same manner as water-solvate-envelopes and thus prevent denaturation by low temperatures (Ullrish and Heber, 1957). However, they were unable to relate the percentage of sugars to cole-resistance. More recently the sugars have been shown to stimulate peroxidase synthesis (Parish, 1969). Therefore, in addition to their role as a protectant for proteins, they may be directly related to enzyme synthesis, and this accelerated or stimulated enzyme synthesis may have a bearing in the cold-resistance phenomenon.

The present studies were initiated to find a substitute for light in an attempt to induce pycnidial production by *P. meliloti* in darkness. Another aim was to investigate the cause of initiation of pycnidia in the light and their characteristic absence from the cultures grown in the dark. Also studied were the biochemical changes occurring during fall, winter, and spring in sweet clover varieties Arctic which is winter-hardy and resistant to *P. meliloti* and Denta which is non-winter hardy and susceptible to *P. meliloti*. In this dissertation will be presented the following investigations: (a) effects of quantity of nutrients on mycelial growth and pycnidial production, (b) role of various inorganic oxidants and some organic compounds on growth and pycnidial production, (c) role of various oxidizing enzymes on mycelial growth and pycnidial production, (d) qualitative studies on the proteins and peroxidase enzyme of *P. meliloti* cultures incubated in light or darkness, (e) quantitative studies, dealing

with seasonal changes of proteins, peroxidase, catalase, and polyphenol oxidase metabolism in roots of sweet clover varieties Arctic and Denta, (f) qualitative studies, dealing with seasonal changes of proteins, peroxidase, and polyphenol oxidase metabolism in roots of sweet clover varieties Arctic and Denta, (g) seasonal changes in the quantities of free and protein amino acids of sweet clover roots of variety Arctic and Denta, (h) seasonal changes in the quality and quantity of soluble sugars in the roots of sweet clover variety Arctic and Denta.

Although a direct host-parasite relationship between the host and the parasite was not established, due to reasons explained in the discussion, an attempt will be made to correlate the metabolic changes in proteins and other oxidative enzymes in sweet clover roots and pathogenicity of *P. meliloti*. Also discussed will be the role of oxidative enzymes and other physical and chemical factors controlling growth and fructification of *P. meliloti*. Some consideration will also be given to quantitative and qualitative biochemical changes in sweet clover roots as related to winter-hardiness and non-hardiness.

MATERIALS AND METHODS

A. STUDIES ON THE PLANT PATHOGEN

a. Organism and the growth medium

The fungus used in these studies was *Plenodomus meliloti* Mark.-Let. which belongs to the form order Sphaeropsidales. The isolates of *P. meliloti* were those used by Netolitzky (1965) and Wu (1965). Their origin and the hosts from which they were isolated are shown in Table 1.

Table 1. Isolates of *P. meliloti*, their hosts, where found, and by whom.

Isolate	Host	Origin	Isolated by
P_{10}	*Melilotus alba*	Alberta and Saskatchewan	Dearness and Sanford, 1930
P_{15}	*Melilotus* sp.	Edmonton, Alberta	Netolitzky, 1965
P_{20}	*Medicago* sp.	Dominion Experimental Farm, Mile 1019, Alaska Highway, Yukon Territory	Netolitzky, 1965
P_{30}	*Trifolium pratense*	Finland	Salonen, 1962

Isolate P_{10} was obtained from the Bureau of Schimmelcultures, Baarn, Netherlands, P_{15} and P_{20} from Netolitzky (1965) and P_{30} from Dr. Salonen*, Helsinki, Finland. The stock cultures of these isolates were maintained on standard potato sucrose agar medium in test tubes at

* Department of Plant Pathology, University of Helsinki, Helsinki, Finland.

3-5°C. The isolate of *P. meliloti*, P_{30} was used for most of the present investigations unless otherwise stated.

The synthetic medium used was described by Ward and Colotelo (1960), except that vitamins were not included. The constituents of the synthetic medium are given in Appendix 1.

b. Inoculum and growth procedures

The inoculum consisted of mycelial plugs, 6 mm in diameter, taken from the periphery of 15-day old colonies grown in Petri plates on the above mentioned synthetic medium plus 1.7% agar agar (Difco) and incubated in light at 15°C. The growth studies were carried out in both light and darkness. Two fluorescent tubes (General Electric F 15 12 CW), suspended about 22 cm above the Petri plates, provided approximately 150 ft-c of illumination at the Petri plate level. The cultures for dark treatment were either placed in an incubator which was then sealed or the inoculated Petri plates were wrapped in two or three folds of aluminum foil and then the cultures placed in appropriate incubators (General Electric, Precision Scientific, Model 805).

c. Effects of various amounts of medium on mycelial growth and pycnidium production in darkness

Fungi are known to reproduce when nutritional conditions are adverse for normal vegetative growth. Coon's (1916) results show that with a decrease in the amounts of various nutrients, to a certain extent, the pycnidia production by *P. fuscomaculans* was enhanced and mycelial growth restricted. It is well known that asexual sporulation occurs when the mycelium of a colony reaches the edge of a Petri plate (Ainsworth and Susman,1966). This could be due to contact stimulus

when the hyphae reach the rim of the plate or to exhaustion of nutrients. In preliminary experiments it was noted that for old cultures of *P. meliloti*, i.e. over 50 days and incubated in the dark, a large number of pycnidia were found scattered throughout the colony. Older pycnidia were located at the periphery of the plates. It was also noted, in a separate experiment, that when small glass beads were dispersed in Petri plates containing synthetic medium, for cultures incubated in the dark, that numerous pycnidia developed only on those portions of the glass beads which had only a thin film of the medium. To further demonstrate a similar effect on the mycelial growth and pycnidial production by *P. meliloti* in darkness, various amounts of synthetic medium were added in the Petri plates. The volumes of the synthetic medium used per Petri plate were 1 ml, 2 ml, 3 ml, 4 ml, 5 ml, 6 ml, 8 ml, and 10 ml respectively. Petri plates having 20 ml of the synthetic medium were used as controls. The cultures were inoculated with 6 mm diameter plug and incubated at 15°C for 15 days in darkness. The results are shown in Table 2; each figure represents the average diameter of three separate colonies.

d. Effects of various inorganic oxidants on mycelial growth and pycnidial production

As mentioned previously, Coons (1916) was able to replace the effect of light to some extent by the addition of H_2O_2 to the medium, which resulted in pycnidium formation by *P. fuscomaculans*. Taking this into consideration, various inorganic oxidants were tried to note their effects on mycelial growth and pycnidial production by *P. meliloti* in light and darkness. The inorganic oxidants incorporated in the medium

were cobaltous nitrate, chromium trioxide, potassium permanganate, calcium hypochlorite, sodium hypochlorite, and hydrogen peroxide. Concentrations selected for these compounds were such that there would be approximately equal amounts of oxygen per mole. Compounds and the concentrations used were:

- cobaltous nitrate, 1.5×10^{-1}, 3.43×10^{-2}, 3.43×10^{-3}M
- chromium trioxide, 1.53×10^{-1}, 3.30×10^{-2}, 3.30×10^{-3}M
- potassium permanganate, 7.28×10^{-2}, 1.58×10^{-2}, 1.58×10^{-3}M
- calcium hypochlorite, 1.07×10^{-1}, 2.3×10^{-2}, 2.3×10^{-3}M
- sodium hypochlorite, 3.09×10^{-2}, 6.71×10^{-3}, 6.71×10^{-4}M
- hydrogen peroxide, 4.06×10^{-1}, 9.65×10^{-2}, 9.65×10^{-3}M.

Solutions containing the above compounds were sterilized by passing through Seitz filters, and then appropriate amounts of each solution were added to the already sterilized semi-cool (40-45°C) synthetic medium. 20 ml of the final medium was used per Petri plate, and inoculated as mentioned previously. Petri plates containing the synthetic medium alone served as controls. The results are presented in Table 3.

e. Effects of various organic compounds on mycelial growth and pycnidial production

Wu (1965) noted that the light-grown cultures of *P. meliloti* always had pigmentation which was characteristically absent from the cultures incubated in the dark. It was concluded that the pigments were important for the initiation of pycnidia, since the pycnidia themselves are highly pigmented. Certain shikimic acid cycle intermediates could be involved in the pigmentation of light-grown cultures, which either

were not produced or their synthesis inhibited in cultures incubated in the dark. This assumption could explain the absence of pycnidia in the dark. Two approaches were taken to determine whether various organic compounds could be involved in pigmentation, and their effects noted in cultures incubated in light or darkness. The first approach was based on the prevention of pigmentation in light-grown cultures by dimethylsulfoxide (DMSO) without affecting pycnidial formation. This procedure was based on the findings of Carley *et al.* (1967) who found that DMSO inhibited pigmentation in *Aspergillus niger* sporulation. Various concentrations: 2.56×10^{-1}, 1.28×10^{-1}, and 1.28×10^{-2}M of DMSO were added to the synthetic medium in an attempt to reduce pigmentation in the cultures incubated in the light and to note if the pigmentation inhibition also inhibited pycnidial production. The results are presented in Table 4.

The second approach was to incorporate in the synthetic medium various organic compounds which could be directly or indirectly involved in the shikimic acid cycle for the synthesis of pigments. Phenylalanine, tyrosine, tryptophan, phenol, catechol, and salicin were such compounds used. The concentrations of these compounds were as follows:

- phenylalanine, 2.42×10^{-2}, 1.21×10^{-2}, 6.05×10^{-3}M
- tyrosine, 2.21×10^{-2}, 1.10×10^{-2}, 5.5×10^{-3}M
- tryptophan, 1.96×10^{-2}, 9.8×10^{-3}, 4.9×10^{-3}M
- phenol, 1.81×10^{-1}, 9.05×10^{-2}, 9.05×10^{-3}, 9.05×10^{-4}, 1.81×10^{-5}M
- catechol, 4.18×10^{-1}, 9.1×10^{-2}, 9.1×10^{-3}M
- salicin, 6.99×10^{-3}, 3.49×10^{-3}, 3.49×10^{-4}M.

Aliquots of synthetic medium each containing one of these com-

pounds were sterilized by autoclaving at 15 lbs pressure for 10 min. Petri plates containing 20 ml of synthetic medium plus each of the above mentioned concentrations were inoculated and placed in light and darkness in a manner previously described. The results are presented in Table 5.

In another experiment *l*-asparagine, normally included in the medium, was excluded and the same concentrations of phenylalanine, tyrosine, and tryptophan as mentioned above and a mixture of 1.96×10^{-2}M tryptophan and 2.21×10^{-2}M tyrosine were incorporated into the medium. Inoculations and incubations were made as usual. The experiment was designed to determine whether these amino acids could substitute for *l*-asparagine as the prime nitrogen source in the medium. The results are presented in Table 6.

To determine whether indole acetic acid (IAA), which has been associated with some of the intermediates of the shikimic acid cycle, may be involved in the pycnidia formation in cultures of *P. meliloti* incubated in darkness, 50 ppm, 100 ppm and 500 ppm of IAA respectively were incorporated in the synthetic medium and the medium sterilized using Seitz filters. IAA was found to substitute for light in the a-sexual reproduction of *Sclerotinia fructigena* by Khan (1966).

f. Influence of various enzymes on mycelial growth and pycnidial production

Since hydrogen peroxide was able to substitute to only a certain extent for light in the pycnidial production by *P. fuscomaculans* (Coons 1916), it was evident that oxidizing agents were not able to replace completely certain physiological effects induced by light.

These physiological effects which appear to be involved in controlling pycnidia production in light-grown cultures are absent in dark-grown cultures. It was then thought that although such compounds may have a role to play in the pycnidial production, an efficient system for the utilization of these compounds may be lacking. Therefore various oxidizing and reducing enzymes were selected to determine their role in the production of pycnidia by *P. meliloti*. Commercial horseradish peroxidase, beef liver catalase, fungal catalase, polyphenol oxidase, cellulase, and β-glucosidase preparations were used for studying their influence on mycelial growth and pycnidial production in light and darkness. The results are presented in Tables 7-10.

Commercial horseradish peroxidase powder (Nutr. Biochem. Corp. activity of 200 purpurogallin units/mg) was dissolved in distilled deionized water (conc: 1 mg/ml) and sterilized by filtration through a sterile GS 0.22-μ Millipore filter. A 2.5 ml aliquot of this preparation was added to cover evenly the surface of the already solidified synthetic medium (20 ml) in each Petri plate (i.d. 90 mm). These Petri plates were allowed to stand for 24 hrs at 15°C before inoculation to allow time for the agar medium to absorb the added peroxidase solution. A hole, 6 mm in diameter, was made in the center of Petri plate into which the inoculum plug, mycelium side down, was placed. The denatured peroxidase enzyme solution was obtained by autoclaving a portion of the above 'active' enzyme solution for 20 mins at 121°C. This autoclaved enzyme solution was not passed through the Millipore filter. 2.5 ml aliquots of the autoclaved horseradish peroxidase solution per Petri plate were aseptically added to the surface of the synthetic medium and

inoculated as described above with isolates P_{10}, P_{15}, P_{20}, and P_{30}.

The effects of the horseradish peroxidase concentration were also studied. In one experiment only 2.5 ml of 'active'* horseradish peroxidase preparation containing 2500, 1250, 625, 312, 156, and 78 pupurogallin units of activity per 2.5 ml respectively were added to the surface of the medium in each Petri plate and inoculated as described previously.

'Active' and denatured forms of *Aspergillus niger* catalase solution (Nutr. Biochem. Corp., activity of 100 catalase units/ml) were used in a similar manner as described for horseradish peroxidase preparations. Two concentrations of the fungal catalase with activities of 250 and 25 catalase units/2.5 ml of solution respectively were used. 'Active' and denatured beef liver catalase (Nutr. Biochem. Corp., activity of 3000 catalase units/mg) solutions with activities of 250, 25, 0.25 units/2.5 ml aliquot respectively were also used. Fungal and beef liver catalases were denatured in a manner similar as described for horseradish peroxidase.

A flocculent precipitate formed in the autoclaved fungal catalase preparation containing 250 units of activity/2.5 ml aliquot. No such precipitate was formed when lower concentrations of fungal catalase, beef liver catalase, or horseradish peroxidase were denatured by autoclaving. This flocculent precipitate was dispersed evenly throughout the liquid by vigorous shaking before it was added to the medium.

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* In this thesis the term 'active' will refer to all the enzyme preparations which were not denatured by autoclaving.

The flocculent material was separated from the liquid portion by centrifugation at 35,000 x g for 30 mins. Both the precipitated flocculent material and the supernatant were used to determine their effects on growth of mycelium and the production of pycnidia in light and darkness. The precipitate was suspended in an aliquot of deionized distilled water. Water was also added to the supernatant and suspended precipitate so that the final volumes of the two fractions were equivalent to the volume of the fungal catalase used for the separation of the precipitate and the supernatant fractions by centrifugation. Each fraction was sterilized by autoclaving and then added to the medium. Petri plates were inoculated as usual.

Theorel (1934, 1935) was able to split the yellow enzyme of Warburg and Christian into its protein part and its riboflavin phosphate part by dialyzing the salt free enzyme against 0.02 N HCl. In an attempt to separate the protein and the heme moieties of intact *A. niger* catalase and to note their effects, if any, on mycelial growth and pycnidial production, Theorel's method (1947) for the separation of protein and coenzyme was used. 100 ml of *A. niger* catalase, concentration of 100 catalase units/ml, was cooled in an ice bath. An acetone-HCl mixture was prepared by adding one ml concentrated HCl per liter of acetone. The mixture was cooled to $-5^{\circ}C$ to $-10^{\circ}C$ in an NaCl-ice mixture, and to 100 ml of this mixture was added 100 ml of cooled enzyme solution. The cream-coloured protein precipitated in a matter of few minutes. The protohemin portion remained in solution. The precipitate and the supernatant were separated by centrifugation at 35,000 x g for 10 mins. The supernatant was decanted off, and the protein precipitate washed twice with distilled

deionized water and then centrifuged again. The protein pellet was dissolved in a minimum volume of 1% Na_2CO_3. The supernatant fraction removed after centrifugation contained the protohemin portion of the enzyme. The acetone from this liquid fraction was evaporated by bubbling nitrogen gas through the solution. The volumes of both protohemin and protein fractions were then brought up to the initial volume, which was 100 ml, with deionized distilled water. Each fraction was used to determine its effect on mycelial growth and pycnidium production in light and darkness. The solutions were sterilized by passing them through AA 0.8-μ Millipore filters. The volume used for each fraction was 2.5 ml/Petri plate.

In another experiment an attempt was also made to regenerate the enzyme from the separated fractions according to the method used by Agner (1935). The purpose of the experiment was to see if the regenerated catalase enzyme had similar effects on mycelial growth and pycnidial production as the original unseparated *A. niger* catalase enzyme. Acetone was removed from the supernatant fraction containing protohemin, separated using Theorel's method (1947), by bubbling nitrogen through the solution. HCl from HCl-acetone mixture used for the separation of the catalase protein and protohemin fractions was neutralized by adding a few drops of Na_2CO_3 solution and then mixing together the protohemin and the protein portions. This solution was sterilized as above and a 2.5 ml aliquot/Petri plate was added to the solidified synthetic medium.

Solutions containing both horseradish peroxidase and *A. niger* catalase were used to determine their combined effects on mycelial growth and pycnidial formation. 50 mg of horseradish peroxidase were dissolved in 50 ml of the fungal catalase solution. This stock solution had a per-

oxidase activity of 500 purpurogallin units plus a catalase activity of 250 units per 2.5 ml. The peroxidase-catalase solution was used in two concentrations:

i. stock solution i.e. 500 units of peroxidase activity + 250 units of catalase activity, and

ii. one tenth dilution of stock solution i.e. 50 units of peroxidase activity + 25 units of catalase activity. Water was used to dilute the stock solution.

'Active' and denatured forms of this enzyme mixture were sterilized by filtration through AA 0.8-μ Millipore filter, and 2.5 ml aliquots/Petri plate were used.

In one experiment only, polyphenol oxidase powder, cellulase, and β-glucosidase (Nutr. Biochem. Corp.) were separately dissolved in distilled deionized water and solution containing 0.5 and 0.05 mg/2.5 ml of the 'active' enzyme were used, as described for the active horseradish peroxidase and catalase enzymes, to determine their effects on mycelial growth and pycnidial production.

Cultures for all these preparations were incubated both in light and darkness for 15 days at 15°C. Petri plates containing synthetic medium flooded with 2.5 ml of sterilized water served as controls.

To determine whether oxidizing enzymes of the fungus could produce peroxides which would result in pycnidia formation in the dark, MnO_2 and haematin were incorporated in the synthetic medium. These compounds have been shown to be involved in peroxide formation in bacteria (Whittenbury, 1964). The sensitivity of this procedure for the detection of hydrogen peroxide was not given. The media were prepared with a modification of the method described by Whittenbury (1964). Synthetic medium was poured (20 ml) into the Petri

plates and then a very thin layer of the same medium (1-2 ml) to which had been added 4% (w/v) MnO_2 was poured on top. Clearing of the MnO_2 under and around the fungal growth would indicate H_2O_2 formation. Haematin medium was prepared by adding 50 µg/ml of haematin from a stock solution (50 mg haematin in 10 ml water and sufficient 0.1 N NaOH to dissolve the haematin) which had been heated at 100°C for 15 min to already sterilized synthetic medium. 20 ml of this medium was then poured per Petri plate for the desired studies.

g. Gel electrophoretic studies

The cultures of *P. meliloti* showed a very diverse physiological behavior depending upon the cultural conditions i.e. the production of pycnidia when the fungus was grown in light and their absence in the cultures incubated in the dark. It was thought that this physiological diversity may show up in the biosynthesis of certain macromolecules which ultimately could throw some light on the mechanism of this physiological diversification. As well, a clue to the nature of triggering pycnidia initiation by light or conversly, the inhibition of these structures in the dark treatment could be provided. Both enzymic and non-enzymic proteins play an important role in metabolism; therefore, experiments were set up to note the qualitative differences in the protein composition of the fungal mycelium and pycnidia at various stages of growth in light or dark treatments. Of particular interest was to note differences at the stage when pycnidia were being initiated in the light grown cultures. The method of disc gel electrophoresis was used to study such differences, since it is a very rapid, sensitive, and a simple method for the separation and characterization of proteins. Peroxidase was found to play a major role in mycelial growth and pycnidial production, therefore a study of

peroxidase isozymes by disc gel electrophoresis was also carried out on the mycelial extracts which were also analyzed for proteins by the same procedure.

1. Harvesting and protein extraction from fungal mycelium

For the purpose of studying protein pattern and peroxidase isozyme changes at various stages of fungal growth, the fungus was cultured on semi-solid synthetic medium (0.2% agar agar instead of 1.7%). At the desired growth period, the central inoculum plug was removed and nearly all of the synthetic medium was removed from the undersurface of the colony by repeated washing under fast running tap water. Only a qualitative study was carried out since the fungus does not show normal growth on liquid medium and the fungal hyphae could not be separated readily from the semi-solid medium.

The extraction procedure was the same as that followed by Sekhon (1969). In this procedure freshly harvested mycelium from 10-, 13-, 15-, 17-, and 20-day old cultures grown in light and darkness were washed to remove, as much as possible, the semi-solid medium. The excess moisture was removed by drying the mycelium between folds of filter paper. Approximately 5 g of this mycelium was frozen at -23°C for 6-8 hrs prior to passing through a chilled Hugh's press to disrupt the mycelium. To the disrupted mycelium 0.1 M Tris-HCl buffer, pH 7.5 was added in a ratio of 1:4 :: w:v. The slurry was thoroughly mixed and centrifuged at 35,000 x g for 30 mins. After centrifugation the supernatant was removed and the precipitate was discarded. The soluble proteins in the supernatant fraction were precipitated with $(NH_4)_2SO_4$ (695 g/liter) and the sample was agitated for three hrs by a magnetic stirrer at 3-5°C. This sample was centrifuged

at 35,000 x g for 10 mins and the supernatant discarded. The pellet, the precipitated protein fraction was dissolved in 5 ml of 0.1 M Tris-HCl buffer, pH 7.5. The $(NH_4)_2SO_4$ was removed by dialysis against the above buffer for 4-6 hrs, the buffer being changed every 30 mins and agitated by magnetic stirrer to hasten dialysis. The dialysis was stopped after the dialyzing buffer gave a negative test for sulphate when $BaCl_2$ was used as the indicator. All extraction procedures were carried out at 3-5°C.

2. Protein determination and the standard curve

Total protein determination of $(NH_4)_2SO_4$-precipitated and dialyzed protein samples were carried out by the Lowry *et al.* method (1951). A standard curve (Fig. 1) was prepared using Bovine Serum Albumin (Nutr. Biochem. Corp.) as the protein source for quantitative estimation of fungal proteins. To one ml solution containing 100, 200, 300, 400, and 500 µg of Bovine Serum Albumin in 0.1 M Tris-HCl buffer, pH 7.5 respectively in a test tube was added 5 ml of a freshly prepared mixture of 50 ml of 2% Na_2CO_3 in 0.1 N NaOH and one ml of 0.5% $CuSO_4 \cdot 5H_2O$ in 1% potassium tartrate. The reaction mixture was allowed to stand for 10 mins, and then 0.5 ml of 1 N Folin- phenol reagent was added. After 30 mins the intensity of the blue colour developed was read at 630 mµ using a Hitachi Perkin-Elmer spectrophotometer, Model 139. The absorbance was plotted against the amounts of protein present in the reaction mixture. Each point in Fig. 1 represents an average value of four readings, and the procedure was repeated twice. Appropriate dilutions of the unknown were made so as to read absorbance between 0.1-0.6 at 630 mµ.

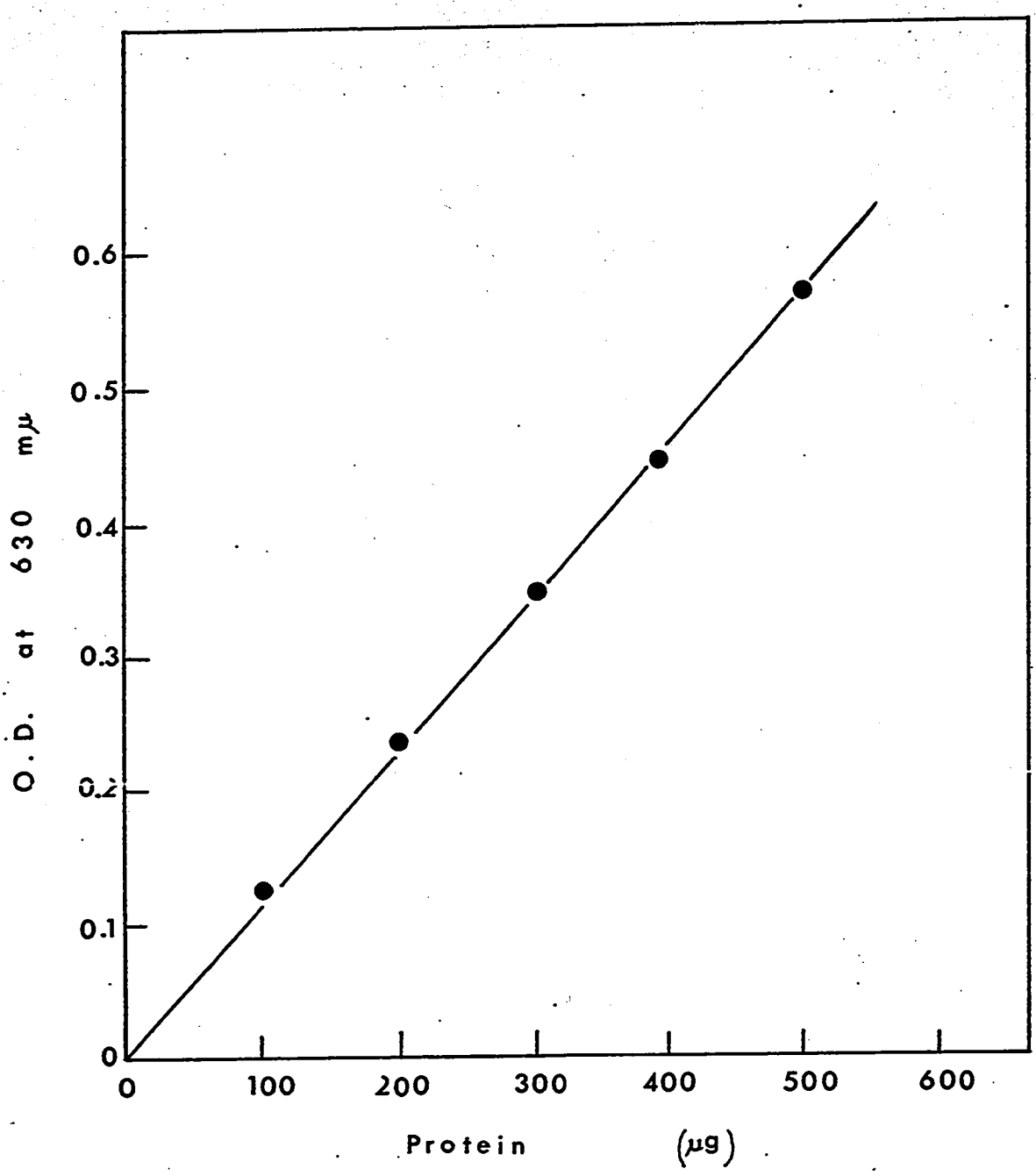

Figure 1. Standard curve for protein determinations.

3. Disc gel electrophoresis of proteins and peroxidase isozymes

Disc gel electrophoresis was carried out using the method described by Davis (1964). 7.5% polyacrylamide gels were used, and $(NH_4)_2SO_4$-precipitated proteins were used for peroxidase isozyme and protein pattern studies. Duplicate and triplicate determinations were made on aliquots of the sample solutions containing 300 µg of protein using a current of 3 mAmps/gel applied for 60-80 min.

Protein bands on the gels were detected by staining with 1% amido black 10-β dissolved in 7% acetic acid. The excess dye was removed by washing in 7% acetic acid. Peroxidase isozymes on the gels, run at the same time as those for proteins, were stained with benzidine dihydrochloride reagent (Bulletin- Special subject, Enzyme analysis, Canal Industrial Corporation, Rockville, Maryland). The electrophoresis experiments for protein patterns and peroxidase isozymes were repeated once and the results are shown in Figs. 38 and 39.

4. Disc gel electrophoresis for protein patterns of horseradish peroxidase, fungal and beef liver catalases

Disc gel electrophoretic protein patterns for horseradish peroxidase, beef liver catalase and fungal catalase used in experiments outlined on pages 19-25 were also made, using the same method as described above. The results are shown in Fig. 40.

B. STUDIES ON THE HOST PLANT

Arctic and Denta varieties of sweet clover were used for these studies.* The Arctic variety is winter-hardy and resistant to *P. meliloti*;

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* Seeds of Arctic and Denta sweet clover varieties were obtained from Dr. B. Goplen, Canada Department of Agriculture, Saskatoon, Saskatchewan.

whereas, Denta is non winter-hardy and susceptible to the fungus.

The seeds of both varieties were sown in flats using a soil-peat-sand (3:1:2) mixture in the greenhouse. After 15 days, seedlings were transplanted to peat pots containing the above soil-peat-sand mixture (two seedlings per pot). Twenty-two-day old seedlings were transplanted in field plots on June 7, 1968 at the University of Alberta Parkland Farm. On August 30, 1968, a soil temperature recorder (Wilh. Lambrecht KG Gottingen type 257 # 342311 Schreibstreifen 82 TT 35-0-45, clock type 252 U4T 7d) was set up in the center of the plot. Two probes of the recorder were buried, one on each side of the recorder, at a depth of about 13 cm in the soil. Temperature recordings were started on August 30, 1968 and continued until June 17, 1969. A summary of these temperature recordings is given in the Appendix (see page A-2).

a. Harvesting and extraction

Lateral roots of the sweet clover varieties were used for analyses. *P. meliloti* generally forms lesions and pycnidia on these roots; however, tap roots are also diseased but were not used for these experiments because a more uniform sample of roots could be obtained using lateral roots. Several plants of each sweet clover variety were dug from the soil on October 8, 15; November 26; December 3, 10, 17, 24, 31; January 7, 14, 21, 28; February 4, 11, 18, 25; March 4, 11, 18, 25; April 1, 8, 15, 29; May 13, 27, and June 17, 1968-1969. The roots were immediately washed in cold running water and dried between paper towels. For the sake of uniformity, those portions of the lateral roots having a diameter of 3-4 mm were removed and were cut into small pieces of 3-4 mm length. 3.0 g fresh weight of this root sample was immediately frozen at -45°C for 4-6 hrs

before further analysis. The remaining root pieces were weighed and oven dried at 95°-100°C for 3-4 days for unit dry weight determinations. The frozen root material was passed through a Hughes press (-45°C) to disrupt the root cells. To the disrupted cells was added 6.0 ml of 0.5 M potassium phosphate buffer, pH 7.0. The slurry was thoroughly mixed and centrifuged at 35,000 x g for 30 mins. The supernatant portion was decanted off and the volume obtained, measured. This crude extract was used without further purification for total protein, peroxidase, catalase, polyphenol oxidase enzyme activity measurements, and disc gel electrophoretic studies for protein, peroxidase, and polyphenol oxidase enzymes. All extraction procedures were carried out at 3-5°C. Results for dry weight are shown in Fig. 47.

b. Total protein determinations

Total protein determinations were made using the method described by Lowry *et al.* (1951). Appropriate dilutions of above cell-free extracts were made so as to give readings between 0.1-0.5 absorbancy at 630 mμ. The amount of protein was calculated from the standard curve (see page 28) and the total protein contents were calculated on the basis of total extractable volume after centrifugation, and also on a dry weight basis. Data for protein analyses are presented in Figs. 48 and 49.

c. Peroxidase activity

Peroxidase activity was determined using the procedure outlined by Gregory (1966). Using this method, the peroxidase activity, in terms of ascorbic acid units, is given by the quantity of ascorbic acid initially present divided by the reaction time in minutes. The unit of peroxidase activity is expressed as μMoles ascorbic acid oxidized per

min.

All the reagents were prepared just prior to carrying out each determination and the reactions were carried out at room temperature. To the reaction mixture was added root extract (diluted 10 or 20 times) and a stop watch immediately started. A sudden development of blue colour indicated the completion of the reaction. The average time for 8-10 readings, in minutes, was used for the calculation of peroxidase units of activity. The time taken for the blue colour to appear is inversly proportional to the activity of peroxidase present in the mixture. The specific activity was calculated per g of protein. The peroxidase activity results are shown in Fig. 50.

d. Catalase activity

The method of Beers and Sizer (1952), as modified by the Worthington Biochemical Corp., Freehold, New Jersy, was used for catalase activity measurements. In this procedure, the disappearance of hydrogen peroxide was followed spectrophotometrically at 240 mμ. The substrate was prepared by diluting 0.3 ml of 30% hydrogen peroxide (superoxol) to 50 ml with 0.05 M potassium phosphate buffer, pH 7.0, resulting in an approximately 5.9×10^{-3} M solution of hydrogen peroxide. The cell-free root extract was diluted 100 times with water. The decomposition of hydrogen peroxide was determined in a reaction mixture containing 2.0 ml of diluted root extract to which 1.0 ml buffered hydrogen peroxide was added at time zero, and readings taken at 240 mμ at 10 sec intervals for 70 secs. The control mixture had water substituted for the root extract. One unit of catalase activity is defined as that amount which catalyzes the decomposition of one micromole of hydrogen peroxide per minute under the

specified conditions as described above. The molar absorbancy index for hydrogen peroxide at 240 mμ in 10 mm cuvettes is 43.6. Specific activity is expressed as units per g protein.

Five to six readings for the extract from each harvest were taken and an average absorbance for sixty sec was used for catalase activity calculations. The results for catalase activity per g protein are shown in Fig. 51.

e. Polyphenol oxidase activity

Polyphenol oxidase activity, as catechol oxidase, was determined using Alberghina's method (1964). Catechol was used as the substrate. When tyrosine was used as substrate instead of catechol, the activity of the enzyme was so low that it could not be measured accurately. Catechol oxidase activity was measured as a function of the increase of absorbancy at 390 mμ during the oxidation of catechol. One ml of 4×10^{-4} M catechol solution and one ml of 0.05 M potassium phosphate buffer, pH 7.0, was added to a cuvette to which was added one ml of cell-free extract. The absorbancy changes were recorded every min for six min. The average of five to six readings for the extracts from each harvest was determined and this average absorbance per min was used for calculating specific activity. One unit of enzyme activity is defined as that amount of the enzyme which catalyzes the oxidation of catechol such that the increase in absorbancy is 0.001 per minute under the specified conditions. Specific activity is expressed as units/g of protein. The results are presented in Fig. 52.

f. Electrophoretic studies

The disc gel electrophoresis process for analyzing root extract was

similar to that used for the fungus mycelium (see page 29). The cell-free extracts from each harvest for both sweet clover varieties were subjected to electrophoresis to note changes in the protein and peroxidase isozyme patterns. Using catechol as substrate, the electrophoretic behaviour of polyphenol oxidase for both varieties at different stages was carried out according to the method described by Stahmann *et al.* (1966).

g. Amino acid analyses

Amino acids have long been implicated in the cold hardiness of many plant species (Pauli and Mitchell, 1960; Wilding, Stahmann and Smith, 1960; Zech and Pauli, 1962; Pauli and Zech, 1964). The fungus *P. meliloti* establishes itself on host plants to form disease lesions and pycnidia during winter and early spring (Netolitzky, 1965). Therefore amino acid analysis was carried out to note whether there was a difference in free and protein amino acids between the two varieties harvested at various times during the fall, winter and spring. Wu (1965) found that media which contained water extracts of root material collected at different times of the year had different effects on mycelial growth and pycnidial formation of P_{20}. However, his studies dealt only with roots harvested in June and August.

It is interesting to note that such a complete amino acid analysis for sweet clover roots has not been previously carried out.

The sweet clover roots harvested each week were air dried over a period of three to four weeks. These dried roots were used for the qualitative and quantitative amino acid and semi-quantitative sugar determinations. Sweet clover roots were ground in an Intermediate Mill so that the ground material passed through a 40-mesh sieve. The root powder was placed in a desiccator having anhydrous calcium chloride as a desiccant.

For each of the following dates, for each variety, 3.000g of the root material was used for analysis: October 8; December 24, 31; January 14, 28; February 11, 25; March 11, 25 and April 8, 1968-1969. The weighed root powder was transferred to 250 Erlenmeyer flasks and 100 ml 80% ethanol added to extract free amino acids. The flasks were put on a gyrorotary shaker and agitated for 24 hrs at room temperature. The ethanol fraction was collected by filtration through Whatman #2 filter paper. The residue was removed from the filter paper and again extracted with 100 ml of 80% ethanol for 24 hrs on the gyro-rotary shaker. The ethanol fraction, obtained after filtration was added to the initial 80% ethanol filtrate.

The ethanol-soluble fractions containing free amino acids were evaporated to dryness using a flash evaporator at 48-52°C. The dried contents were dissolved in 20 ml of 6 N constant boiling HCl (B.P. 106-108°C). The free amino acid fractions were transferred quantitatively to a 25 ml ampule, which was sealed under vacuum. All the samples were similarly prepared and then hydrolyzed for 18 hrs at 121°C at 15 lbs pressure in the autoclave. The hydrolyzate was filtered using Whatman #2, 4.25 cm diameter filter paper and the residue washed thoroughly with approximately 100 ml of deionized distilled water. The filtrate was evaporated to dryness repeatedly using a flash evaporator (48-52°C) to remove the HCl. The dried material was dissolved in a dilutor buffer, which consisted of citric acid·H_2O, 21 g; NaOH 97%, 8.4 g; concentrated HCl, 16 ml; caprylic acid, 0.1 ml; thiodiglycol, 20.0 ml; Brij-35 solution, 2.0 ml; and distilled deionized water to bring the volume to one liter. The dilutor had a sodium concentration of 0.20 N and the pH was adjusted

to 2.2 ± 0.03 (Instruction Manual and Handbook, Beckman/Spinco Model 120 amino acid analyzer, Beckman Instruments Inc., Spinco Division, Palo Alto, California, 1963). The pH of the sample solution was adjusted to approximately 2.2 with N KOH. The volume of the solution was brought up to 25 ml with sample dilutor. One ml of this solution was further diluted 5 times, and 200 µl of this diluted sample was used for free amino acid analysis using a Beckman/Spinco amino acid analyzer Model 120C, employing columns having 8% cross linked, sulphonated styrene copolymer resins, PA-35 (Beckman) for basic and PA-28 (Beckman) for acidic amino acids. The amino acid analyses were carried out by Mr. M. Batory.* The free amino acid quantities were calculated as mg/g dry weight. The bound amino acids of the residue, obtained after the free amino acids were extracted with 80% ethanol, were also quantitatively determined. The residues were oven dried at 95-100°C for 8-12 hrs and after cooling, 0.5 g was placed into each 25 ml ampule to which 25 ml of constant boiling HCl mixture was added. The procedures for hydrolysis and preparation of the hydrolyzate for bound amino acid analyses were similar to that described for the free amino acids, except that the final volume after pH adjustment was 15 ml instead of 25 ml. Further dilutions and the amounts used for bound amino acid analyses were also the same as described above. The quantities of bound amino acids are expressed as mg/g dry weight. The results for free and bound amino acids are shown in Tables 11 & 12.

h. Sugar analyses

Wu (1965) observed that the naturally occuring sugars in alfalfa roots were important factors influencing pycnidium production. Wu found

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that a combination of glucose, fructose, and sucrose was most favourable for pycnidial production. Wilding *et al.* (1960) found that fructose was absent when alfalfa roots were collected in the summer; however, they detected fructose, glucose, and sucrose in roots sampled in December. Wu (1965) also noted the absence of fructose in alfalfa root samples harvested in the summer. Since pycnidia are most abundant during periods shortly after winter than summer, it would appear that carbohydrates could play an important role in the pycnidium production. The results obtained by Parish (1969) were also very pertinent to the present investigation, since Parish found that sucrose, glucose and fructose stimulated peroxidase synthesis in senescing tobacco leaf discs. Because of the interesting findings of the above researchers, it was decided to determine semi-quantitatively the changes in the quantities of various sugars in the sweet clover roots during the period from October through April, 1969 in an attempt to find any correlation between such changes and pycnidium production by the fungus, peroxidase activity of the plant roots and their relationship with cold hardiness of sweet clover.

The procedures used for extraction of sugars and subsequent analyses by one dimensional paper chromatography are outlined below.

One g of root powder, the same as used for amino acid analyses, was extracted twice with 80% ethanol in the same manner as described for extraction of free amino acids. The extracted ethanol fractions were dried using a flash evaporator at 45-48°C. The dried material was dissolved with 10% isopropanol and the volume brought up to 6 ml. For chromatography, Whatman paper #1 sheets which had been previously washed with water and dried were used. On each of the two sheets 2 15-µl aliquots

respectively of the unknown sugar extract were applied. Next to each spot containing the unknown sugar mixture, 30 µl of a mixture containing 30 µg of fructose, 30 µg of glucose, and 30 µg of sucrose were also applied on the paper for reference standards.

The irrigant solvent was the top portion of a n-butanol-acetic acid-water (4:1:5) mixture. Chromatograms were run for 24 hr at room temperature. The chromatograms were dried at room temperature in a fume hood and sprayed with the aniline hydrogen phthalate reagent and heated for 15 min at 118°C to bring out the spots for glucose, fructose, and sucrose (Block *et al.*, 1955). Strips containing all the sugar spots for a particular sample were removed. The density and area of spots for a particular sample (unknown) and known sugars were determined using a Chromoscan MK II employing reflectance system and a blue filter #5265. With this instrument the area under the curve was automatically integrated. The mixture of known sugars with different concentrations (10, 20, 30, 40, 50, and 60 µg of each sugar) were also chromatoscanned and the area under the curve for each sugar and each concentration was integrated. A plot of area under the curve versus sugar concentration was drawn and used as a standard curve for calculating the quantity of individual sugars in the unknown sugar mixture. In these semi-quantitative determinations the total amounts of sugars were calculated and expressed as mg/g dry weight. The data for these experiments is presented in Table 13. Figs. 2, 3, and 4 show the standard curves for sucrose, glucose, and fructose respectively.

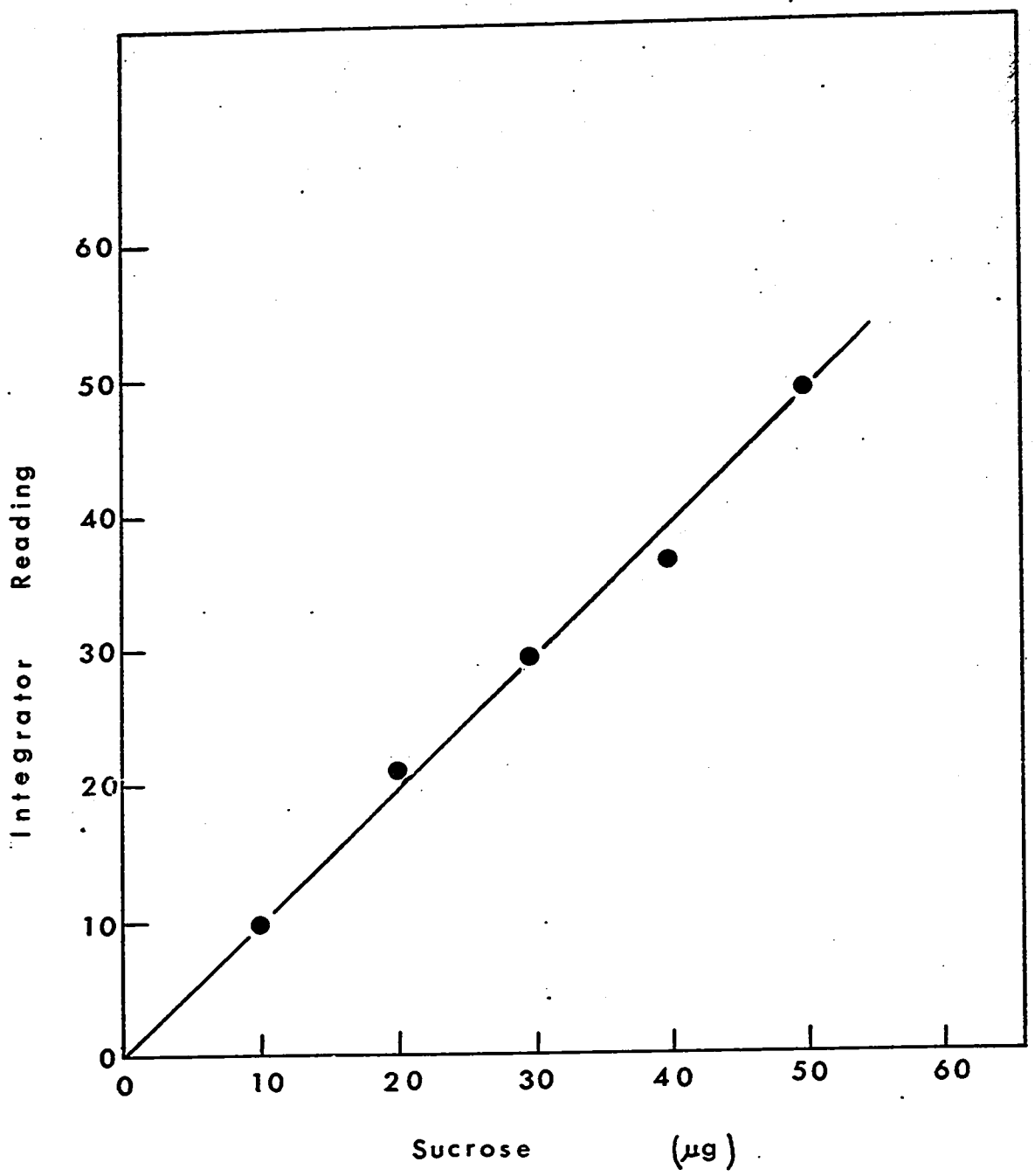


Figure 2. Standard curve for sucrose determination.

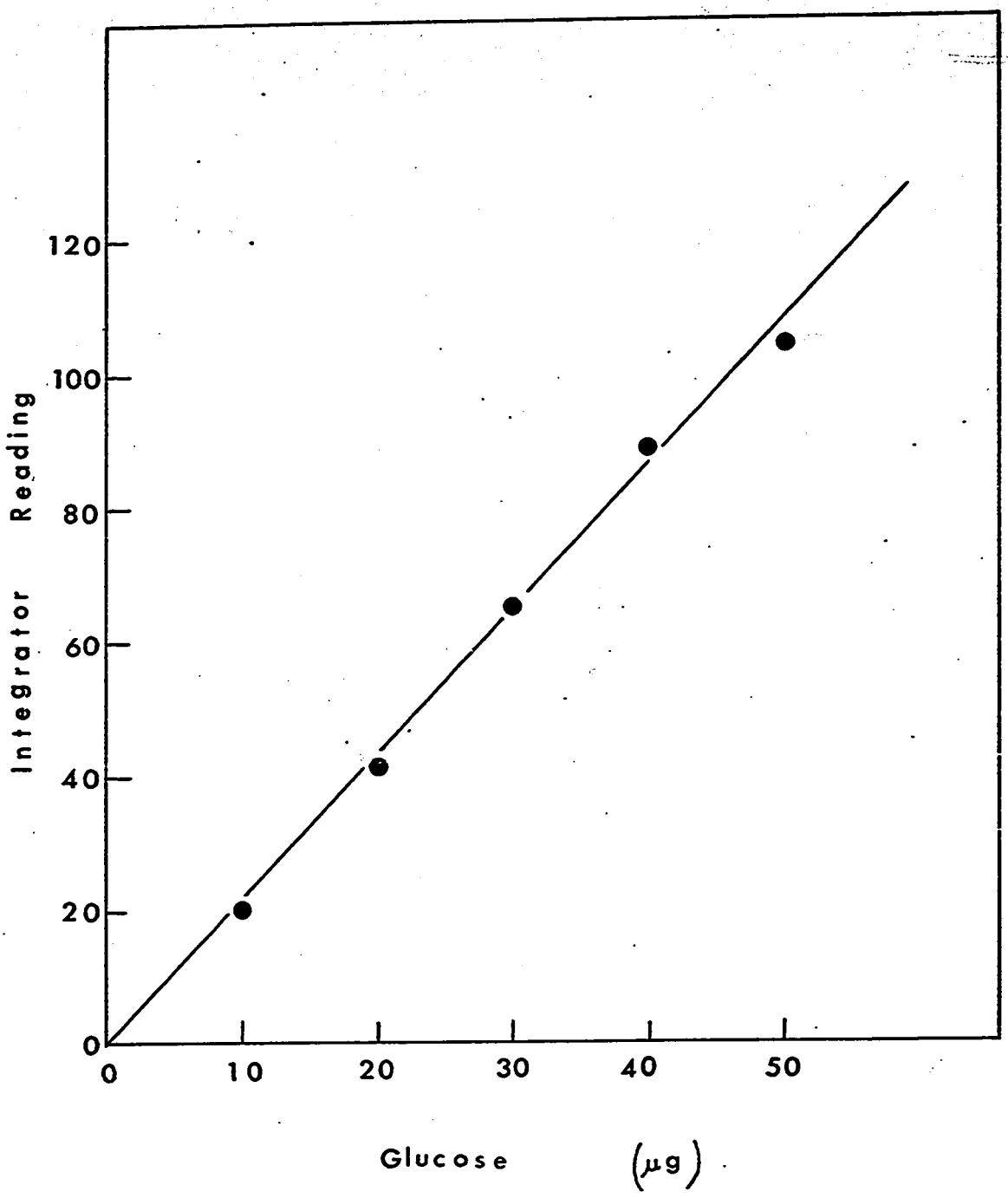


Figure 3. Standard curve for glucose determination.

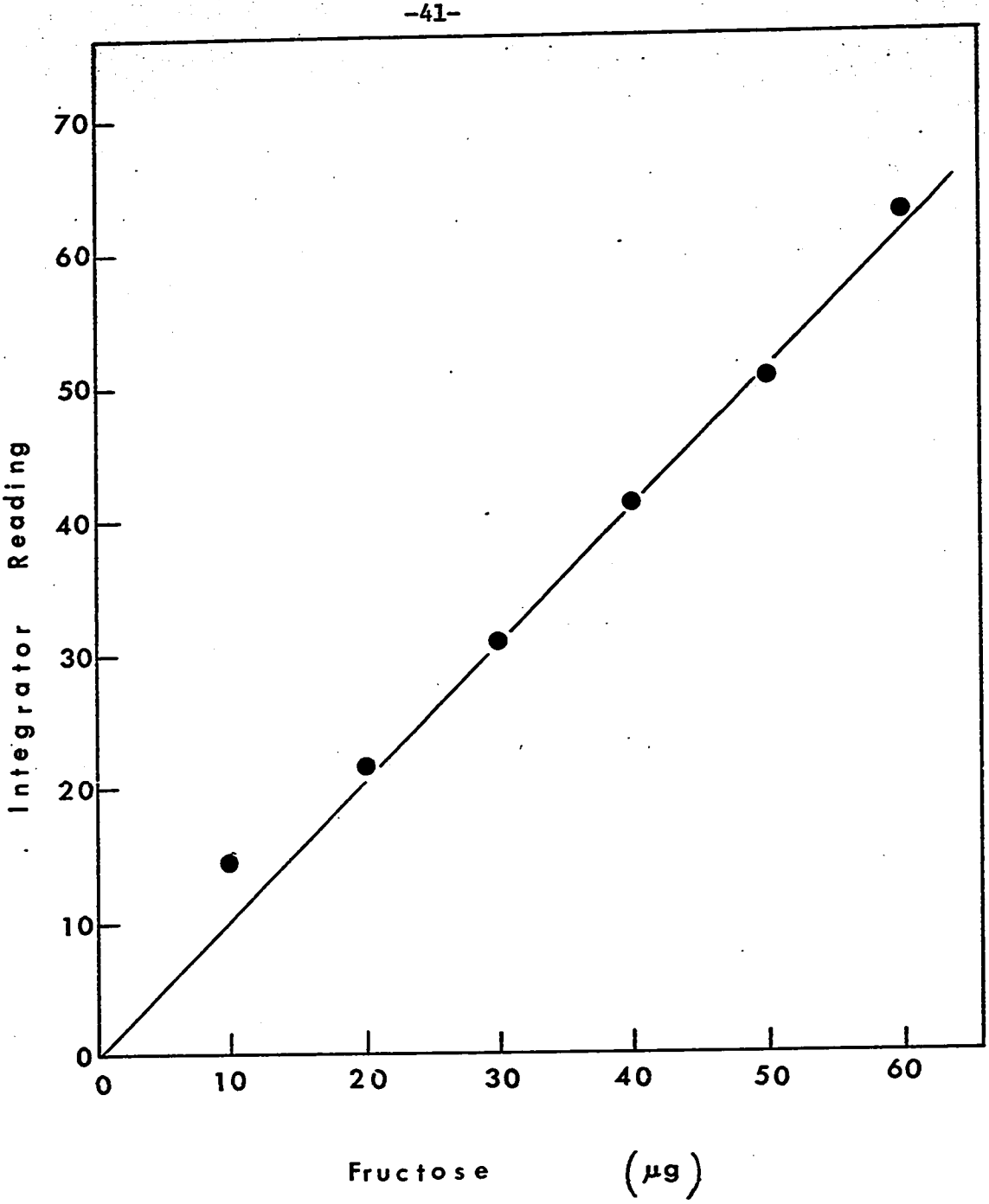


Figure 4. Standard curve for fructose determination.

RESULTS

A. STUDIES ON THE PLANT PATHOGEN

a. Effects of Various Amounts of Available Medium on Mycelial Growth and Pycnidial Production in Darkness

A large number of pycnidia were formed in those cultures incubated in the dark when the amounts of medium relative to the controls (Petri plates containing 20 ml medium) were reduced (Table 2). The type of mycelial growth was like the controls incubated in light; whereas, with increasing amounts of the medium the colonies resembled the controls grown in darkness. The controls in dark had well pronounced aerial growth whereas cultures incubated in the light had little aerial growth. Less than three ml of medium resulted in a greatly restricted growth and pycnidial formation. Pycnidia were not formed in cultures grown in Petri plates containing one ml of medium. Maximum numbers of pycnidia resulted when three ml of medium was used.

b. Effects of Various Inorganic Oxidants on Mycelial Growth and Pycnidial Production

The effects of various inorganic oxidants incorporated in the synthetic medium on the growth of mycelium and production of pycnidia by *P. meliloti* as observed for 15-day old cultures in light or darkness, are presented in Table 3 . The results of this study indicate that:

(i) Chromium trioxide, at the highest concentration, inhibited mycelial growth. The lower concentrations had no effect on the fungal growth. Pycnidial formation in the dark was not induced by any chromium trioxide concentration.

(ii) Potassium permanganate, at the lowest concentration, caused a

TABLE 2

Effects of different amounts of synthetic medium on mycelial growth, pycnidial production, and colony types of *Plenodomus meliloti*, isolate P_{30}, cultured in darkness for 15 days at 15°C as compared to light-grown cultures.*

	Amounts of synthetic medium per Petri plate									
	20 ml (control)		10 ml	8 ml	6 ml	5 ml	4 ml	3 ml	2 ml	1 ml
	Light	Dark	Dark	Dark	Dark	Dark	Dark	Dark	Dark	Dark
Growth (dia. of colony cm)	6.3	6.3	6.5	6.3	6.2	6.5	6.5	6.1	4.2	1.9
Pycnidia/Petri plate	>100	Few on inoc. plug	43	57	54	80	79	170	15	Nil
Type of colony**	++ Grey-white	++++ White	++++ White	++++ White	++++ White	+++ Grey-white	+++ Grey-white	++ Grey-white	++ Grey-white	+ Light-grey

* Average of three Petri plates for one replication.

** + Aerial growth drastically reduced.

++ Aerial growth very little.

+++ Aerial growth abundant.

++++ Aerial growth very abundant.

TABLE 3

Effects of various inorganic oxidants on mycelial growth and pycnidial production by *Plenodomus meliloti*, isolate P_{30}, cultured in light and darkness for 15 days at 15°C.

Medium + inorganic oxidants	Concentration (M)	Growth* (dia. of colony - cm) Light	Dark	Pycnidia per Petri plate Light	Dark	Type of mycelial growth Light	Dark
Control	-	6.1	6.15	>100	Nil	Aerial growth little, grey-white	Abundant aerial hyphae, cotton-white
Chromium trioxide	1.53×10^{-1}	Nil	Nil	Nil	Nil	-	-
	3.30×10^{-2}	1.5	0.5	Nil	Nil	Growth suppressed	Growth suppressed
	3.30×10^{-3}	6.3	6.4	>100	Nil	As for controls	As for controls
Potassium Permanganate	7.28×10^{-2}	6.0	6.4	>100	40-50**	As for controls	Little aerial growth, otherwise as for controls
	1.58×10^{-2}	6.1	6.3	>100	Nil	As for controls	As for controls
	1.58×10^{-3}	6.5	6.4	>100	Nil	As for controls	As for controls
Cobaltous nitrate	1.58×10^{-1}	Nil	Nil	-	-	-	-
	3.43×10^{-2}	Nil	Nil	-	-	-	-
	3.43×10^{-3}	Nil	Nil	-	-	-	-
Sodium hypochlorite	3.09×10^{-2}	1.5	3.0	50-60	10-12**	Growth suppressed, darker in the center and paler towards the periphery	Growth suppressed, darker in the center and paler towards the periphery
	6.71×10^{-3}	1.7	2.9	50-60	10-12**	Growth suppressed, darker in the center, paler towards the periphery	Growth suppressed, darker in the center, paler towards the periphery
	6.71×10^{-4}	6.1	6.0	>100	16-20**	As for controls	Growth suppressed, darker in the center, paler towards the periphery
Calcium hypochlorite	1.07×10^{-1}	Nil	Nil	Nil	Nil	Growth inhibited, little stunted growth only along the inoc. plug	No growth even on the inoc. plug
	2.30×10^{-2}	0.2	0.4	Nil	Nil	Growth suppressed, deep grey in colour	Growth suppressed, hardly any aerial growth
	2.30×10^{-3}	6.3	5.9	>100	10-12**	As for controls	As for controls
Hydrogen peroxide	4.06×10^{-1}	5.9	6.2	>100	5-7**	As for controls	Aerial growth a little suppressed, otherwise as for controls
	9.65×10^{-2}	6.2	6.3	>100	Nil	As for controls	As for controls
	9.65×10^{-3}	6.1	6.2	>100	Nil	As for controls	As for controls

* Average of four Petri plates for one replication.

** Pycnidia not visible through the dense aerial growth, but the immature pycnidia could be felt by rubbing off the aerial mycelial growth.

slight increase in growth; the other concentrations had no effect on the type and size of the fungal colony. The highest concentration of potassium permanganate induced pycnidial formation in cultures incubated in the dark.

(iii) Cobaltous nitrate, at all the concentrations, was found inhibitory to fungal growth.

(iv) Sodium hypochlorite and calcium hypochlorite, at higher concentrations, were found inhibitory to mycelial growth. Sodium hypochlorite, regardless of concentration, induced pycnidial formation in cultures incubated in dark; however, calcium hypochlorite induced pycnidia formation in the dark only at the lowest concentration.

(v) Hydrogen peroxide did not have any effect on the size and type of mycelial growth, but at the highest concentration few pycnidia were produced in the dark.

It may be noted that generally when pycnidia were induced the type of mycelial growth resembled more the control incubated in light than the control incubated in the dark.

c. Effects of Various Organic Compounds on Mycelial Growth and Pycnidial Production

There was no inhibition of pigmentation due to the presence of dimethyl sulfoxide (DMSO). Mycelial growth was inhibited at higher concentrations of DMSO. The type of mycelial growth was like the respective controls. In the cultures kept in darkness, the fungus produced pycnidial initials at the lowest concentration of DMSO. The results of incorporating DMSO and various other organic compounds in the synthetic medium on pigmentation, pycnidial production and mycelial growth are presented in Tables 4-6.

TABLE 4

Effects of various concentrations of dimethyl sulfoxide (DMSO) on mycelial growth, pycnidial production, and pigmentation of *Plenodomus meliloti*, isolate P_{30}, cultured in light and darkness for 15 days at 15°C.

	Controls		2.56×10^{-1}		1.28×10^{-1}		1.28×10^{-2}	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
Growth* (dia. of colony, cm)	6.2	6.2	3.8	3.8	5.1	4.8	6.5	6.1
Pycnidia per Petri plate	>100	Nil	>100	Nil	>100	Nil	>100	35**
Pigmentation***	+	-	+	-	+	-	+	-

* Average of four Petri plates for one replication.

** Pycnidia not visible through the dense aerial growth, but immature pycnidia could be felt by rubbing off the aerial mycelial growth.

*** + Present.

\- Absent.

TABLE 5

Effects of various organic compounds on mycelial growth and pycnidial production by *Plenodomus meliloti*, isolate P_{30}, cultured in light and darkness for 15 days at 15°C.

Medium + organic compound	Concentration (M)	Growth* (dia. of colony - cm) Light	Dark	Pycnidia per Petri plate Light	Dark	Pigmentation Light	Dark
Control	-	6.3	6.2	>100	Nil	Colony light-grey, undersurface dark-grey	Colony white, undersurface cream-colour
Phenol	1.81×10^{-1}	Nil	Nil	-	-	-	-
	9.05×10^{-2}	Nil	Nil	-	-	-	-
	9.05×10^{-3}	Nil	Nil	-	-	-	-
	9.05×10^{-4}	Nil	Nil	-	-	-	-
	1.81×10^{-5}	Nil	Nil	-	-	-	-
Catechol	4.81×10^{-1}	Nil	Nil	-	-	-	-
	9.10×10^{-2}	Nil	Nil	-	-	-	-
	9.10×10^{-3}	6.1	6.2	>100	5-7**	As for control	As for control
Salicin	6.99×10^{-3}	6.0	6.0	>100	Few**	As for control	As for control
	3.49×10^{-3}	6.1	6.15	>100	Few**	As for control	As for control
	3.49×10^{-4}	6.9	6.2	>100	Numerous**	As for control	As for control
Phenyl-alanine	2.42×10^{-2}	6.1	6.3	>100	Nil	As for control	As for control
	1.21×10^{-2}	6.2	6.1	>100	Nil	As for control	As for control
	6.05×10^{-3}	6.2	6.2	>100	Nil	As for control	As for control
Tyrosine	2.21×10^{-2}	5.9	6.05	>100	40**	Colony light-grey, under-surface black	Colony white, undersurface black
	1.10×10^{-2}	6.4	6.2	>100	Few**	Colony light-grey, under-surface black	Colony white, undersurface black
	5.50×10^{-3}	6.3	6.4	>100	12**	Colony light-grey, under-surface black	Colony white, undersurface light-grey
Tryptophan	1.96×10^{-2}	6.1	6.4	>100	14**	As for control	As for control
	9.80×10^{-3}	5.9	6.0	>100	34**	As for control	Colony white, undersurface dark brown
	4.90×10^{-3}	6.3	6.1	>100	53**	As for control	Colony white, undersurface pale brown

* Average of four Petri plates for one replication.

** Pycnidia not visible through the dense aerial growth, but the immature pycnidia and pycnidial initials could be felt by rubbing off the aerial mycelial growth.

TABLE 6

Effects of various amino acids on mycelial growth and pycnidial production by *Plenodomus meliloti*, isolate P_{30}, cultured in darkness for 15 days at 15°C on synthetic medium with and without *l*-asparagine.*

Amino acids	Concentration (M)	Media plus *l*-asparagine: Growth (dia. of colony, cm)	Media plus *l*-asparagine: Pycnidia / Petri plate**	Media plus *l*-asparagine: Remarks	Media minus *l*-asparagine: Growth (dia. of colony, cm)	Media minus *l*-asparagine: Pycnidia / Petri plate**	Media minus *l*-asparagine: Remarks
Control	-	6.2	Nil	Abundant aerial growth, hyphae white, undersurface of colony cream-coloured	4.3	Nil	Growth very sparce, hardly any aerial growth, thread-like hyphae growing along surface of medium
Phenyl-alanine	2.42×10^{-2}	6.1	Nil	"	4.9	Nil	"
	1.21×10^{-2}	5.9	Nil	"	5.9	Nil	As for above control, but undersurface pale yellow
	6.05×10^{-3}	6.2	Nil	"	6.0	Nil	As for above control, but undersurface from pale to dark green
Tyrosine	2.21×10^{-2}	6.0	45**	As for above control except undersurface of colony appears black	6.2	Nil	Aerial growth sparse, colony colour grey
	1.10×10^{-2}	6.1	30**	"	5.9	Nil	"
	5.50×10^{-3}	6.2	16**	As for above control except for slightly more pigmentation	6.0	Nil	"
Tryptophan	1.96×10^{-2}	6.2	10**	As for above control	6.1	Nil	Aerial growth sparse, mycelium light-grey, undersurface light-brown
	9.80×10^{-3}	6.1	27**	Mycelium white, undersurface of colony dark brown	6.1	Nil	"
	4.90×10^{-3}	6.0	50**	Mycelium white, undersurface of colony pale brown	6.2	Nil	"
Tyrosine + Tryptophan	2.21×10^{-2} + 1.96×10^{-2}	-	-	-	6.2	Nil	Growth abundant, aerial hyphae well developed, light-grey. Undersurface of colony light-brown

* Average of four Petri plates for one replication.

** Pycnidia not visible through the dense aerial growth, but the immature pycnidia and pycnidial initials could be felt by rubbing off the aerial mycelial growth.

It is evident from these results that:

(i) Phenol, at all the concentrations tried, inhibited mycelial growth to the extent that even the mycelium on the inoculum plug was killed.

(ii) Catechol at higher concentrations was inhibitory, but at lower concentrations, the type of mycelial growth and the colony size was like controls, and some pycnidia were produced in the dark.

(iii) Salicin did not have any effect on the mycelial growth of the fungus; however, in its presence, numerous pycnidia were produced in the dark.

(iv) Phenylalanine had no effect on mycelial growth, pycnidial production, and pigmentation of the fungus.

(v) Tyrosine and tryptophan induced pycnidial formation and pigmentation in the cultures incubated in the dark; however, these amino acids did not alter the size of the colony.

Growth was very much suppressed when the described synthetic medium excluding *l*-asparagine was used. When the amino acids tyrosine, tryptophan, and phenylalanine were substituted for *l*-asparagine, growth was greatly affected. The colony size was similar to those of controls; however, aerial growth was reduced drastically. A mixture of tyrosine and tryptophan induced greater pigmentation as compared to the controls and other cultures grown on a medium containing only one of these amino acids as the nitrogen source.

The growth substance indole acetic acid did not have any effect on mycelial growth and pycnidial production for cultures grown in darkness.

d. Influence of Various Enzymes on Mycelial Growth and Pycnidium Production

The effects of horseradish peroxidase, *Aspergillus niger* catalase,

and beef liver catalase on the growth of mycelium and production of pycnidia of 15-day old cultures of *P. meliloti*, isolate P_{30}, incubated in light and darkness in relation to controls are presented in Tables 7-10, and Figs. 5-37.

Several significant factors should be emphasized. In 15-day old cultures grown in the light (Fig.5) numerous pycnidia formed first on and around the disc of inoculum and then progressively outwards from the inoculum. Pycnidia were not formed in the peripheral regions of the growing colonies. Control cultures, incubated in the dark (Figs. 6 and 7), often had several pycnidia only on the inoculum plug. These pycnidia may be accounted for when one considers that the inoculum was taken from colonies incubated in the light. Mycelia of the inoculum would therefore have been influenced by light to give an effect similar to that of colonies grown in the light. However, the influence was not transferred to mycelium which formed in the dark.

Much of the mycelium of cultures grown in the dark was aerial and cotton-white whereas the mycelium of cultures grown in the light was more prostrate and grey-white in colour.

In the presence of 'active' horseradish peroxidase, in light or dark treatments, the fungus, in contrast to the respective controls, produced numerous pycnidia which were randomly scattered throughout the culture (Figs. 11-13). Pycnidia in colonies cultured in the dark, although not as numerous or as large as those in cultures incubated in the light, were evident after the dense aerial mycelium was rubbed or brushed (Fig. 13). When the denatured peroxidase preparation was added, the colonies grown in light and dark were similar in growth rate, type, and colour of mycelium, and pycnidial production to those of respective controls

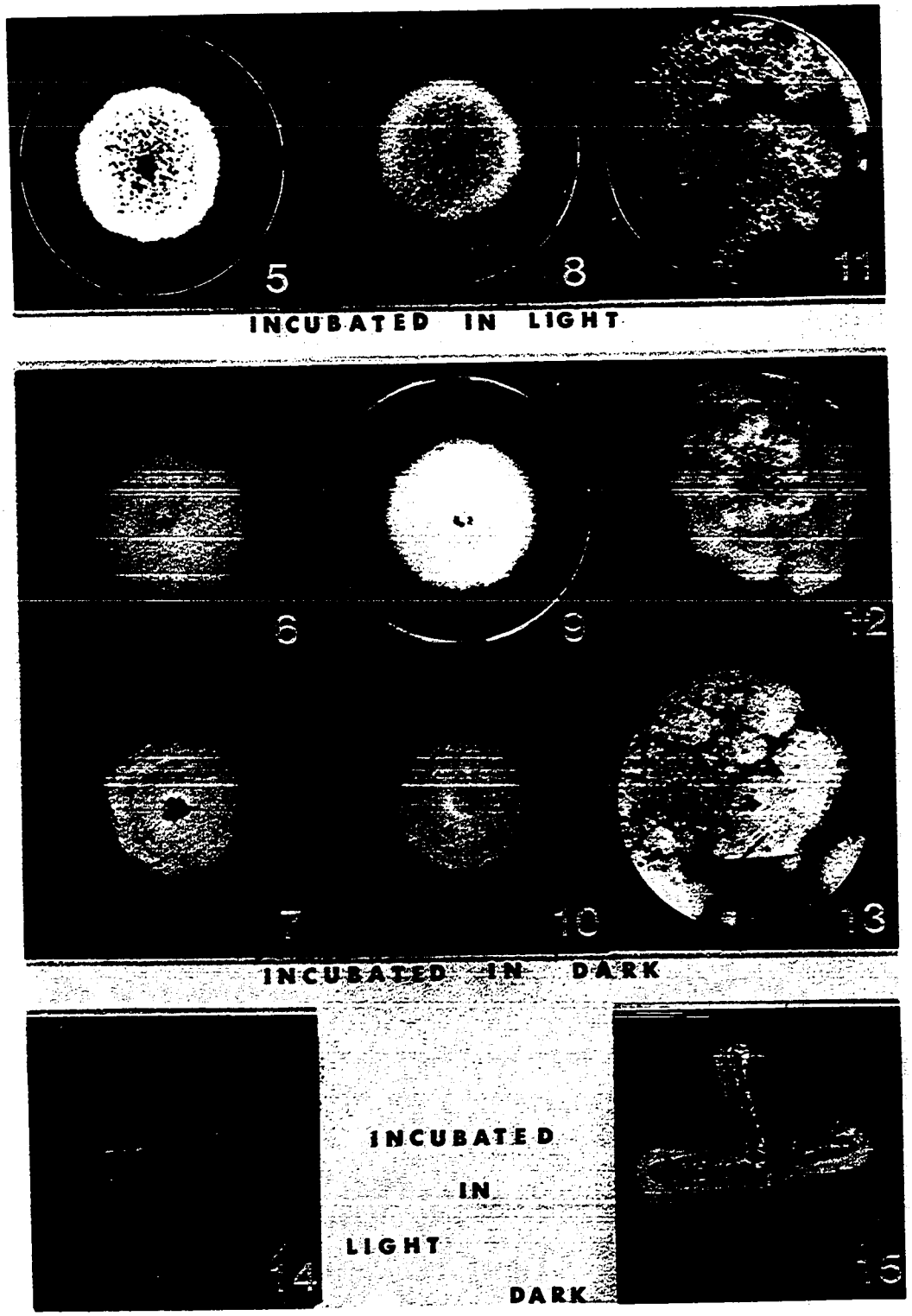
TABLE 7

Mycelial growth and pycnidial production by *P. meliloti*, isolate P_{30}, cultured in light and darkness for 15 days at 15°C on synthetic medium and synthetic medium plus 'active' and denatured horseradish peroxidase, *Aspergillus niger* catalase, and beef liver catalase preparations.

	Medium + H_2O (control)		Medium + peroxidase* activity 500 units/2.5 ml per Petri plate		Medium + fungal catalase* activity (units/2.5 ml per Petri plate)				Medium + beef liver catalase** activity (units/2.5 ml per Petri plate)					
					250		25		250		25		0.25	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark
Growth (dia. of colony, cm)	6.2	6.2												
'Active' enzyme		—	Pp. completely covered		Nil	Nil	0.8	1.3	6.1	6.2	Irregular, larger than controls		Irregular, larger than controls	
Denat. enzyme		—	6.1	6.4	2.7	1.7	6.4	6.1	6.3	6.1	6.0	6.2	6.0	6.1
Pycnidia per Petri plate	>100	Few on inoc. plug												
'Active' enzyme		—	>100	>100	Nil	Nil	6-8	Nil	>100	As for controls	>100	20-40	>100	40
Denat. enzyme		—	>100	Few on inoc. plug	Nil	Nil	>100	Nil	>100	"	>100	As for controls	>100	As for controls
Type of mycelial growth	Little aerial hyphae, grey-white	Abundant aerial hyphae, white												
'Active' enzyme		—	As for controls		Nil	Nil	Very little aerial hyphae, grey-white		As for controls		As for controls		As for controls	
Denat. enzyme		—	As for controls		Very little aerial hyphae grey-white		As for controls		As for controls		As for controls		As for controls	

* Average of three Petri plates for three replicates.

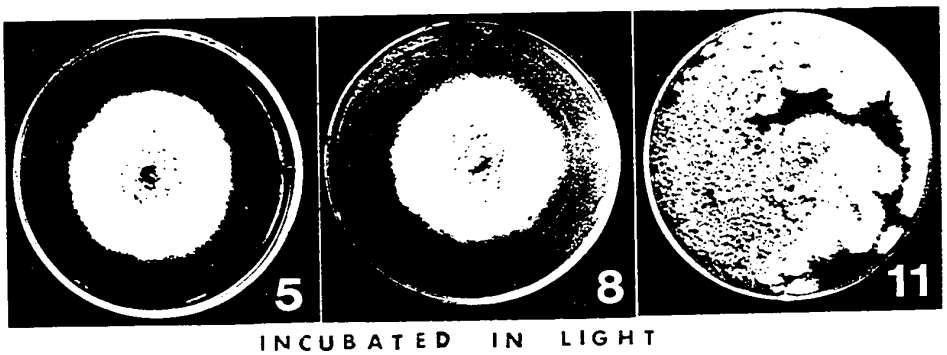
** Average of three Petri plates for two replicates.



Figs. 5-13. Effects of 'active' and denatured horseradish peroxidase on mycelial growth and pycnidia production by *P. meliloti* in light and darkness, relative to controls. Figs. 5,8,11. Light: control,with denatured and 'active' peroxidase respectively; numerous pycnidia present. Figs. 6,9. Dark: control and with denatured peroxidase respectively; pycnidia absent in growing colonies. Fig. 12. Dark: with 'active' peroxidase;large mycelial colony, numerous pycnidia under aerial hyphae. Figs. 7,10. Dark: control and with denatured peroxidase respectively; aerial hyphae removed by rubbing. Pycnidia absent in growing colonies. Fig.13. Dark: with 'active' peroxidase; colony rubbed to remove aerial hyphae to reveal pycnidia.

Fig.14. Light: 'active' peroxidase added to horizontal depression. Numerous pycnidia present.

Fig. 15. Dark: 'active' peroxidase added to three arms of depressions. Depressions rubbed to remove aerial hyphae to show numerous pycnidia.



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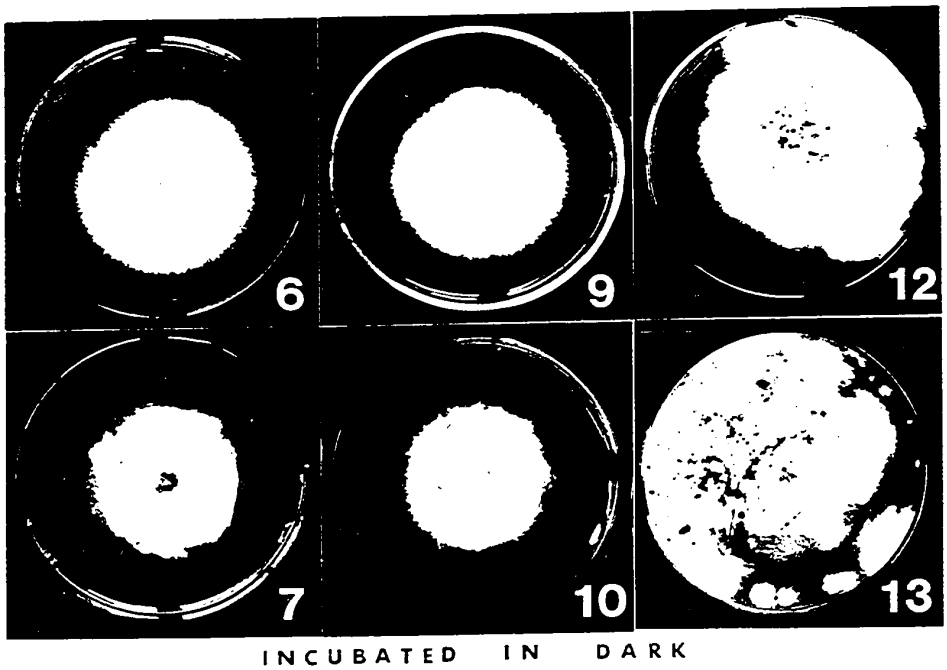

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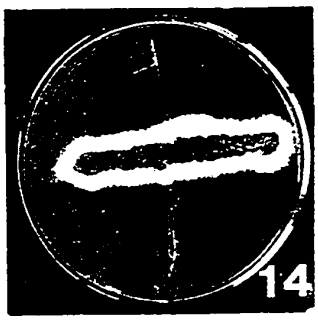

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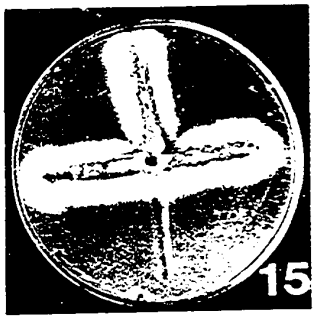


(Figs. 8-10).

To demonstrate further the effects of 'active horseradish peroxidase on the growth of mycelium and pycnidium production, intersecting depressions were made in the medium and 0.5 ml of 'active' horseradish peroxidase solution pipetted along each of either two or three arms of depressions. After time had elapsed for the peroxidase to be absorbed by the medium, an inoculum plug, 6 mm in diameter, taken from a culture grown in the light was placed at the intersection of the depressed areas. The Petri plates were kept at 15°C in the light or dark for 9 days. Mycelium and numerous pycnidia and numerous pycnidial initials were found principally along the depressions to which the peroxidase had been applied (Figs. 14 and 15). In Fig. 15, representing the dark treatment, the depressed area was rubbed with the forefinger to reveal the pycnidia which had been covered with a dense growth of aerial mycelium whereas in the light treatment (Fig. 14), numerous pycnidia and very little mycelium formed in the depressions to which peroxidase was added.

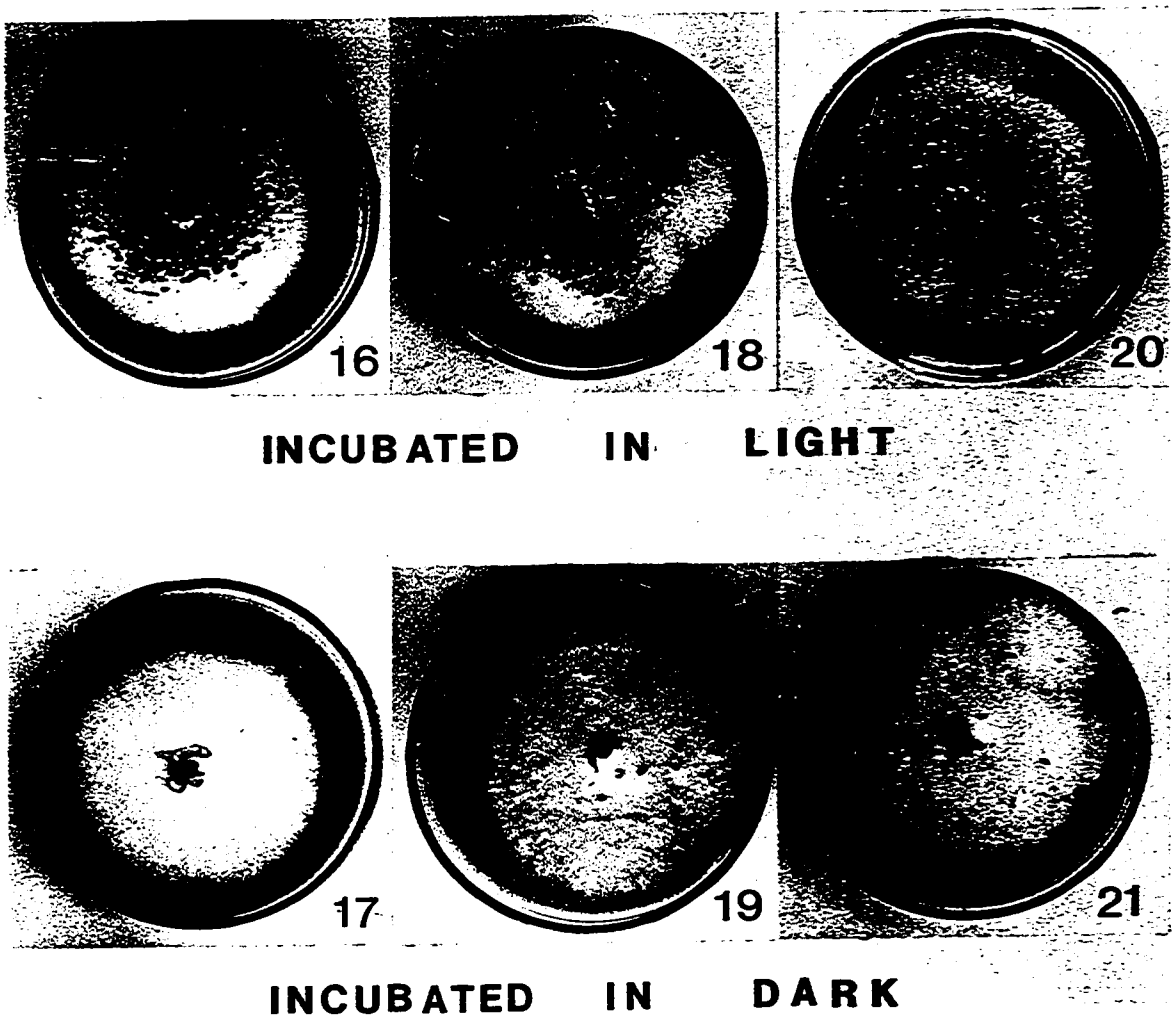
The concentration of horseradish peroxidase was found to affect production of pycnidia. Approximate numbers of pycnidia per Petri plate (average of three Petri plates) observed after 15 days incubation in the dark for preparations containing 2500, 1250, 625, 312, 156, and 78 units of activity per 2.5 ml per Petri plate were 50, 50, 100, 1100, 30, and 6 respectively. Type of mycelial growth for the above preparations was similar to that of controls and 'active' horseradish peroxidase for cultures incubated in darkness.

Mycelial growth and pycnidial production of isolate P_{20} was similar to that of P_{30} in the presence of 'active' horseradish peroxidase. Isolate P_{15} produced numerous pycnidia in the dark in the presence of 'active'

horseradish peroxidase; however, colony size was similar to the controls of P_{30} grown in light or darkness. The mycelial growth of isolate P_{10} was similar to that of P_{30} when cultured in the presence of 'active' horseradish peroxidase. P_{10} no longer produces pycnidia in light or darkness, but in the presence of 'active' horseradish peroxidase, it formed numerous parenchymatous knot-like bodies which resembled pycnidial initials. These structures did not show any further development but when cultures were kept for a long time, thus undergoing a natural desiccation, these areas were found to be full of salt crystals.

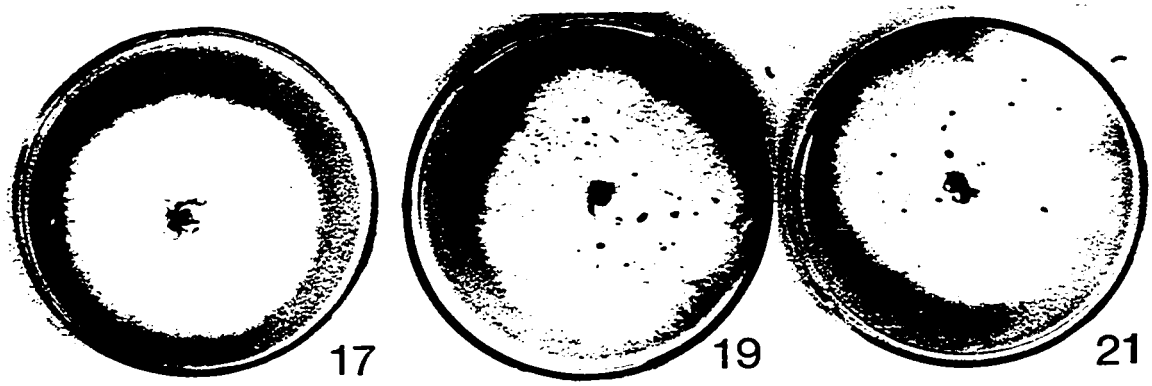
All colonies in the light produced numerous pycnidia in the presence of 'active' beef liver catalase (Figs. 18 and 20). Fewer pycnidia were formed in the dark than in the light when the lower concentrations of beef liver catalase were used (Figs. 19 and 21). At the highest concentration, 250 units per 2.5 ml of preparation and in the dark, a few pycnidia formed only on the inoculum plug. When the denatured enzyme was used, numerous pycnidia formed in all colonies in the light treatment, but only a few, if any, were observed in the dark treatment, and these were on the inoculum plugs. Surprisingly, mycelial colonies in the light and dark treatment, at the lower concentrations of the 'active' beef liver catalase, were slightly larger than those of the controls (Figs. 16-21), but not when the denatured enzyme was used.

Commercial *A. niger* catalase adversely affected mycelial growth and pycnidial production (Table 7 and Figs. 22-31). The mycelium on the inoculum plug appeared to be killed by 250 units per 2.5 ml aliquot of the 'active' fungal catalase in cultures incubated in the light or darkness (Figs. 24 and 25). There was only a slight growth of mycelium and no pycnidia formed in the presence of the denatured enzyme (Figs. 26 and 27). If the



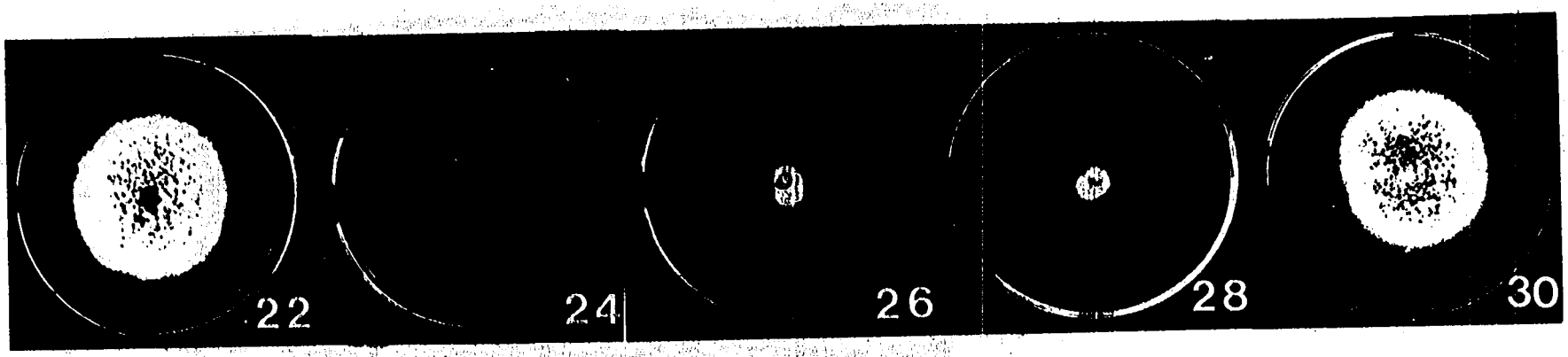
Figs. 16-21. Effects of 'active' beef liver catalase on mycelial growth and pycnidia production by *P. meliloti* in light and darkness, relative to controls. Figs. 16,17. Controls: light and dark respectively; numerous pycnidia in light, absent in growing colony in dark. Figs 18,19. Incubated in light and dark respectively with 'active' catalase (25 units/2.5 ml per Petri plate); numerous pycnidia in light, few in dark. Figs. 20,21. Light and dark respectively with 'active' catalase (0.25 units/2.5 ml per Petri plate); numerous pycnidia in light, few in dark. In Figs.19 and 21 aerial mycelium removed by rubbing to show pycnidia.

INCUBATED IN LIGHT



INCUBATED IN DARK

Figs. 16-21. Effects of 'active' beef liver catalase on mycelial growth and pycnidia production by *P. meliloti* in light and darkness, relative to controls. Figs. 16,17. Controls: light and dark respectively; numerous pycnidia in light, absent in growing colony in dark. Figs 18,19. Incubated in light and dark respectively with 'active' catalase (25 units/2.5 ml per Petri plate); numerous pycnidia in light, few in dark. Figs. 20,21. Light and dark respectively with 'active' catalase (0.25 units/2.5 ml per Petri plate); numerous pycnidia in light, few in dark. In Figs.19 and 21 aerial mycelium removed by rubbing to show pycnidia.



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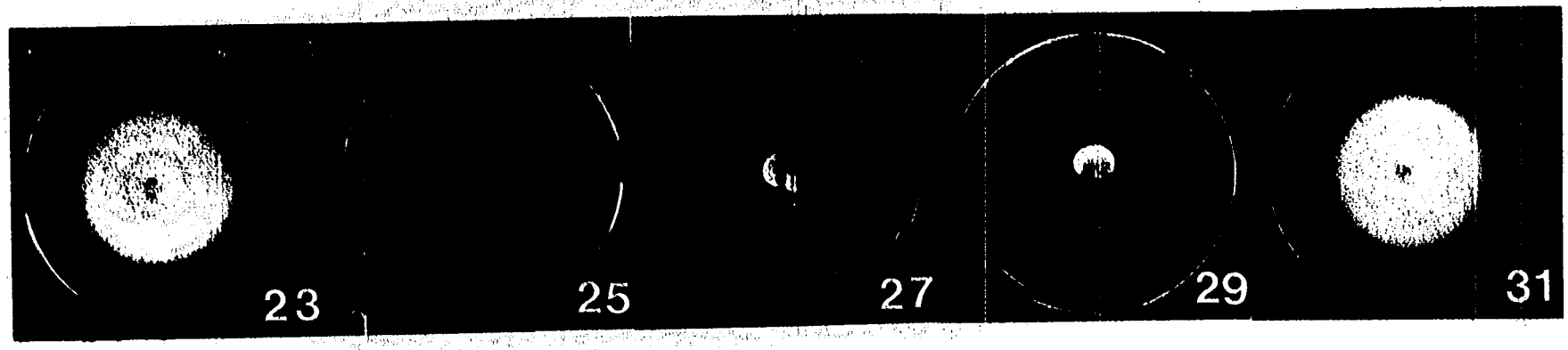
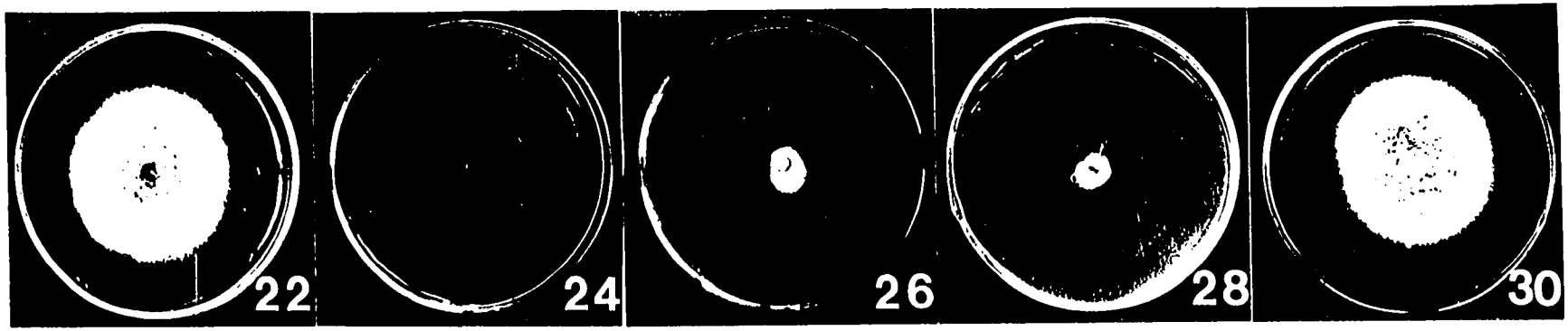
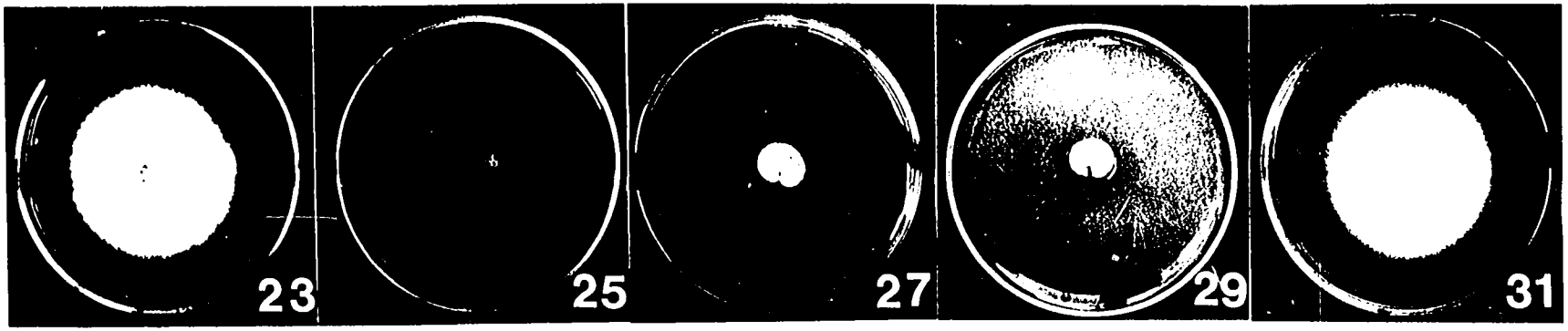


INCUBATED IN DARK

Figs. 22-31. Effects of 'active' and denatured *A. niger* catalase on mycelial growth and pycnidia production by *P. meltlott* in light and darkness, relative to controls. Figs. 22,23. Controls: light and dark respectively; numerous pycnidia in light, absent in growing colony in dark. Figs. 24,26,28,30. Incubated in light with corresponding treatments in Figs. 25,27,29,31 but incubated in darkness. Fig.24.With 'active'catalase (250 units/2.5 ml per Petri plate); growth inhibited. Fig. 26. With denatured catalase (autoclaved solution used for Fig.24); slight growth, pycnidia absent. Fig. 28. With 'active' catalase (25 units/2.5 ml per Petri plate); slight growth, few pycnidia on inoculum plug. Fig.30. With denatured catalase (autoclaved solution used for Fig.28); similar to control.



INCUBATED IN LIGHT



INCUBATED IN DARK

Figs. 22-31. Effects of 'active' and denatured *A. niger* catalase on mycelial growth and pycnidia production by *I. melleti* in light and darkness, relative to controls. Figs. 22,23. Controls: light and dark respectively; numerous pycnidia in light, absent in growing colony in dark. Figs. 24,26,28,30. Incubated in light with corresponding treatments in Figs. 25,27,29,31 but incubated in darkness. Fig.24.With 'active'catalase (250 units/2.5 ml per Petri plate); growth inhibited. Fig. 26. With denatured catalase (autoclaved solution used for Fig.24); slight growth, pycnidia absent. Fig. 28. With 'active' catalase (25 units/2.5 ml per Petri plate); slight growth, few pycnidia on inoculum plug. Fig.30. With denatured catalase (autoclaved solution used for Fig.28); similar to control.

protein of the 'active' catalase enzyme was solely responsible for the inhibition of growth of mycelium then Figs. 26 and 27, representing colonies grown on media containing the denatured enzyme, should be comparable to Figs. 30 and 31, which represent the growth of cultures on denatured catalase but at a lower catalase concentration. Therefore, some factor other than protein in the denatured concentrated preparation was involved in the inhibition of mycelial growth. At the lower concentration of 25 units per 2.5 ml of 'active' fungal catalase, slight growth of mycelium occurred (Figs. 28 and 29). Several pycnidia were found around the inoculum plugs of colonies grown in light (Fig. 28) but none in the dark treatment (Fig. 29). Mycelial growth and pycnidial production for colonies grown on medium containing the dilute denatured catalase preparation in light or dark treatments (Figs. 30 and 31) were similar to those for the corresponding controls (Figs. 22 and 23).

When the flocculent material, noted in the denatured concentrated *A. niger* catalase preparation, was separated from the liquid portion by centrifugation and each used to determine its effect on mycelial growth and pycnidial production, the inhibitory factor was found to be in the supernatant fraction (Table 8). However, the degree of inhibition of mycelial growth was not as great as when the combined fractions were used. Pycnidia were not formed in either the light or dark treatments. In the presence of the precipitate, the cultures were similar regarding the growth of mycelium and production of pycnidia to those of controls (Figs. 5 and 6). The inhibitory factor, at the concentration used, may be the co-enzyme since the co-enzyme of a bacterial catalase was found to be thermostable while the apoenzyme was thermolabile (Bingold and Lang, 1955).

TABLE 8

Mycelial growth and pycnidium production by *Plenodomus meliloti*, isolate P_{30}, cultured in light and darkness for 15 days at 15°C on synthetic medium and synthetic medium plus autoclaved supernatant and precipitate fractions of *Asperfillus niger* catalase.*

	Medium + H_2O (control)		Medium + autoclaved supernatant				Medium + autoclaved precipitate			
			Stock soln.**		Stock soln. diluted 10 X		Stock soln.**		Stock soln. diluted 10 X	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark
Growth (dia. of colony, cm)	6.3	6.3	4.0	4.2	6.1	6.4	6.4	6.4	6.3	6.4
Pycnidia/ Petri plate	>100	Nil	Nil	Nil	>100	Nil	>100	Nil	>100	Nil
Type of mycelial growth	Aerial hyphae little, grey-white	Aerial hyphae abundant, white	Little growth, dirty white	As for control	As for controls	As for controls	As for controls	As for controls	As for controls	As for controls

* Average of three Petri plates for one replicate.

** Stock soln. - *Aspergillus niger* catalase activity of 250 units per 2.5 ml prior to autoclaving.

The effects of the protein and co-enzyme fractions of commercial *A. niger* catalase, separated using Theorel's method (1947), on mycelial growth and pycnidial production were also noted. The results are presented in Table 9 and Figs. 32-37. From these it may be noted that in the presence of fungal catalase protein (Figs. 34 and 35) the growth pattern was like controls (Figs. 32 and 33), although the rate of growth was a little slower. However, growth was not completely inhibited as it was when the intact enzyme was added on top of the medium. In the presence of protohaematin (Figs. 36 and 37), in comparison to the controls, the growth rate was slower, the mycelium appeared stunted and the production of pycnidia was completely inhibited, even when the cultures were incubated in light. When an attempt was made to regenerate the enzyme by combining the protein and protohaematin fractions, the type of mycelial growth closely resembled the cultures grown in the presence of protohaematin alone (Figs. 36 and 37). In these treatments which included intact catalase enzyme protein, protohaematin, and a solution containing both the protein and protohaematin another point of interest noted was the greater growth in cultures incubated in the dark than in the light (Table 9).

'Active' horseradish peroxidase stimulated production of pycnidia in the dark even in the presence of the fungal catalase at the lower enzyme concentrations (Table 10). On the other hand, fungal catalase inhibited mycelial growth but the degree of inhibition was not as great as that observed when catalase alone was used (Tables 7 and 10).

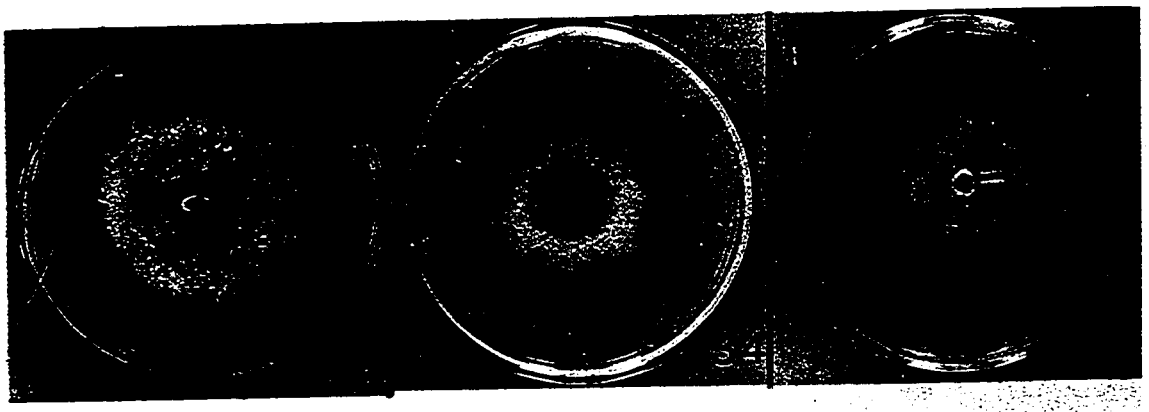
Polyphenol oxidase also caused the formation of many pycnidia in cultures incubated in darkness. However, the numbers were fewer than observed for cultures containing horseradish peroxidase. In the presence of cellulase no pycnidia were produced in the dark and the colony characteris-

TABLE 9

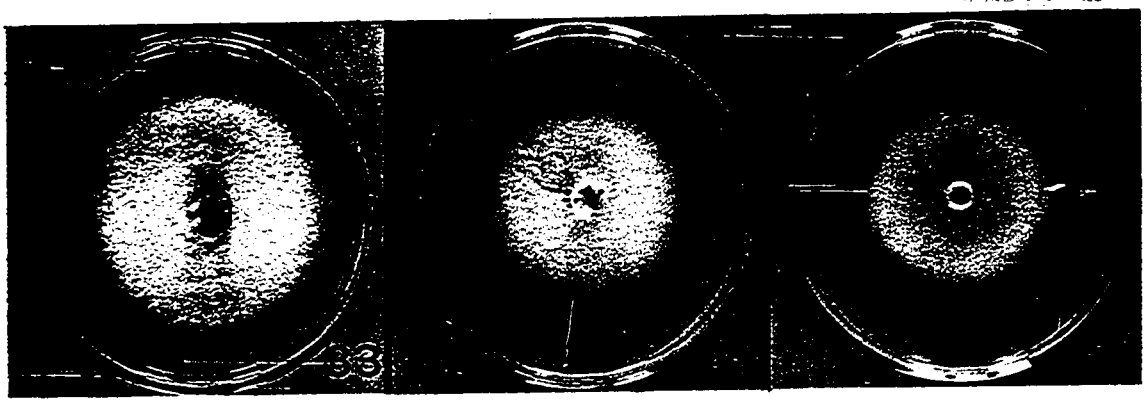
Mycelial growth and pycnidial production by *Plenodomus meliloti*, isolate P_{30}, cultured in light and darkness for 15 days at 15°C on synthetic medium and synthetic medium plus fungal catalase protein, protohaematin, and protein + protohaematin fractions.

	Medium + H_2O (control)		Medium + 'active' enzyme		Medium + protein		Medium + protohaematin		Medium + protein + protohaematin	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark
Growth* (dia. of colony, cm)	6.35	6.3	Nil	Nil	4.9	5.6	4.5	5.4	4.6	5.4
Pycnidia/ Petri plate	>100	Nil	Nil	Nil	>100	Few on inoc. plug	Not even on inoc. plug	Not even on inoc. plug	Not even on inoc. plug	Not even on inoc. plug
Type of mycelial growth	Aerial hyphae little, grey-white	Aerial growth abundant, white	-	-	As for control	As for control	Limited aerial growth, wrinkle-like depressions in colony	Little aerial growth, looks stunted	Little aerial growth, looks stunted	Little aerial growth, looks stunted

* Average of three Petri plates for one replication.

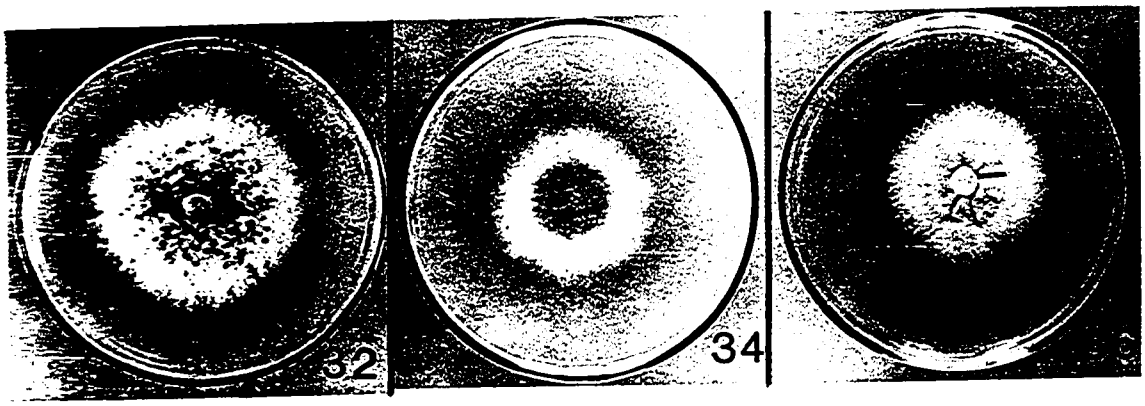


INCUBATED IN LIGHT



INCUBATED IN DARK

Figs. 32-37. Effects of protein and protohaematin fractions of *A. niger* catalase on mycelial growth and pycnidia production by *P. meliloti* in light and darkness, relative to controls. Figs. 32,33. Controls: light and dark respectively; numerous pycnidia in light, absent in growing colony in dark. Figs. 34,35. Incubated in light and dark respectively with protein fraction of *A. niger* catalase; numerous pycnidia in light, absent in growing colony in dark. Figs. 36,37. Incubated in light and dark respectively with protohaematin fraction of *A. niger* catalase; pycnidia absent in light as well as in dark.



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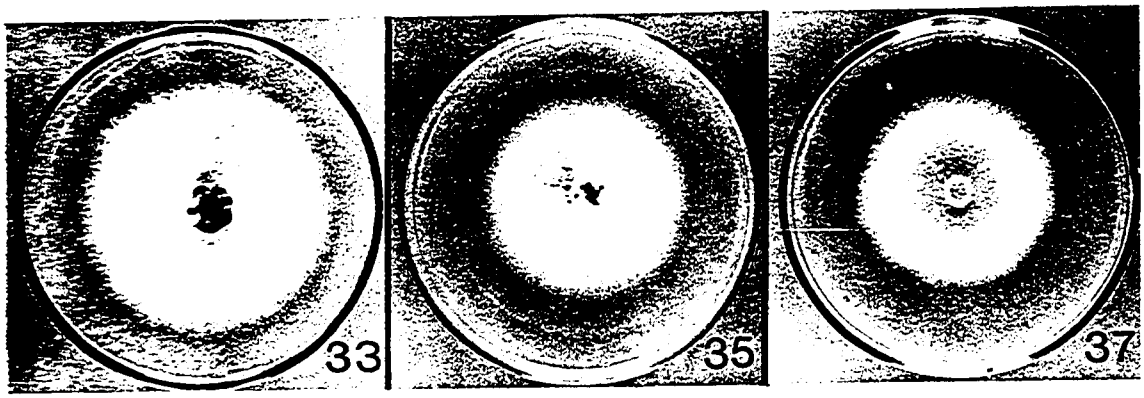


INCUBATED IN DARK

Figs. 32-37. Effects of protein and protohaematin fractions of *A. niger* catalase on mycelial growth and pycnidia production by *P. meliloti* in light and darkness, relative to controls. Figs. 32,33. Controls: light and dark respectively; numerous pycnidia in light, absent in growing colony in dark. Figs. 34,35. Incubated in light and dark respectively with protein fraction of *A. niger* catalase; numerous pycnidia in light, absent in growing colony in dark. Figs. 36,37. Incubated in light and dark respectively with protohaematin fraction of *A. niger* catalase; pycnidia absent in light as well as in dark.

TABLE 10

Mycelial growth and pycnidium production of *P. meliloti*, isolate P_{30}, cultured in light and darkness for 15 days at 15°C on synthetic medium and synthetic medium plus a mixture of horseradish peroxidase and *Aspergillus niger* catalase.*

	Medium + H_2O		Medium + peroxidase and catalase			
			concn.: stock** sol'n		concn.: stock sol'n dil. 10 X	
	Light	Dark	Light	Dark	Light	Dark
Growth (dia. of colony, cm)	6.4	6.3				
'Active' enzymes		-	1.7	2.1	5.0	5.0
Denat. enzymes		-	4.3	4.2	6.2	6.2
Pycnidia/Petri plate	>100	Few on inoc. plug				
'Active' enzymes		-	Nil	Nil	>100	40-50
Denat. enzymes		-	Nil	Nil	>100	Few on inoc. plug
Type of mycelial growth	Grey-white	White abundant aerial hyphae				
'Active' enzymes		-	White	White	As for controls	As for controls
Denat. enzymes		-	Yellow brown	Pale brown	As for controls	As for controls

* Average of three Petri plates for two replicates.

** Peroxidase activity, 500 units/2.5 ml per Petri plate. Catalase activity, 250 units/2.5 ml per Petri plate.

tics were similar to controls. β-glucosidase also did not affect rate of growth, but did induce pycnidial formation in the dark to a very little extent. The maximum number of pycnidia observed was 37.

Negative results for the presence of hydrogen peroxide were obtained in cultures incubated in either light or darkness using Whittenbury's method (1964), which was used for detecting peroxide in bacterial cultures. In the presence of this medium which contained MnO_2 the mycelial growth was slightly greater than the controls (0.3-0.5 cm); otherwise, the type of mycelial growth was similar to controls, and 5-6 pycnidia were present in cultures incubated in darkness. Similar results were obtained in the presence of haematin medium prepared according to the method described by Whittenbury (1964). The colonies had 8-12 pycnidia per Petri plate. One very significant observation noted for cultures grown in the haematin medium was that the pycnidia matured much faster than the controls. In 15-day old cultures the majority of the pycnidia had already released pycnospores whereas, in 15-day old control cultures rarely did the pycnidia produce pycnospores.

e. Disc Gel Electrophoresis of Proteins and Peroxidase Isozymes of Fungal Extracts

The protein and peroxidase patterns of cell-free mycelial extracts obtained on polyacrylamide gels are shown in Figs. 38 and 39 . Proteins and isozyme bands varied in their staining intensities; therefore, an arbitrary scheme was used to designate the relative stain uptake by various protein and isozyme bands. Patterns on the gels are shown in line drawings and not as photographs because good photographic definitions of the lightly staining protein and isozyme bands in the photographic prints was not possible. Prominent bands are shown as solid lines, faint bands as broken lines, and diffuse zones as diagonal lines.

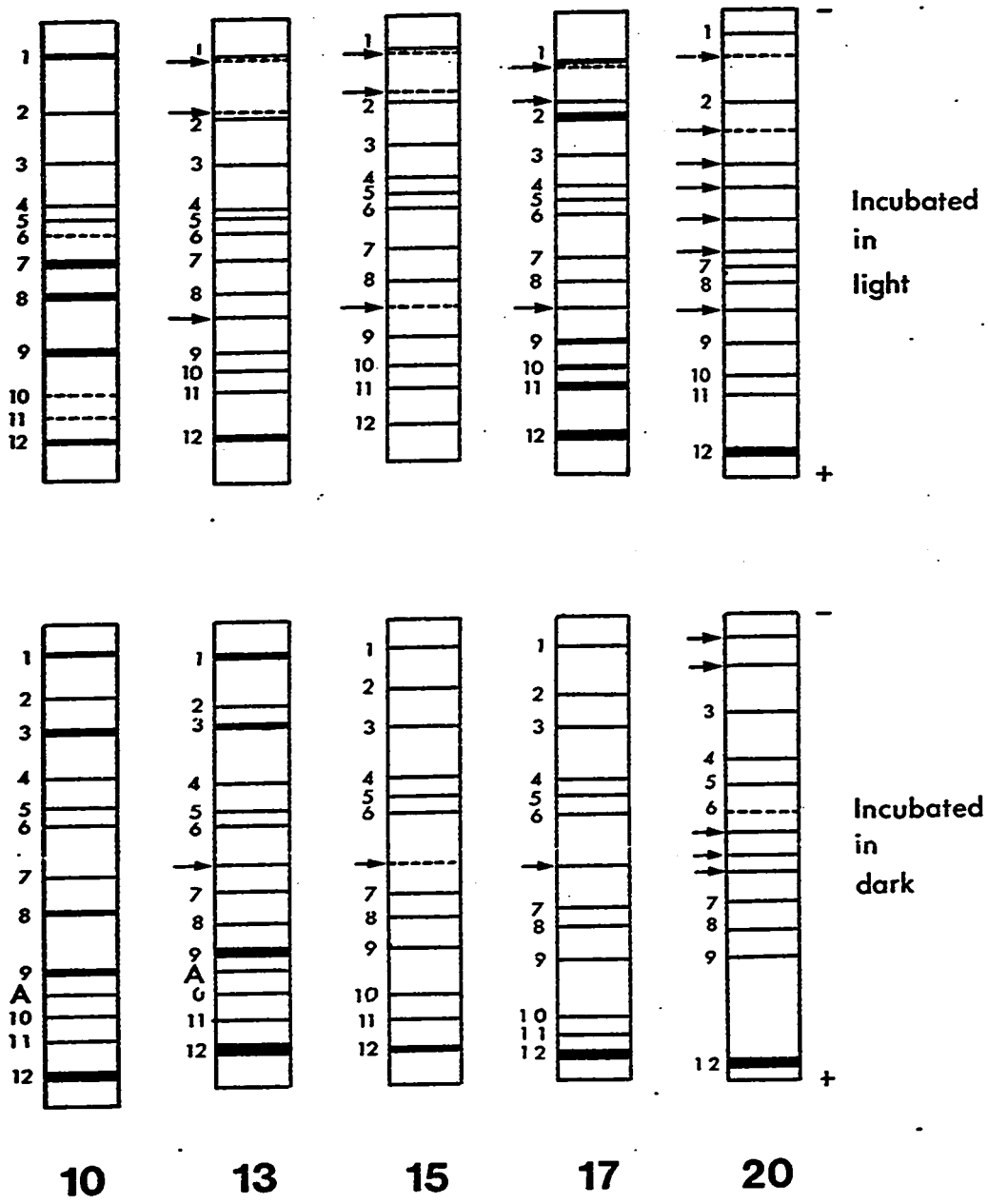


Figure 38. Disc gel electrophoresis of soluble proteins of *P. meliloti* cultures incubated in light or darkness in relation to their age. Numbered bands appeared consistently; arrows indicate bands not found in 10-day old cultures. Band A present only in cultures incubated in the dark. Relative intensities of stain uptake: Solid lines, prominent bands, broken lines, faint bands.

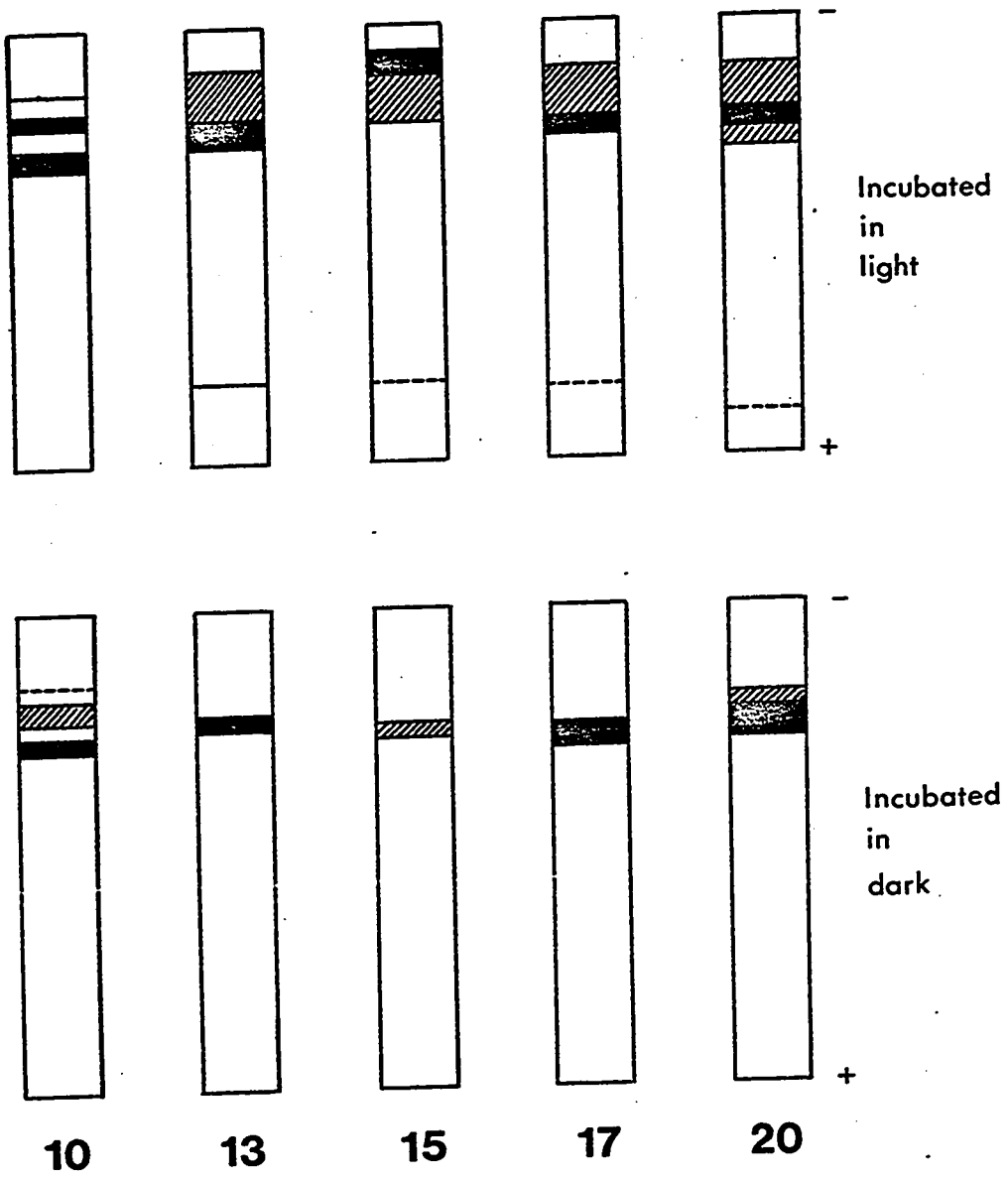


Figure 39. Disc gel electrophoresis patterns of peroxidase isozymes of *P. meliloti* cultures incubated in light and darkness with relation to their age.

To help interpret the protein patterns on the gels, the different protein bands were numbered with Arabic numerals (Fig. 38). All those bands which appeared to have moved the same distance and had the same position relative to other bands on the different gels were tentatively given the same number for both light and dark treatments and from the mycelial extracts obtained from the different ages of cultures.

It may be noted that mycelial extracts from cultures incubated in the light show more changes in the numbers of bands and their positions than did the cultures incubated in darkness. The numbers of protein bands from mycelial extracts of cultures grown at 15°C and harvested 10, 13, 15, 17, and 20 days after inoculation were 12, 15, 15, 15, and 15 for light and 13, 14, 13, 13, and 13 for dark treatments respectively. It may be mentioned here that, excepting 10-day old cultures, all the other harvests from cultures incubated in the light consisted of a mixture of fungal mycelium and pycnidia. Pycnidia were not present in cultures incubated in darkness. The protein patterns for 10-day old cultures, for both light and dark treatments, revealed similar protein patterns except the presence of an additional protein band marked A in mycelial extracts from cultures incubated in the dark. 13-, 15-, and 17-day old cultures incubated in light had three bands more than the 10-day old cultures, and there was an increase of only one protein band for cultures of the same age incubated in the dark. Protein bands of 20-day old cultures kept in light or darkness differed considerably from those already described. Protein bands present in the younger cultures could not be seen on the gels, and new protein bands appeared which were not noticed previously. Therefore, corresponding numbers could not be assigned to protein bands with certainty due to the appearance and disappearance of one or more protein bands in cultures of various ages. All those bands which

were not present in 10-day old culture mycelial extracts and those whose position could not be established are denoted by arrows.

Disc gel electrophoretic patterns for peroxidase isozymes, as indicated by staining with benzidine·2HCl reagent for cultures incubated in light or darkness for the same periods as mentioned for protein pattern determinations, are shown in Fig. 39. 10-day old cultures for both light and dark treatments had a similar isozyme pattern except for a less intense reaction with extracts from dark- than light-incubated cultures. Cultures older than 10 days, and incubated in the light, always had more isozyme bands than the cultures grown in the dark.

f. Disc Gel Electrophoresis for Protein Patterns of Horseradish Peroxidase, Fungal Catalase, and Beef Liver Catalase

The disc gel electrophoretic patterns for horseradish peroxidase, fungal catalase, and beef liver catalase are shown in Fig. 40. These analyses show that all these enzymes have different protein patterns and even the protein patterns of the two catalases differ considerably.

B. STUDIES ON THE HOST PLANT

a. Temperature Recordings

Minimum and maximum soil temperatures at a depth of 13 cm from the period August 30, 1968 to June 17, 1969 are shown in tabular form in Appendix (see page A-2). Except for October 17, and 18, 1968 the minimum soil temperature was $0^{o}C$ or below continuously for six months from October 14, 1968 to April 15, 1969. The lowest soil temperatures observed during the period January 8, 1969 to February 2, 1969 were between $-8.5^{o}C$ to $-9.5^{o}C$. On January 23, 29, 30, and 31, 1969, minimum soil temperature reading of $-9.5^{o}C$ was recorded. The first snowfall (0.8 inch) was on

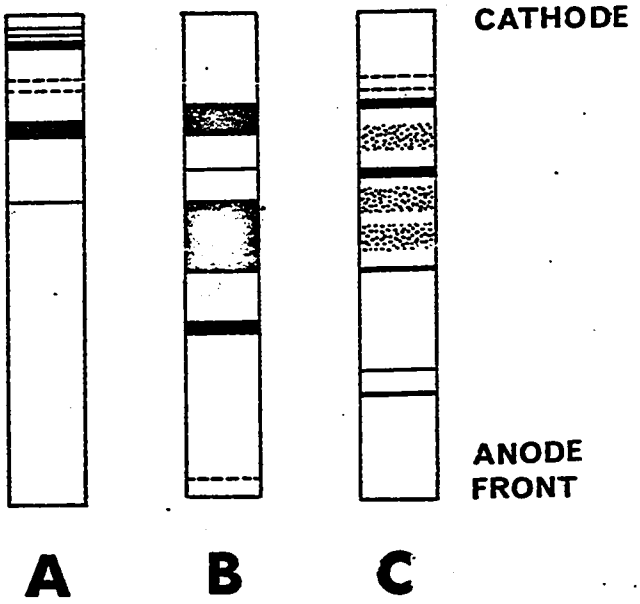


Figure 40. Disc gel electrophoretic protein patterns of (A) commercial horseradish peroxidase. (B) *Aspergillus niger* catalase. (C) beef liver catalase.

September 21, 1968 and subsequent snowfalls until December 5, 1968 amounted from trace amounts to 0.5 inch, but none of which stayed permanently on the ground. On December 5, a snowfall of 1.8 inches was recorded, and the plants were covered permanently with snow from this date until April 8, 1969.

b. Morphological Observations

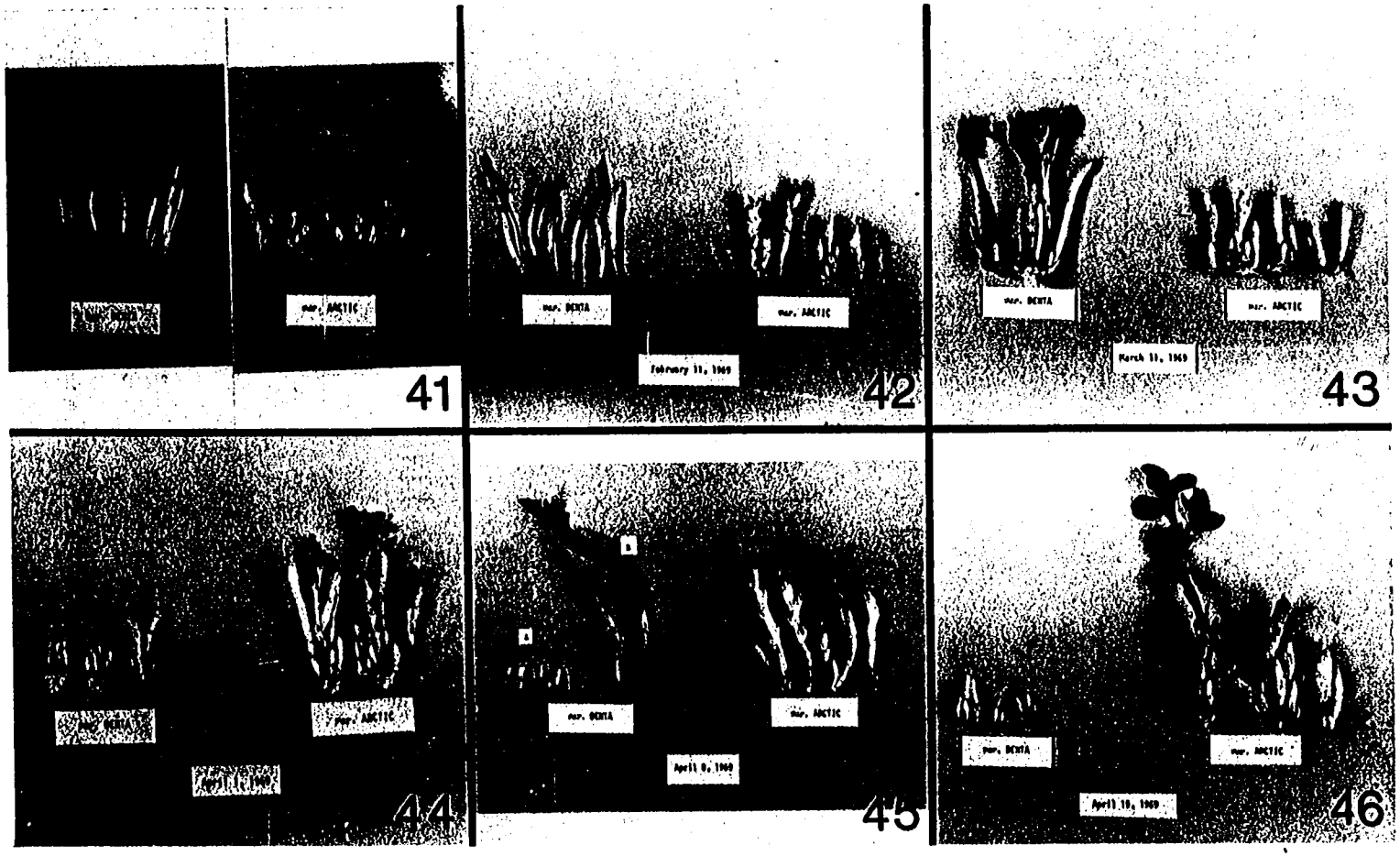
Morphological changes of the buds of both sweet clover varieties occurred during the winter months. The buds of variety Denta (non-winter hardy and susceptible to *P. meliloti*) were found to be growing i.e. elongating and differentiating in January, 1969, whereas, those of the variety Arctic (winter-hardy and resistant to *P. meliloti*) remained dormant until much later (Figs. 41 - 46). On April 1, 1969, for the first time, the buds of the variety Arctic were noticed to be elongating (Fig. 44). At this time the growth of Denta resembled young shoots, and a second set of buds had started to emerge (Figs. 44 and 45). The growth of these new buds was much slower as compared to those of the variety Arctic.

c. Dry Weight Determinations

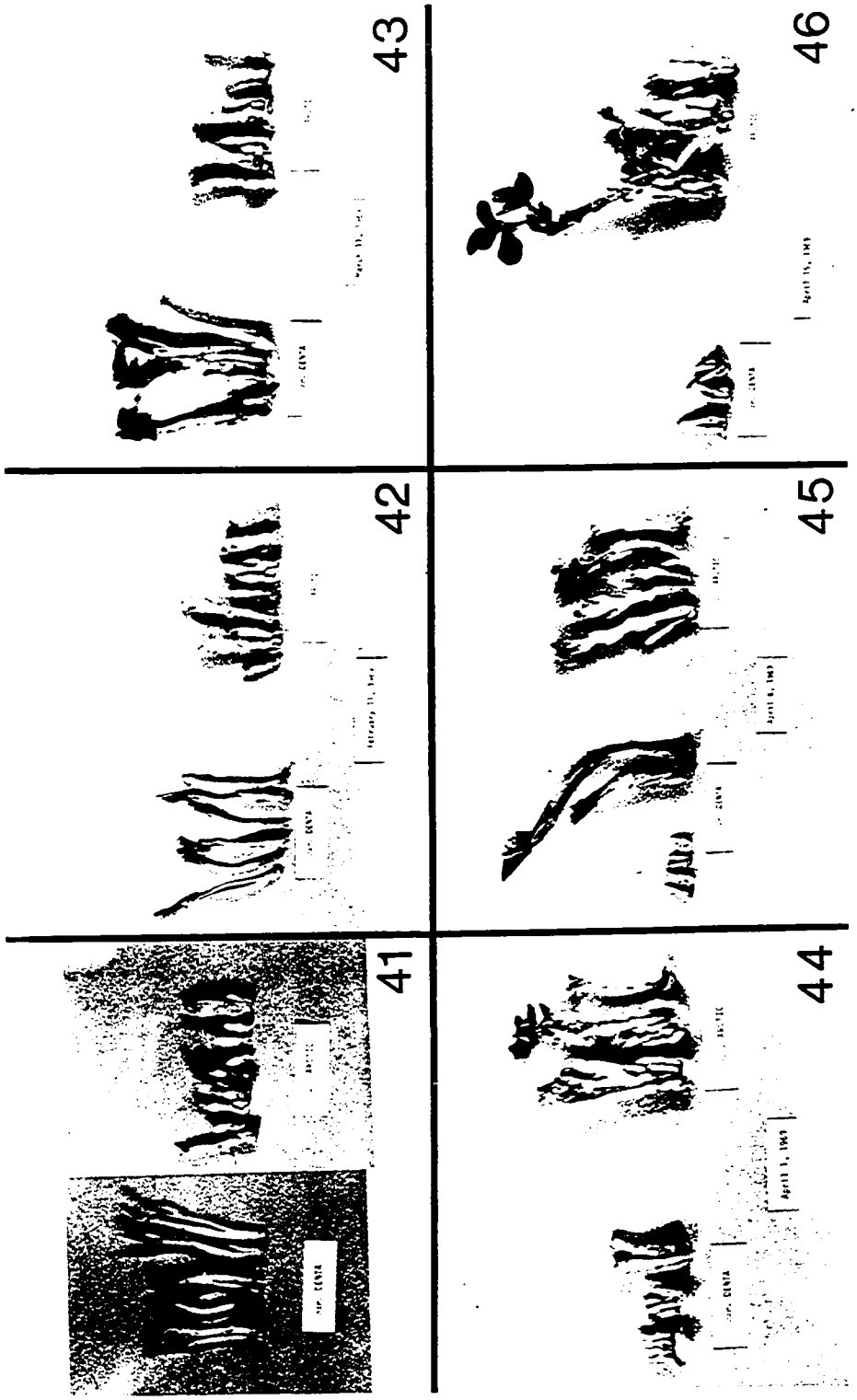
The results of dry weight determination, on a unit basis, of secondary roots of both sweet clover varieties showed that the dry weights were higher during the winter than the spring periods (Fig. 47).

d. Total Protein Determinations

The results of total protein determinations for cell-free extracts of sweet clover roots harvested at various time intervals during 1968-69, are presented in Figs. 48 and 49. When the quantitative protein determinations were based on dry weight or fresh weight, the total protein values for Arctic were always found to be much higher than for Denta. However, when the calculations were based upon dry weights, the differences in the



Figs. 41-46. Buds of field-grown sweet clover varieties Denta and Arctic at various stages of growth. Figs. 41,42,43. Buds harvested on January 14, February 11, and March 11,1969. Figs. 44,45,46. Buds harvested on April 1,8,15,1969; buds of variety Denta in two stages of growth, A- showing no morphological change over the previous harvests, B- already transformed into young shoots.



Figs. 41-46. Buds of field-grown sweet clover varieties Denta and Arctic at various stages of growth. Figs. 41,42,43. Buds harvested on January 14, February 11, and March 11,1969. Figs. 44,45,46. Buds harvested on April 1,8,15,1969; buds of variety Denta in two stages of growth, A- showing no morphological change over the previous harvests, B- already transformed into young shoots.

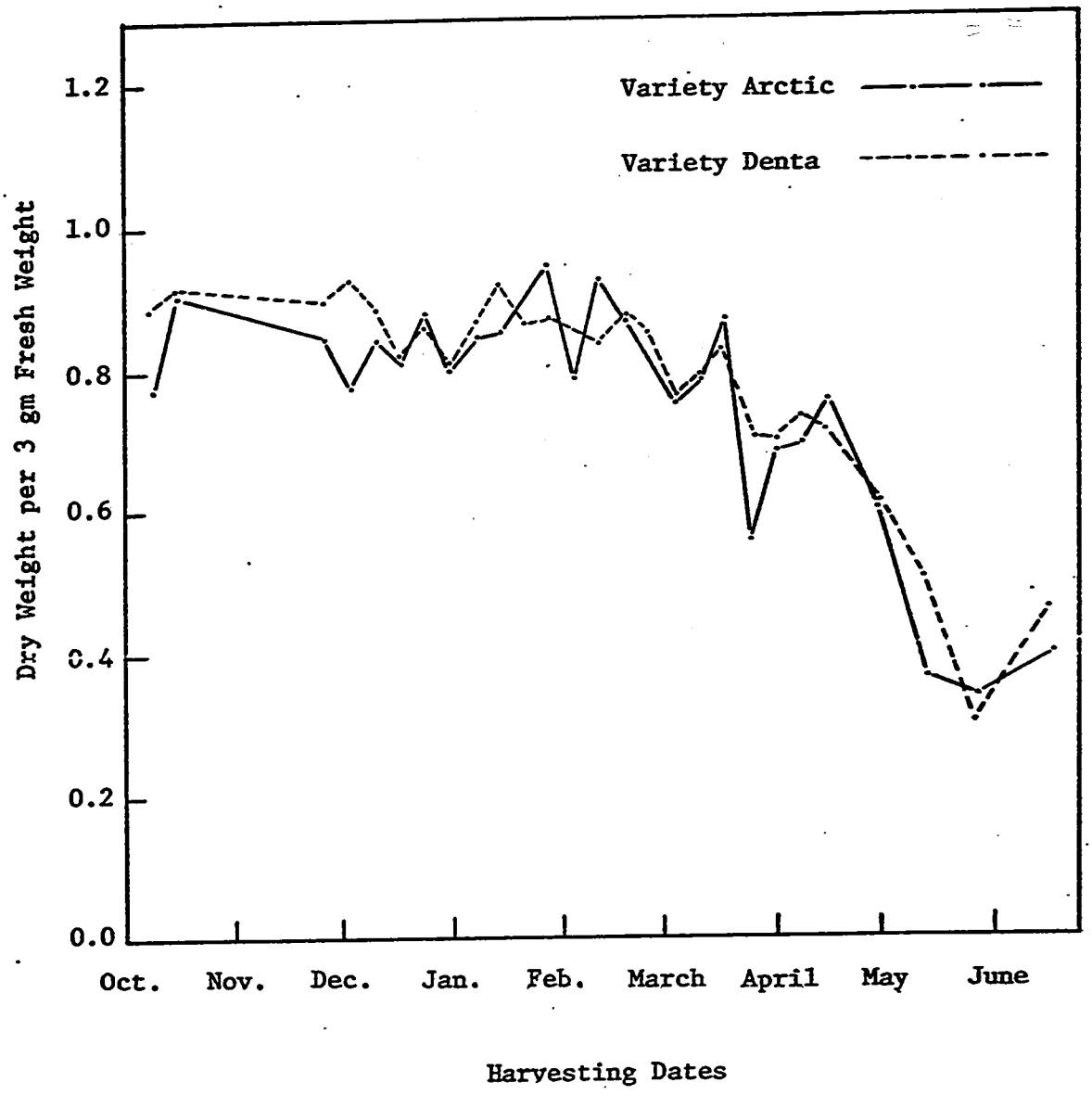


Figure 47. Dry weight changes per unit fresh weight in sweet clover roots of varieties Arctic and Denta between October 8, 1968 and June 17, 1969.

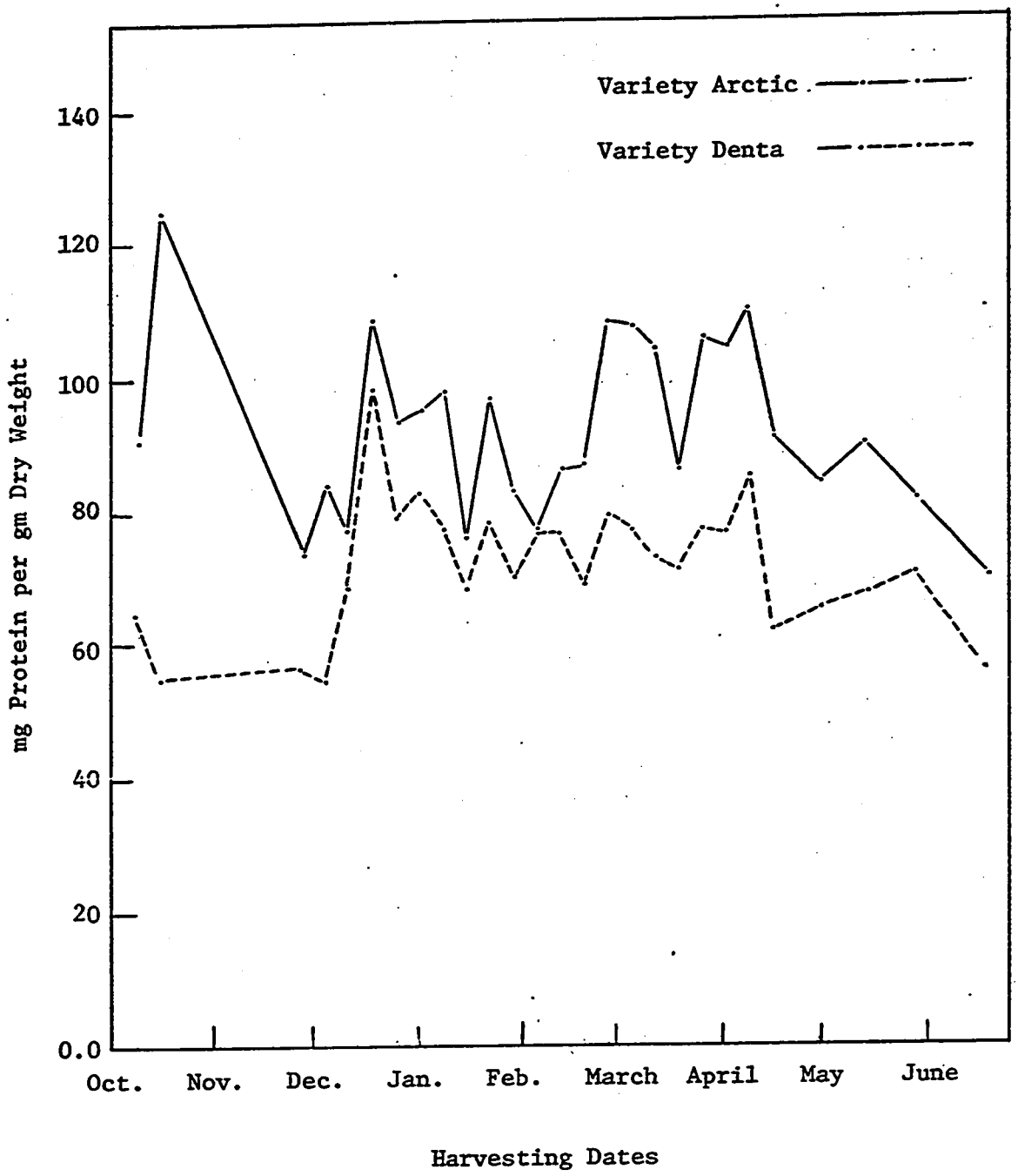


Figure 48. Levels of protein per gram dry weight in extracts from sweet clover roots of varieties Arctic and Denta harvested at various intervals between October 8, 1968 and June 17, 1969.

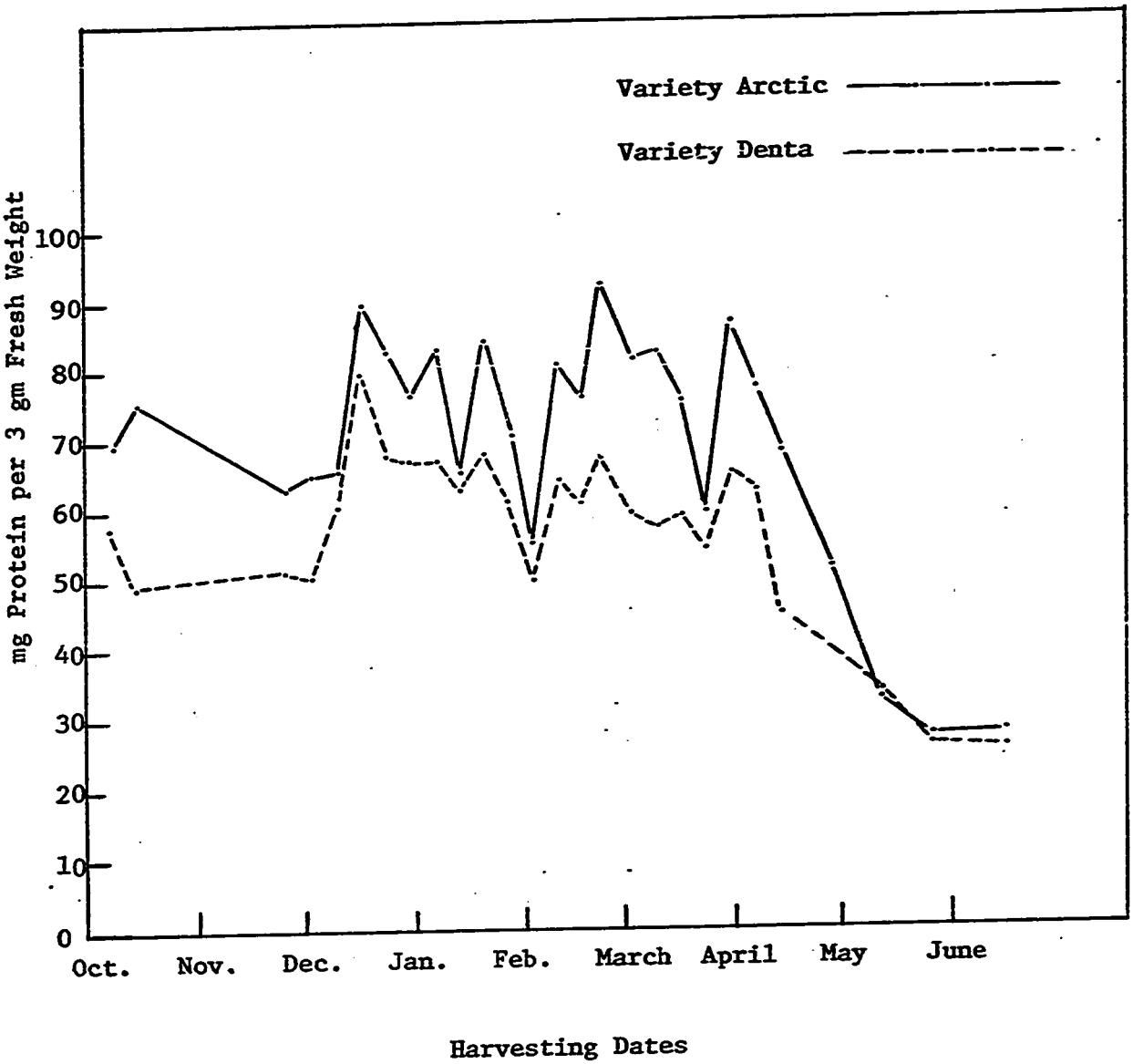


Figure 49. Levels of soluble proteins per gram fresh weight in extracts from sweet clover roots of varieties Arctic and Denta harvested at various intervals between October 8, 1968 and June 17, 1969.

total protein values for roots of both varieties harvested during the winter and those harvested in the spring were not very appreciable. On the other hand when the calculations were based on fresh weight basis, the protein values were found to be higher during winter and much lower during the spring.

e. Peroxidase Activity

The results for peroxidase activity in the cell-free extracts of sweet clover roots at various intervals from October 8, 1968 to June 17, 1969 are shown in Fig. 50. Peroxidase activities were found to be only slightly higher in variety Denta as compared to the variety Arctic. Twice during the study peroxidase activity increased much above that generally observed. The first sharp increase coincided at the time when the buds of Denta first started to grow i.e. a marked elongation of the buds was noted over the previous harvest and the second increase occurred with a similar growth of the buds of Arctic.

f. Catalase Activity

Several significant factors may be emphasized from the data on catalase activity of cell-free extracts of both varieties of sweet clover roots harvested at various intervals during 1968-69 (Fig. 51). Catalase activity was much higher in roots of Arctic as compared to Denta. Catalase activity for roots of both varieties harvested during winter was higher than for roots which were harvested either during the fall or the spring periods. A sharp increase of catalase activity was noticed during mid-March in the variety Arctic, and for this period the variety Denta did not show any such increase.

g. Polyphenol Oxidase Activity

Polyphenol oxidase activities (as catechol oxidase) were always

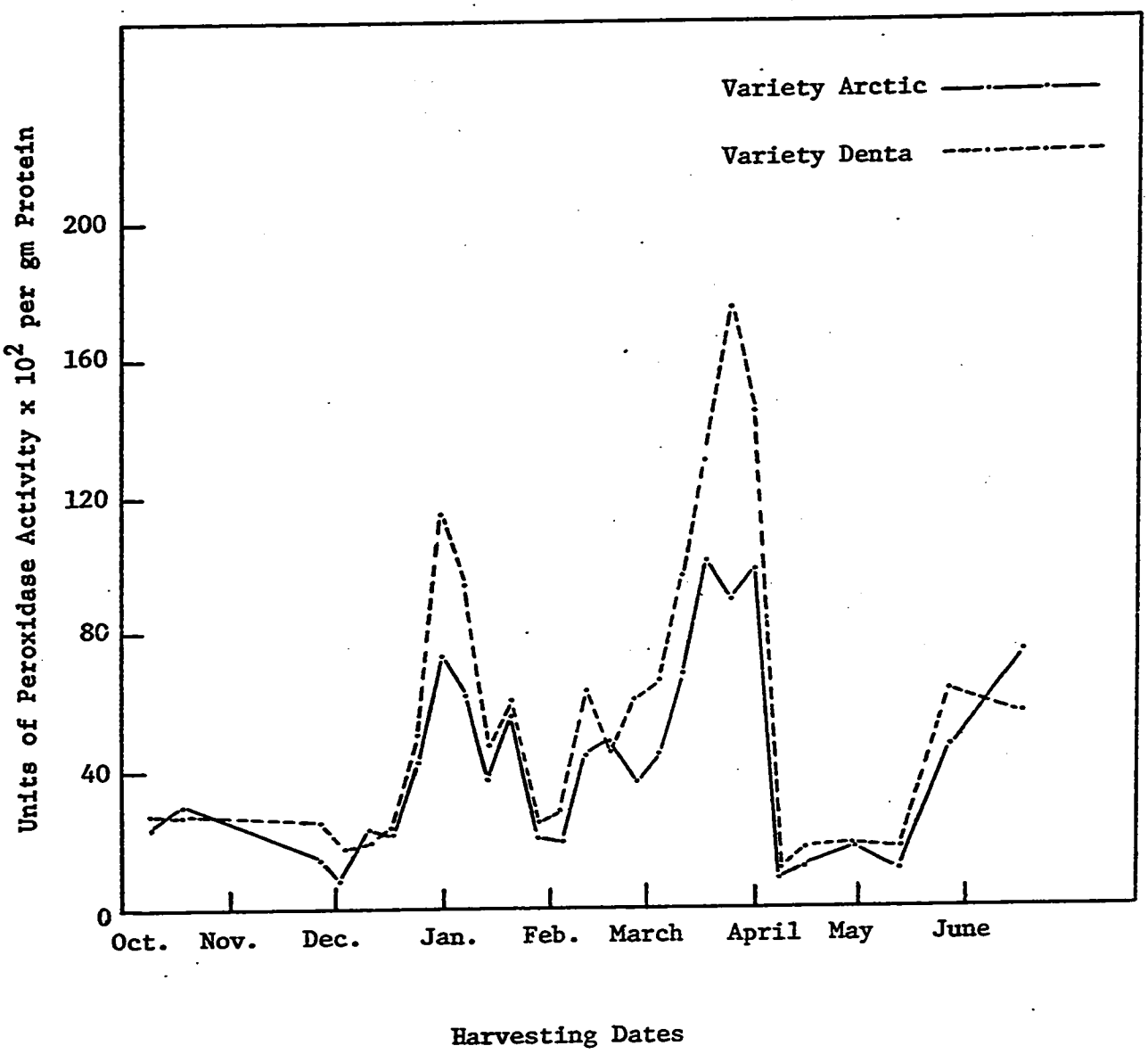


Figure 50. Peroxidase enzyme activity in extracts from sweet clover roots of varieties Arctic and Denta harvested at various intervals between October 8, 1968 and June 17, 1969.

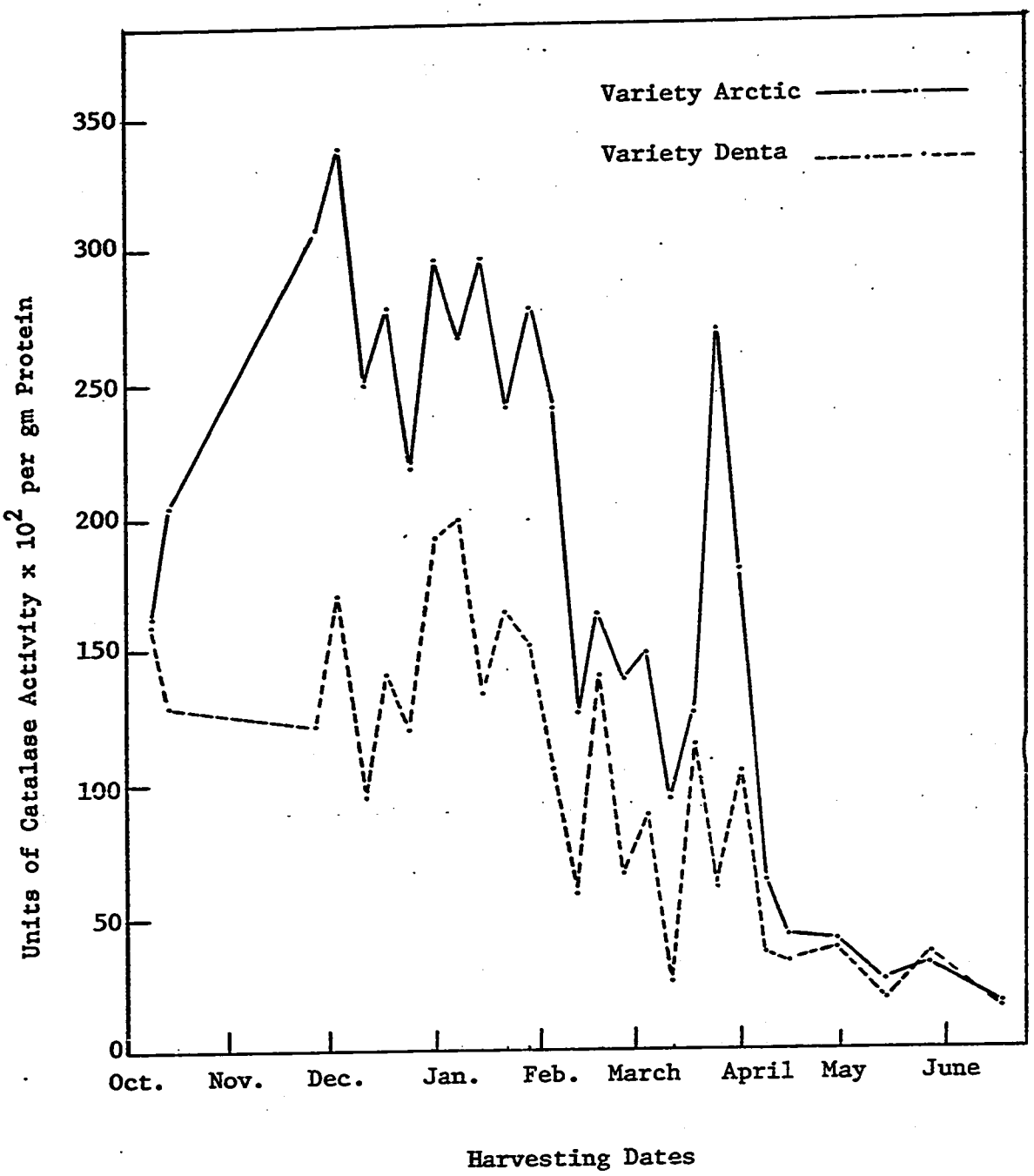


Figure 51. Catalase enzyme activity in extracts from sweet clover roots of varieties Arctic and Denta harvested at various intervals between October 8, 1968 and June 17, 1969.

slightly higher in roots of Arctic than in Denta (Fig. 52). On the whole, the enzyme levels did not fluctuate very much during the period under investigation except between March and mid-April when there was an increase in the activity similar to that observed for peroxidase and catalase enzymes.

h. Gel Electrophoretic Patterns of Sweet Clover Root Proteins

The electrophoretic protein patterns of cell-free extracts of sweet clover roots of Arctic and Denta varieties harvested at various time intervals are shown in Fig. 53. Protein bands varied in their staining properties, therefore an arbitrary scheme, similar to that described for fungal protein studies (see page 63) was adopted.

To help interpret the protein patterns on the gels, the gels were divided into sections, designated as A, B, C, and D. The numbers of bands in each section for each gel were noted (Fig. 53). The division into four sections was based upon the consistent occurrence of certain protein bands in a specific place on the gels e.g. in section A and C there were two and three bands respectively, and in D there was little variation in the numbers or changes in patterns of protein bands.

More changes in protein patterns occurred in Denta, the susceptible variety, than were noticed for Arctic which is resistant to *P. meliloti* and more winter-hardy than Denta. These changes were more erratic i.e. there were greater variations in the number of protein bands in the variety Denta than in Arctic. On the whole, most of the changes in the protein patterns occurred during the fall and early winter than the latter part of the winter and spring. During the period December 17, 1968 to February 18, 1969, 30 or 31 protein bands were observed in root extracts from Arctic variety; however, in the variety Denta this higher number of protein bands (32 or 30) did not occur until a week later i.e. on December 24, 1968.

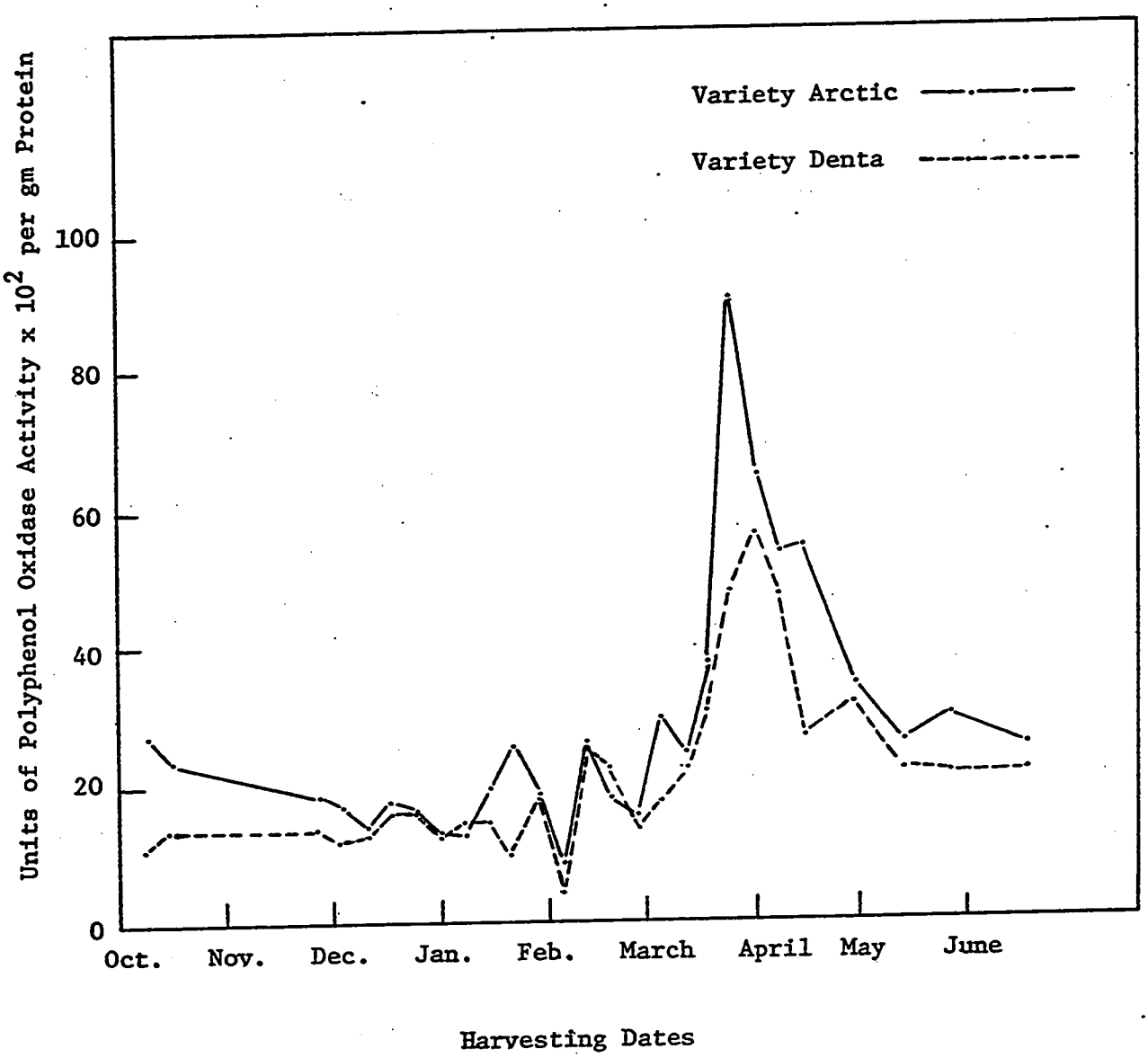


Figure 52. Polyphenol oxidase enzyme activity in extracts from sweet clover roots of varieties Arctic and Denta harvested at various intervals between October 8, 1968 and June 17, 1969.

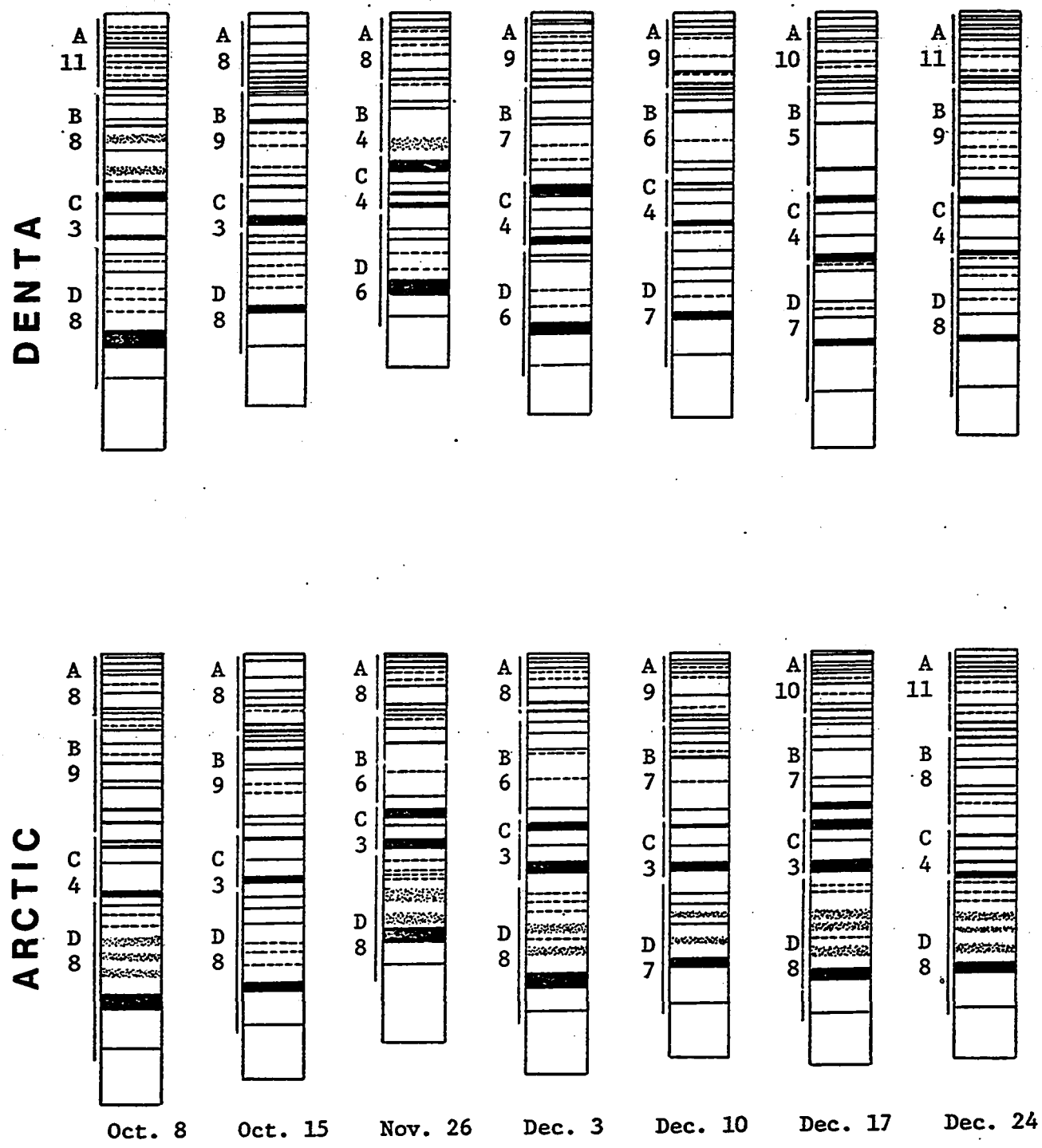


Figure 53. Disc gel electrophoresis patterns of soluble proteins in extracts from sweet clover roots of varieties Arctic and Denta at various intervals between October 8, 1968 and June 17, 1969.

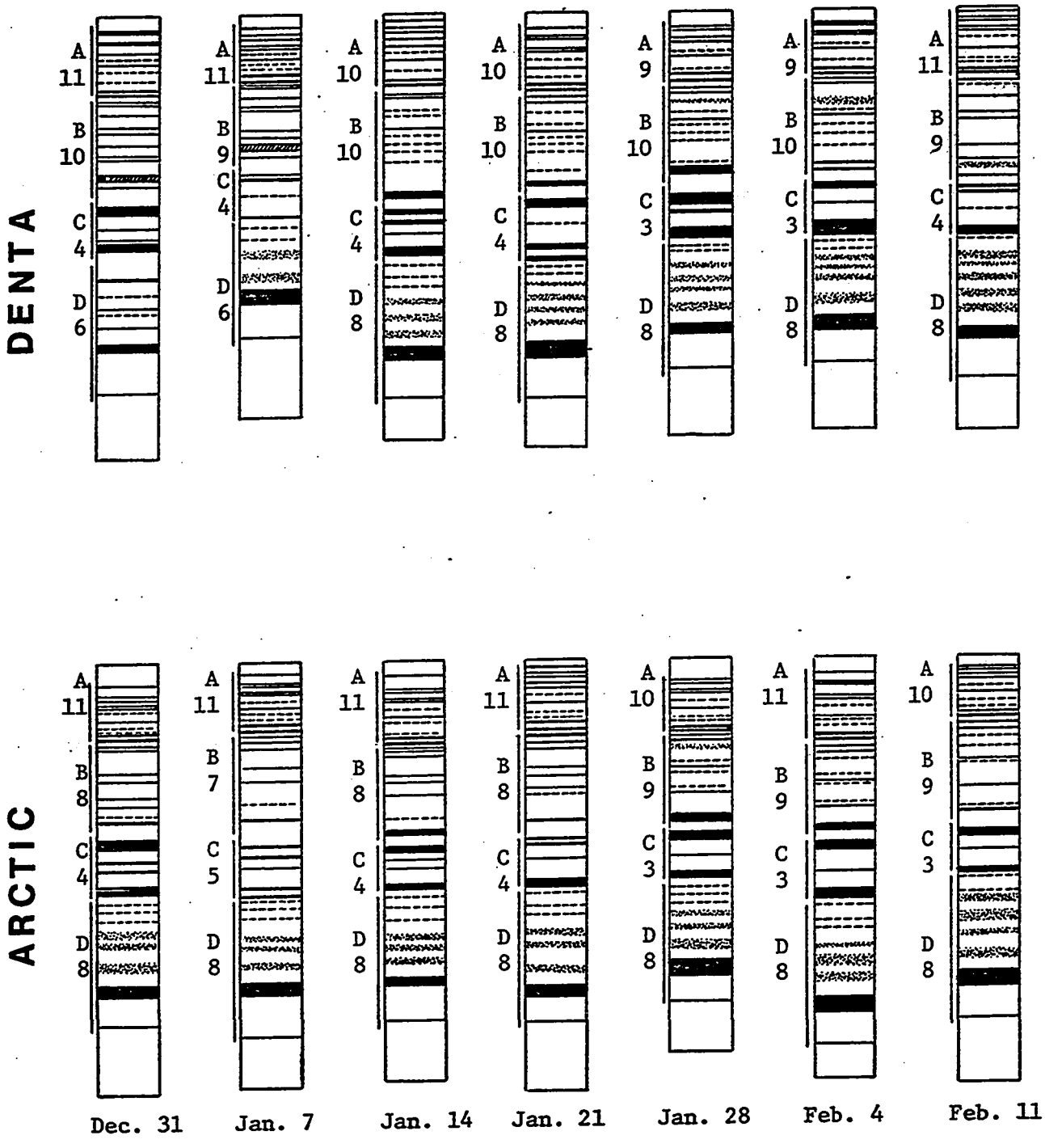


Figure 53. Continued

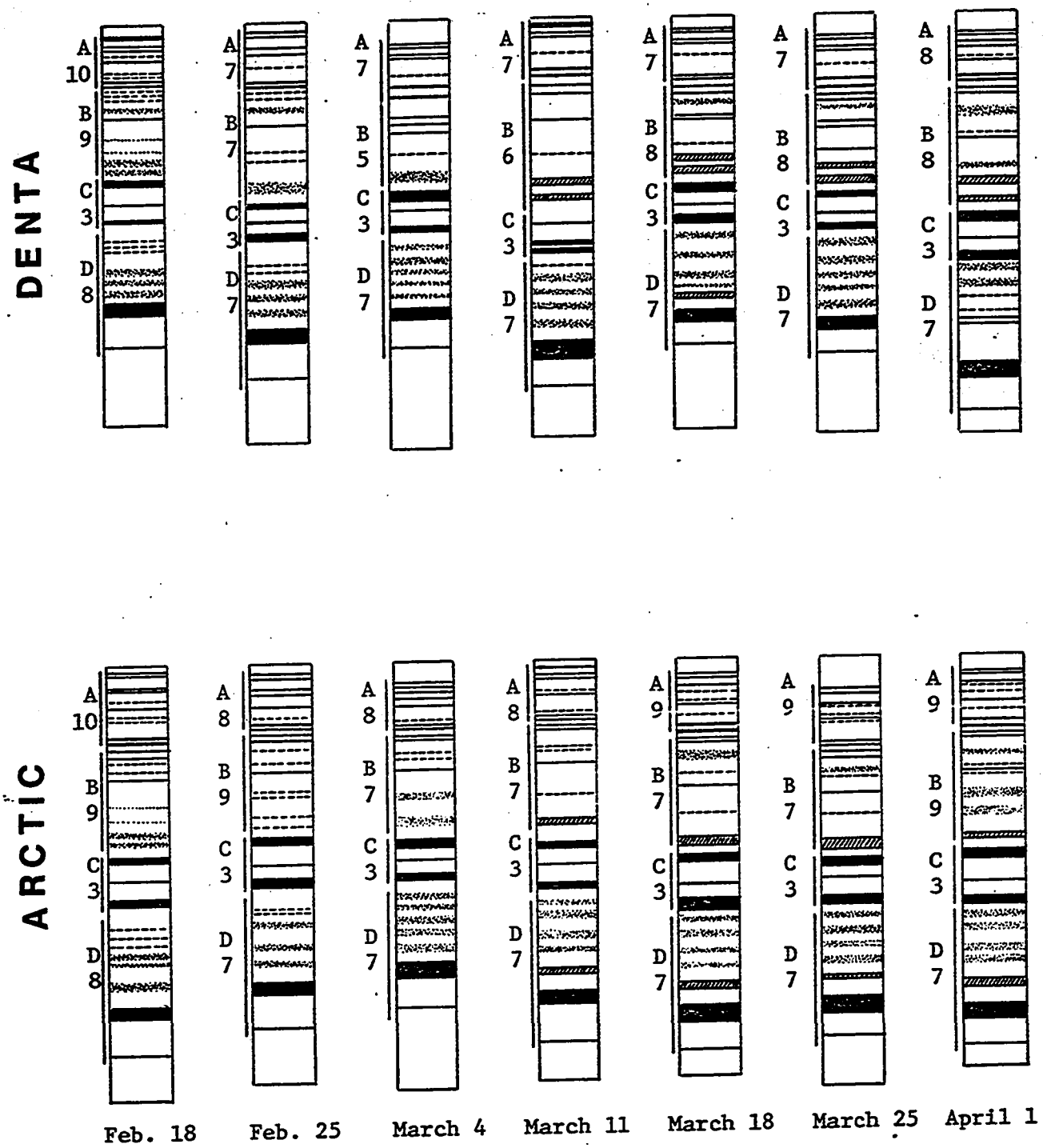


Figure 53. Continued

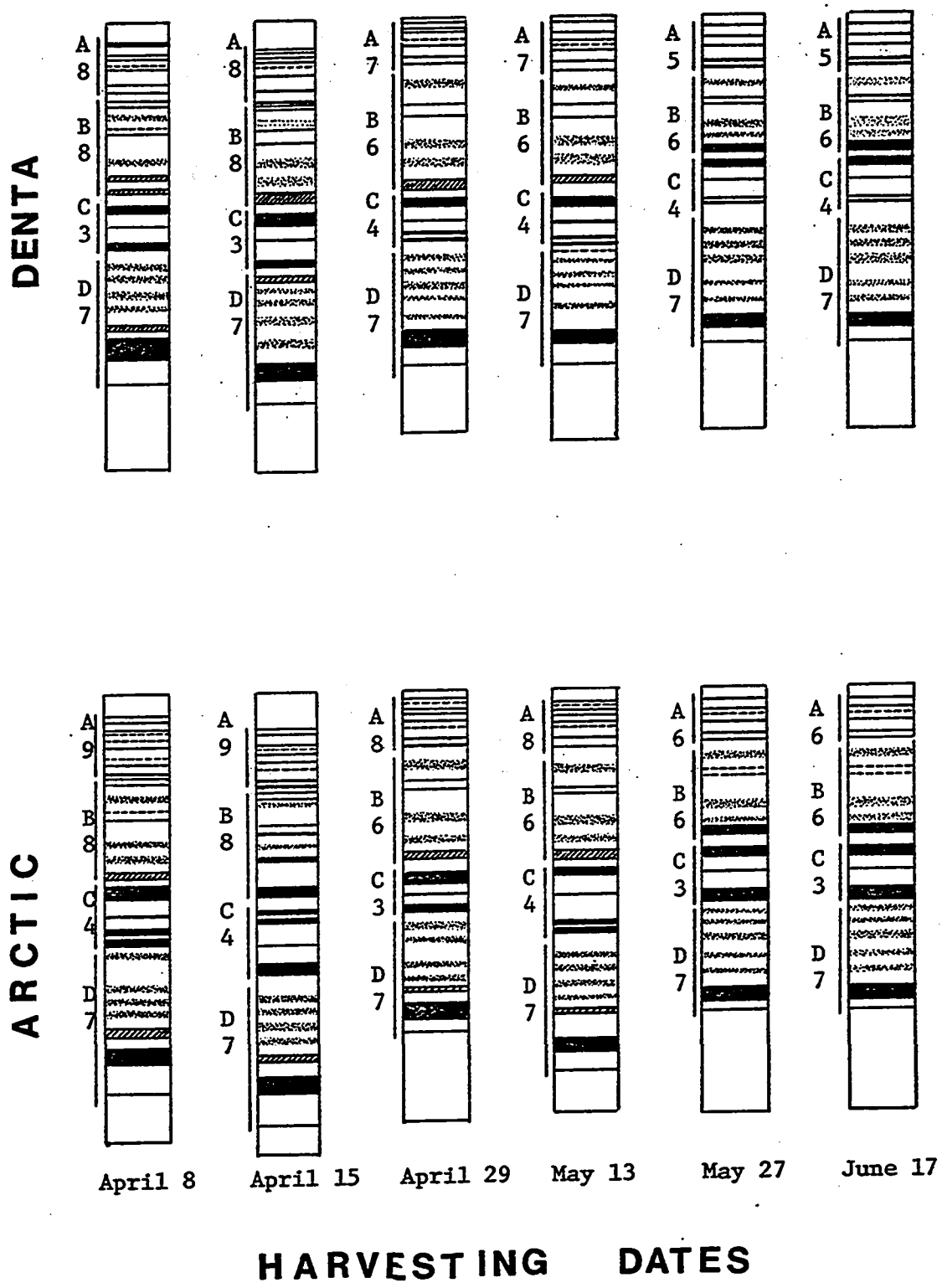


Figure 53. Continued

Prior to this period there were considerable variations in the numbers of protein bands, generally below 30. After February 18, 1969, the number of protein bands for both varieties varied but decreased to 24 by June 17, 1969 when the experiment was terminated. A summary of readily-detectible changes in protein patterns and numbers of protein bands for each section are presented as follows:

Section A. - The patterns of protein bands for the variety Denta were quite similar to that of Arctic, but the fluctuations in the numbers of protein bands were greater for Denta than noted for the variety Arctic. These fluctuations were more common during the first part than the latter part of the studies. Maximum numbers of protein bands in this section occurred during December 17, 1968 to February 18, 1969, which was the same period during which maximum numbers of protein bands were observed when the gel was taken as a whole.

In relation to time, the numbers of protein bands in this section of the gel for the variety Arctic varied from eight to ten from October 8, 1968 to December 17, 1968, and then either ten or eleven protein bands were present until February 18, 1969. From February 25, 1969 to May 13, 1969 only eight or nine protein bands were observed, and then the number decreased to six. For the variety Denta for the above time sequence the following numbers of protein bands were observed, seven to eleven, nine to eleven, seven or eight, and five respectively.

Section B. - Except during the latter part of the experiment, no set protein band pattern in this section was evident for both varieties. The numbers of protein bands were fairly constant in the variety Arctic, ranging from seven to nine except for the first six harvestings when the number of protein bands varied from six to nine and for the last four harvests when the number

of protein bands was six. In Denta for the same periods the variations in the numbers of protein bands were much greater than in Arctic. For the first six harvests the number of protein bands varied from four to nine and then from five to ten for the rest of the harvestings except the last four. For the last four harvests the number of protein bands was six which was the same as that observed for the variety Arctic in this section during this period. Also, the protein pattern for the last four harvests in this section were similar in the two varieties, however, in general the protein patterns were not alike for the two varieties for the previous harvestings.

Section C. - This section consisted of three prominent protein bands in both the varieties which had consistent migration rates. In several instances a fourth protein band appeared but there was no consistency as to its position on the gel in this section with respect to the other three protein bands. In one instance five protein bands were observed in the variety Arctic. It may be noted that the frequency of occurrence of three or four bands in the variety Denta was the same.

Section D. - In the variety Arctic there was a fairly constant protein pattern which was not evident in the variety Denta until January 14, 1969. Except in one instance (seven bands for December 10, 1968), there were eight bands present until February 18, 1969, after which seven bands were observed. In Denta for the same periods the variations in the number of bands was from six to eight and then consistently seven for the latter part of the experiment.

i. Peroxidase Isozyme Patterns

Changes in the peroxidase isozyme patterns for Arctic and Denta from October 8, 1968 to June 17, 1969 are shown in Fig. 54. As noted for protein bands, the peroxidase isozyme bands also varied in their staining

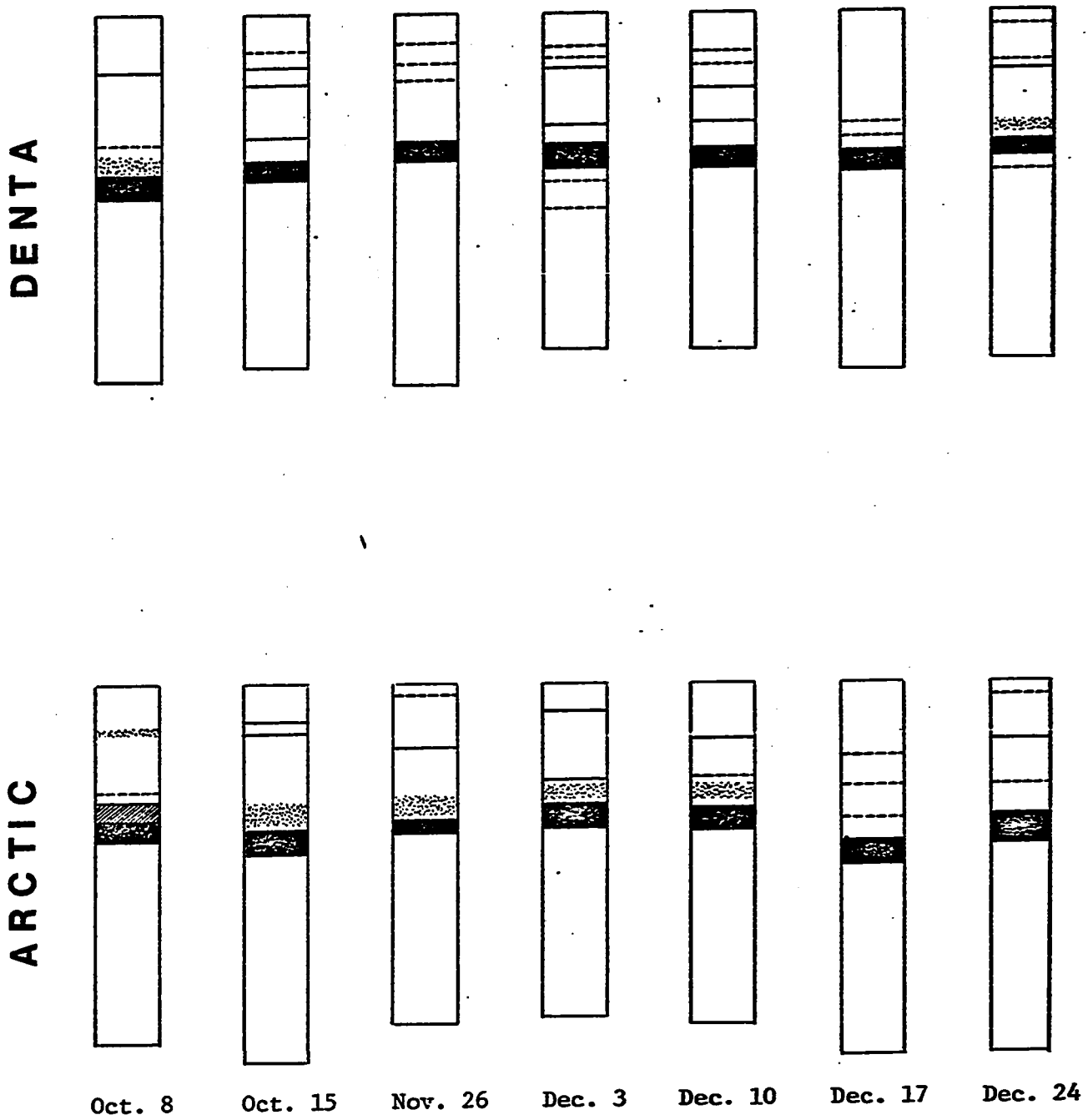


Figure 54. Disc gel electrophoresis patterns of peroxidase isozymes in extracts from sweet clover roots of varieties Arctic and Denta harvested at various intervals between October 8, 1968 and June 17, 1969.

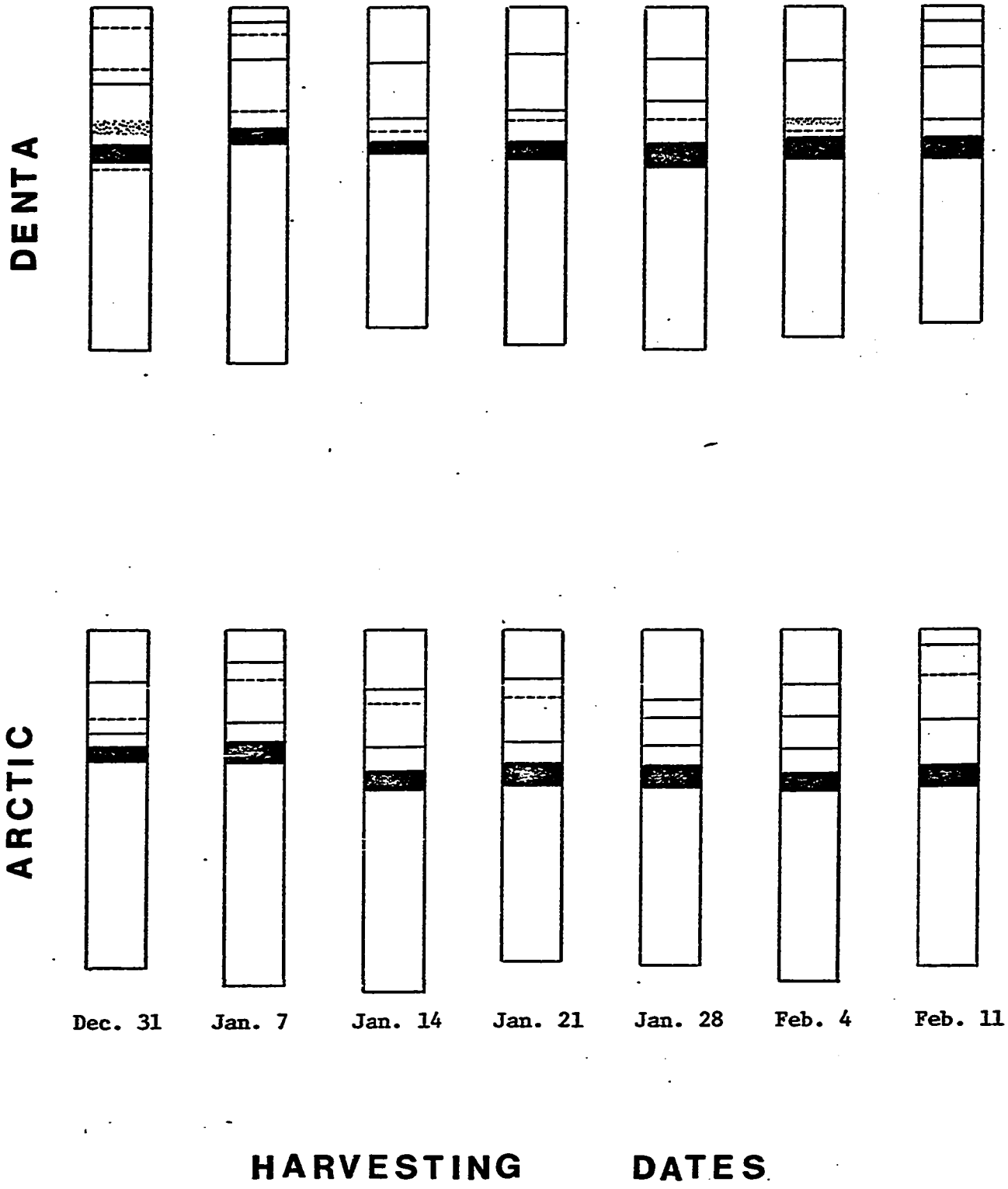


Figure 54. Continued

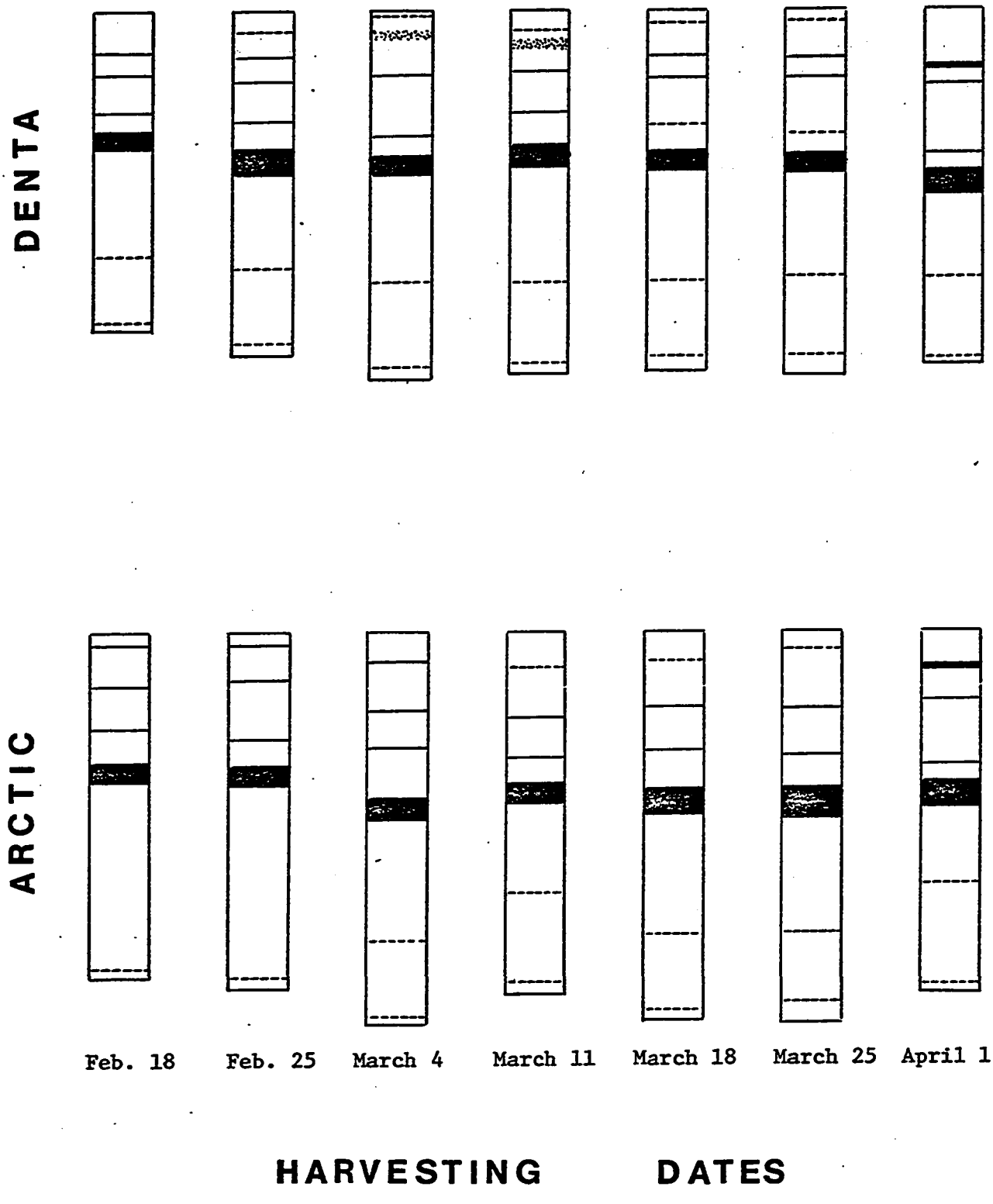


Figure 54. Continued

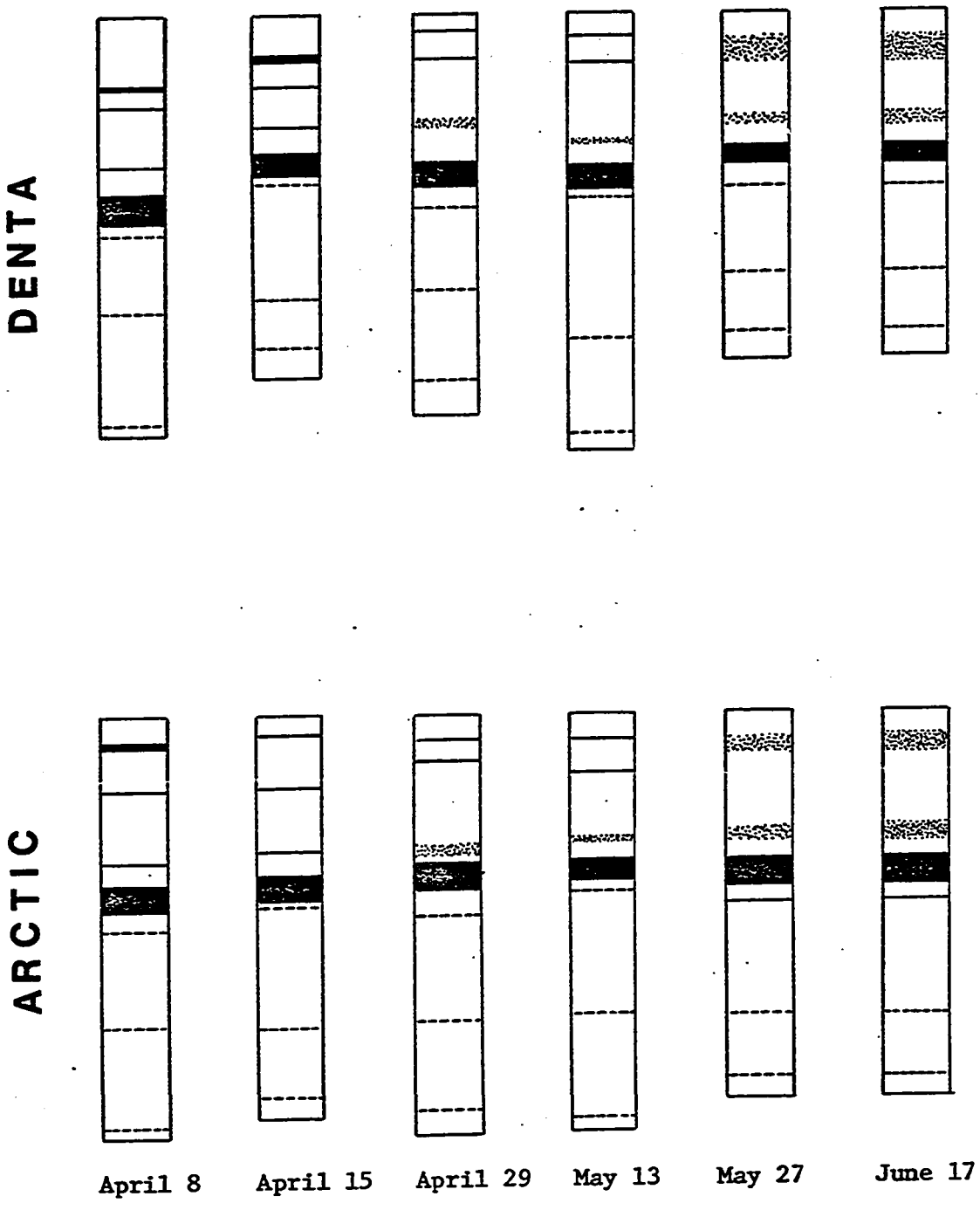


Figure 54. Continued

properties, therefore an arbitrary scheme was used to designate the relative intensities of colour development. Prominent bands are shown as solid lines, faint bands as broken, faint but wide and with well-defined boundaries with diagonal lines, and diffuse zones as dotted areas.

A greater variation in the number of peroxidase isozymes was noted for Denta than for Arctic. A prominent isozyme band, in the central portion of the gel, was present in both varieties throughout the study. In Arctic three isozymes were always present above this band, however for Denta, the number of isozyme bands above the prominent band varied from two to four. The bands present above the above-mentioned prominent band also showed quantitative changes from one harvest to the other for both varieties; however, no consistency in these changes was apparent. One to two faint isozyme bands occurred occasionally below the prominent band in the variety Denta but none in the variety Arctic until February 18, 1969, when both varieties showed the presence of two more faint isozyme bands below this heavy band. For both varieties these two bands were present below the prominent band until April 1, 1969, after which three bands were always present below the prominent band.

j. Gel Electrophoretic Studies on Polyphenol Oxidase

Gel electrophoretic studies for polyphenol oxidase for both varieties showed the presence of this enzyme; however due to the development of a pinkish hue of the gel, shortly after incubation in the reaction mixture no definite polyphenol oxidase isozyme band(s) on the gel could be observed.

k. Amino Acid Analyses

1. Free amino acids:

The results of free amino acid analyses of roots of sweet clover varieties, Arctic and Denta, are shown in Table 11. The amino acid amounts are expressed as mg per gram dry root powder. Fig. 55 shows a typical eleutogram for free amino acids of an HCl hydrolysate of an 80 per cent ethanol extract from sweet clover roots. Among the free amino acids, arginine, aspartic acid, glutamic acid, and proline were found in much greater quantities than the other amino acids. Consistently present in very low amounts were lysine, glycine, cystine, methionine, leucine, tyrosine, and phenylalanine. Several amino acids increased greatly with the initial decrease in the soil temperature, whereas many others did not show such a trend. The values for arginine, glutamic acid, proline, and isoleucine were, 1.5, 2.4, 3.0, and 2.0 times respectively greater on December 24, 1968 than on October 8, 1968. Threonine, glycine, alanine, valine, tyrosine, and phenylalanine did not show any appreciable change; however, histidine, aspartic acid, and serine increased markedly during the latter part of the studies. The amounts of leucine from the roots harvested on December 24, 1968, were approximately one third that observed for samples harvested on October 8, 1968, and then remained at this low level.

When the amounts of free amino acids are totalled and compared, these totals are much higher for the December 24 harvest as compared to the roots harvested on October 8. For Arctic these high values stayed at this high level until February 25 and then another increase occurred. A similar increase for Denta was observed a little earlier, on February 11, 1969.

2. Protein amino acids:

The results of protein amino acid analyses for the various harvests are shown in Table 12. These results are expressed as mg per gram dry

TABLE 11

Amounts* of free amino acids in roots of sweet clover varieties Arctic (winter hardy) and Denta (non-winter hardy) as determined by column chromatography. Roots harvested from October 8, 1968 to April 8, 1969.

		Dates of Harvest									
		October	December		January		February		March		April
Amino Acid	Variety	8	24	31	14	28	11	25	11	25	8
Lysine	Arctic	0.89	0.76		0.73	0.82	0.81	0.74	0.65	0.69	1.77
	Denta	1.03	0.94	0.85	0.78	0.96	0.86	1.02	0.79		0.95
Histidine	Arctic	1.46	1.23		2.07	2.71	2.12	3.35	3.17	2.45	2.93
	Denta	0.85	0.79	0.77	1.86	1.18	1.83	0.90	0.98		1.66
Arginine	Arctic	15.32	22.85		19.06	19.57	19.59	27.76	28.13	20.95	22.80
	Denta	14.09	10.41	19.15	18.60	11.06	16.15	26.38	20.59		23.75
Aspartic Acid	Arctic	26.42	22.75		21.72	22.52	23.08	34.62	34.12	21.81	27.19
	Denta	23.16	21.88	20.24	27.19	22.52	25.93	25.28	31.39		23.43
Threonine	Arctic	1.33	0.59		0.66	0.97	0.81	0.91	0.88	0.64	0.53
	Denta	0.79	0.60	0.36	0.74	0.91	0.51	0.53	0.51		0.55
Serine	Arctic	0.93	0.81		1.14	1.04	1.43	2.33	1.04	1.03	1.24
	Denta	1.11	0.59	0.58	1.04	1.08	0.99	1.43	1.14		0.87
Glutamic Acid	Arctic	2.83	6.87		10.09	7.25	7.19	7.85	8.51	10.07	6.00
	Denta	2.37	6.40	6.93	6.21	4.50	7.49	7.64	8.97		5.52
Proline	Arctic	4.43	13.20		12.08	18.72	17.75	25.23	22.76	12.02	18.91
	Denta	6.48	13.15	10.00	8.10	8.90	16.74	13.80	15.08		20.45
Glycine	Arctic	0.98	0.30		0.41	0.36	0.28	0.35	0.37	0.41	0.29
	Denta	1.12	0.32	0.22	0.34	0.59	0.31	0.33	0.32		0.27
Alanine	Arctic	1.27	0.88		1.24	1.16	1.00	1.08	0.86	1.23	0.72
	Denta	1.17	0.92	0.82	0.82	0.88	1.01	0.81	0.81		0.57
Half Cystine	Arctic	0.06	0.06		0.12	-	0.15	-	-	0.11	0.06
	Denta	0.06	0.11	-	0.06	-	0.08	-	-		-
Valine	Arctic	1.04	1.02		1.29	1.47	0.86	1.01	0.98	1.24	1.03
	Denta	0.84	0.88	0.82	1.13	1.26	0.88	0.74	0.90		0.82
Methionine	Arctic	0.08	0.18		0.04	0.72	0.40	-	0.10	-	0.70
	Denta	-	0.20	-	T	-	-	-	0.03		0.22
Isoleucine	Arctic	1.00	1.84		2.03	1.61	0.96	0.92	0.99	1.54	0.69
	Denta	0.69	1.17	0.92	1.12	1.06	0.92	0.73	0.73		0.73
Leucine	Arctic	0.99	0.38		0.50	0.32	0.30	0.41	0.44	0.48	0.47
	Denta	1.01	0.39	0.24	0.41	0.34	0.34	0.34	0.33		0.31
Tyrosine	Arctic	0.31	0.39		0.48	0.37	0.31	0.37	0.33	0.34	0.37
	Denta	0.27	0.31	0.11	0.14	0.18	0.20	0.18	0.19		0.22
Phenylalanine	Arctic	0.52	0.48		0.36	0.32	0.31	0.27	0.49	0.62	0.68
	Denta	0.44	0.20	0.28	0.21	0.36	0.34	0.26	0.43		0.24
Total Amino Acid	Arctic	59.86	74.59		74.02	79.93	77.35	107.20	103.82	75.63	85.38
	Denta	55.48	53.50	62.29	68.75	55.78	74.58	80.37	83.19		80.56

* The results are expressed as mg/g dry weight.

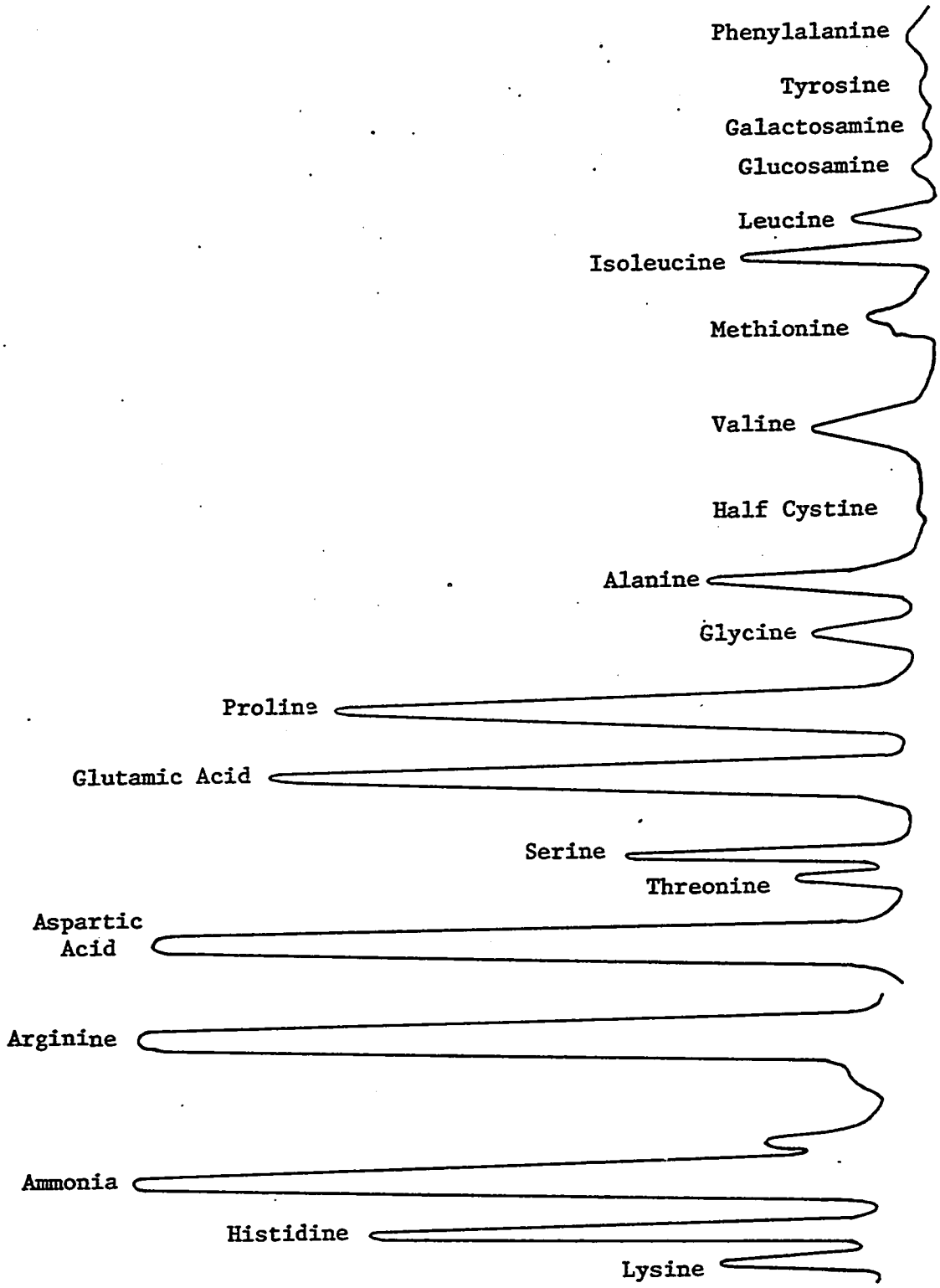


Figure 55. A typical eleutogram of free amino acids found in 80 per cent ethanol extracts from roots of sweet clover variety Arctic harvested on February 25, 1969.

TABLE 12

Amounts* of protein amino acids in roots of sweet clover varieties Arctic (winter hardy) and Denta (non-winter hardy) as determined by column chromatography. Roots harvested from October 8, 1968 to April 8, 1969.

		Dates of Harvest									
		October	December		January		February		March		April
Amino Acid	Variety	8	24	31	14	28	11	25	11	25	8
Lysine	Arctic	11.11	17.71	18.33	15.82	19.45	15.02	20.18	19.86	16.07	16.48
	Denta	8.13	13.08	12.06	15.35	14.52	8.88	7.71	14.09	15.13	12.08
Histidine	Arctic	4.14	5.95	6.27	5.18	6.47	7.09	6.98	6.67	6.57	5.14
	Denta	2.60	4.11	4.46	4.37	4.67	4.81	2.50	7.02	5.31	4.20
Arginine	Arctic	24.34	45.48	25.97	19.90	24.65	33.58	33.62	24.59	22.79	23.78
	Denta	19.99	39.20	23.13	29.79	35.85	27.96	11.99	18.13	22.73	23.97
Aspartic Acid	Arctic	17.90	33.07	23.37	17.95	31.11	30.51	34.71	23.57	31.65	27.73
	Denta	12.11	20.49	26.99	29.57	34.05	33.80	12.66	16.38	29.97	27.78
Threonine	Arctic	5.12	8.69	9.02	7.66	11.36	9.92	9.55	9.84	6.94	7.55
	Denta	3.98	6.03	5.73	6.29	6.49	9.01	6.08	6.48	7.18	6.23
Serine	Arctic	4.84	8.84	9.29	8.57	9.96	10.23	11.27	10.14	8.07	7.88
	Denta	3.92	5.00	6.19	6.73	7.15	9.97	7.18	6.98	7.49	7.09
Glutamic Acid	Arctic	12.67	20.71	21.23	20.06	21.23	23.51	23.28	22.20	17.76	22.88
	Denta	8.74	14.53	14.64	15.34	15.96	22.25	16.09	15.56	16.88	14.81
Proline	Arctic	5.43	8.92	9.33	8.83	9.15	11.04	10.36	10.00	8.30	7.86
	Denta	3.80	8.50	6.65	7.53	6.85	10.37	8.20	7.20	7.99	7.08
Glycine	Arctic	5.47	10.39	10.72	9.37	11.47	11.59	11.43	11.66	9.23	9.07
	Denta	3.97	6.64	6.93	7.15	7.27	10.19	7.54	6.41	8.11	7.36
Alanine	Arctic	5.47	9.86	10.47	9.46	11.58	11.55	11.37	11.28	9.25	8.82
	Denta	4.28	7.22	7.22	6.17	7.43	10.98	8.13	7.85	8.42	7.44
Half Cystine	Arctic	0.22	0.30	0.27	0.54	0.51	0.56	0.70	0.58	0.35	0.75
	Denta	0.30	-	-	-	-	0.43	0.25	0.22	0.30	0.48
Valine	Arctic	8.40	12.89	14.82	12.80	16.20	15.55	15.18	14.97	11.65	11.07
	Denta	8.87	9.21	9.13	10.01	10.71	14.45	10.42	10.40	13.26	9.50
Methionine	Arctic	0.32	0.16	0.25	0.21	0.44	0.17	0.27	0.25	0.44	0.19
	Denta	0.27	0.17	0.17	0.32	0.38	0.11	0.11	0.17	-	0.26
Isoleucine	Arctic	6.22	9.77	10.79	11.02	10.11	12.06	11.79	12.29	9.25	8.96
	Denta	4.02	7.57	7.47	7.49	8.04	11.14	8.04	8.00	8.20	7.65
Leucine	Arctic	8.80	14.87	15.35	14.23	16.97	17.46	17.12	17.67	14.45	13.09
	Denta	5.06	10.88	11.02	11.22	11.34	16.19	11.51	11.98	11.81	11.03
Tyrosine	Arctic	3.26	5.79	5.67	5.78	6.61	6.60	6.85	5.88	5.11	5.37
	Denta	2.30	4.27	3.89	4.13	4.78	5.79	4.53	4.43	4.50	4.32
Phenylalanine	Arctic	5.29	8.82	9.07	8.67	10.56	10.35	10.48	10.54	8.03	8.09
	Denta	3.58	6.58	6.31	6.73	6.76	9.66	7.21	7.00	7.15	6.78
Total Amino Acids	Arctic	129.00	222.22	200.22	176.05	217.83	226.79	235.14	211.99	185.91	184.71
	Denta	95.92	163.48	151.99	168.19	182.25	205.99	129.85	148.30	174.43	158.06

* The results are expressed as mg/g dry weight.

root powder. A typical eleutogram for amino acids from the HCl hydrolysates of the protein fractions of sweet clover roots is shown in Fig. 56. The quantities of cystine and methionine were extremely low and an accurate quantitative determination could not be made. Except for valine, the amounts of the other amino acids were much less in Denta as compared to Arctic on October 8, 1968. There was a greater increase in the amounts of all the protein amino acids when the initial decrease in the soil temperatures occurred in samples harvested in December than on October 8, 1968. Depending upon the amino acid, the increase varied from a fraction to as much as two times. Most of the protein amino acids were present in larger amounts during the period from January 28, 1969 to February 25, 1969. After this period there was a gradual decrease in the protein amino acids. On April 8, 1969, when the experiment was terminated, the amounts of various amino acids were still much higher than that found on October 8, 1968.

When the amounts of protein amino acids were totalled and compared, the totals were much higher in the variety Arctic than found for the variety Denta. The total values for protein amino acids followed a similar trend as observed for most of the protein amino acids.

1. Sugar Analyses

In Fig. 13 are presented two typical paper chromatograms sprayed with aniline hydrogen phathalate reagent showing five spots for both Arctic and Denta varieties from extracts obtained from samples harvested on October 8, 1968. Three of the spots were identified as sucrose, glucose, and fructose on the basis of their Rf values, while the other two spots were not identified. The unidentified sugars are designated as sugar A and sugar B for this study. The results of sugar analyses are shown in Table 13, and the results for sucrose, glucose, and fructose are expressed as mg per gram

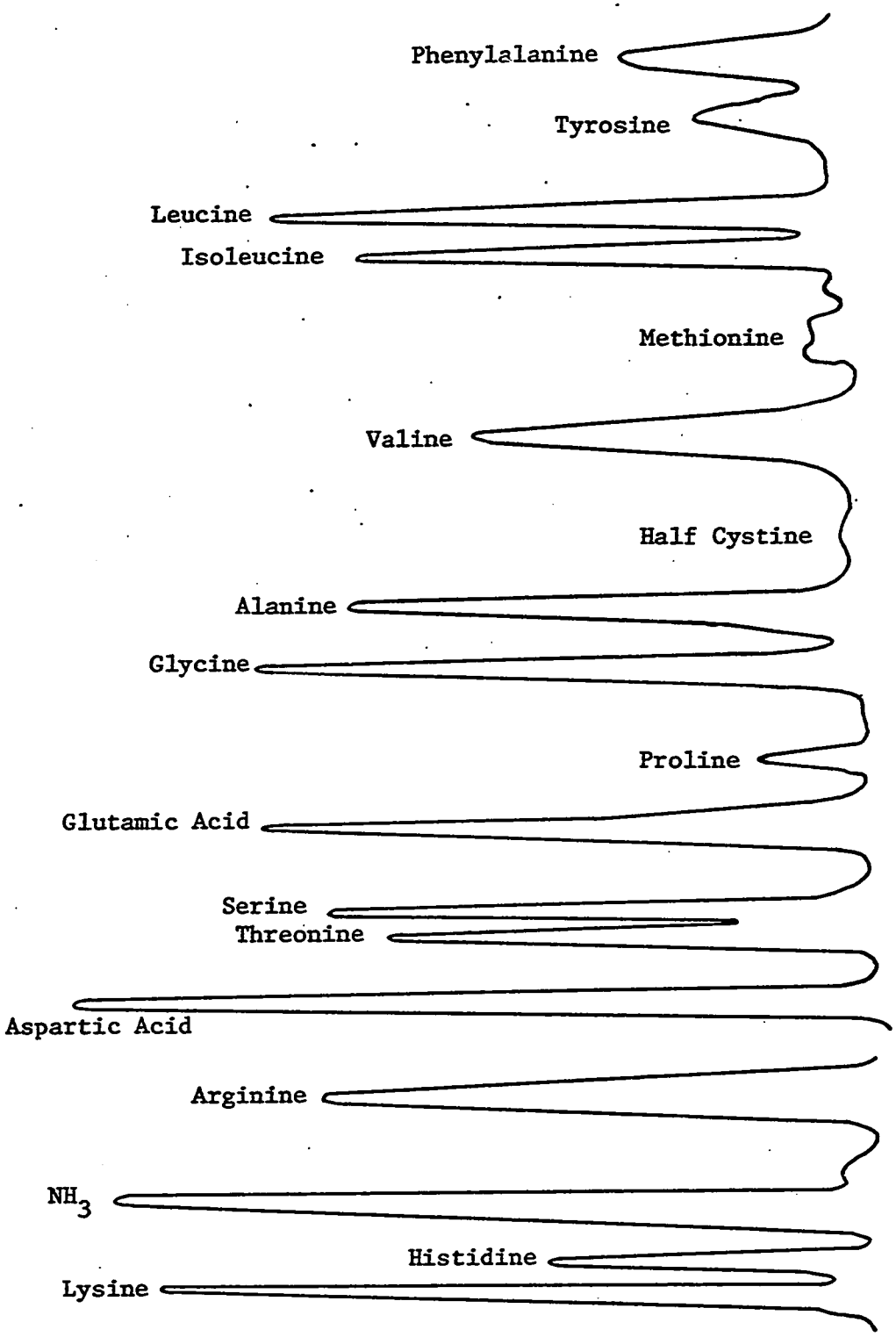


Figure 56. A typical eleutogram of protein amino acids found in extracts from roots of sweet clover variety Arctic harvested on February 25, 1969.

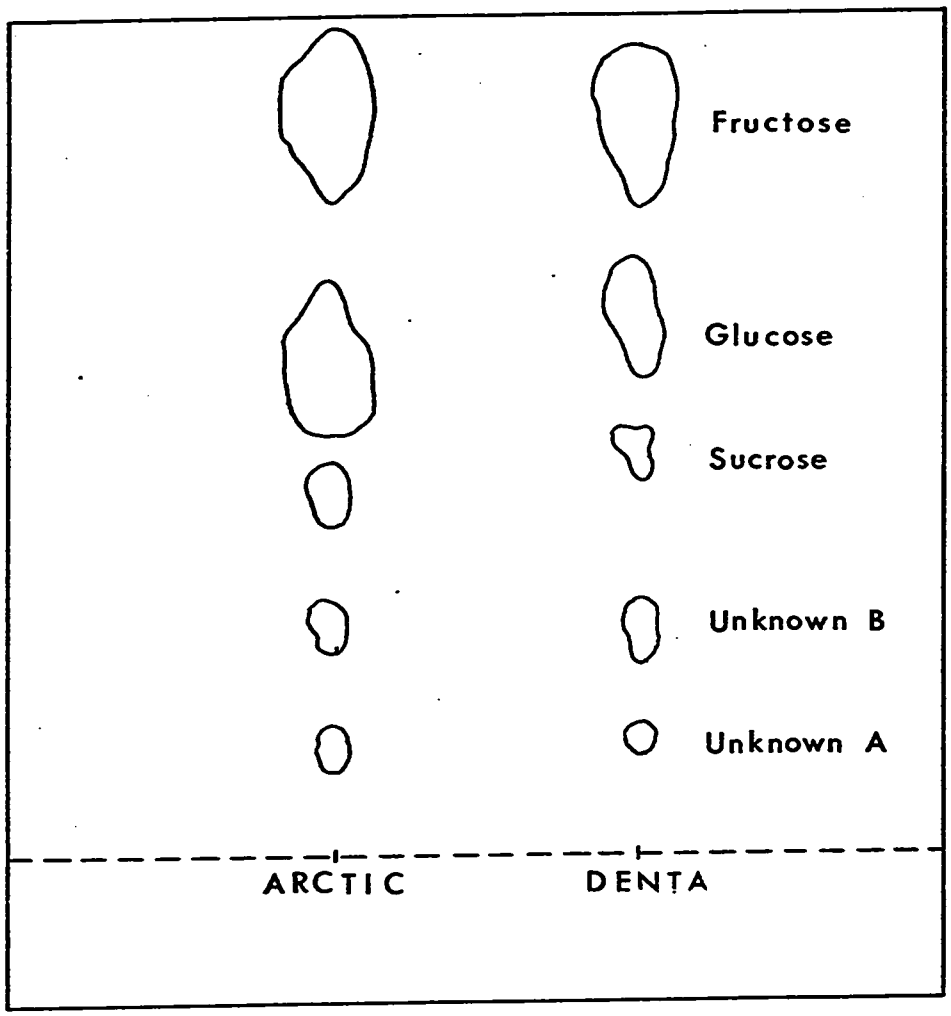


Figure 57. Paper chromatogram of different sugars present in 80 per cent ethanol extracts from roots of sweet clover varieties Arctic and Denta harvested on October 8, 1968.

TABLE 13

Amounts* of sucrose, glucose, and fructose, and the unknown sugars A and B, in roots of sweet clover varieties Arctic (winter hardy) and Denta (non-winter hardy). Roots harvested from October 8, 1968 to April 8, 1969.

		October	December		January		February		March		April
Sugar	Variety	8	24	31	14	28	11	25	11	25	8
Sucrose	Arctic	29.0	23.8	25.2	23.6	24.8	26.2	28.0	23.6	24.0	25.2
	Denta	31.2	20.4	22.8	19.2	25.2	23.8	23.6	12.0	18.0	27.2
Glucose	Arctic	33.6	65.6	74.4	71.4	75.2	73.6	57.6	60.2	64.0	52.0
	Denta	25.6	57.6	59.2	46.4	49.6	29.6	52.8	56.8	49.6	42.4
Fructose	Arctic	78.4	35.2	39.2	39.8	36.8	28.8	47.6	52.4	64.0	67.2
	Denta	76.8	31.2	38.8	38.4	34.4	24.0	33.3	34.2	57.6	64.8
Unknown Sugars											
Unknown Sugar A	Arctic	20.0	-	-	-	-	-	-	-	-	-
	Denta	20.5	-	-	-	-	-	-	-	-	-
Unknown Sugar B	Arctic	28.0	27.0	28.5	27.0	26.5	28.0	30.0	23.5	27.0	16.0
	Denta	26.0	27.0	29.5	26.5	22.0	27.0	24.0	16.5	16.0	14.5

* The results for sucrose, glucose, and fructose are presented as mg/g dry weight and for unknowns as their relative amounts.

dry weight root powder. In Table 13 the relative amounts of sugars A and B for the two varieties and between different harvests are also presented. The data for sugars A and B are presented as relative amounts since sugars A and B are unknown.

Sucrose was present in about the same amounts in the two varieties. Except for a few instances when decreases in the amounts of sucrose occurred, there were no appreciable quantitative changes.

Glucose values were always higher in Arctic than in Denta. The amounts of glucose present in the roots of both varieties on December 24, 1968 were twice as much as found on October 8, 1968. The high values for glucose prevailed throughout the period of study, although a trend showing slight decrease was noticed on February 25, 1969 for the variety Arctic. No such trend was apparent in the variety Denta.

The values for fructose were always higher for Arctic than for Denta. Fructose showed a trend entirely opposite to that observed for glucose. The amounts of fructose observed for roots harvested on December 24, 1968, were less than one half that noticed for samples harvested on October 8, 1968. For the variety Arctic the fructose values began to increase as of February 25, 1969 and glucose values began to decrease. This trend for increases in the values of fructose did not occur until March 25, 1969 for the variety Denta.

Sugar A was present in about equal amounts in the extracts from roots of both the varieties Arctic and Denta harvested on October 8, 1968. In subsequent harvests sugar A was not detected. The amounts of sugar B in the two varieties did not alter appreciably throughout the period of harvesting until February 25, 1969. On March 11, 1969, a marked decrease in the values for the variety Denta was noticed, whereas such a decrease

in the variety Arctic did not occur until April 8, 1969.

DISCUSSION

The importance of light to fungal sporulation is well known. Many fungi which do not sporulate in the dark have been reported to do so when cultured in light. The effects of light on fungi with regard to reproduction, morphology, pigmentation, and phototropic phenomenon have been reviewed by Marsh, Taylor, and Bassler (1959), and Carlile (1965). Of the fungi mentioned in these two reviews, those which produce pycnidia do so under natural conditions on the above-ground plant parts where light could have a direct affect on fructification. Recently, Knox-Davies (1965) found that light was essential for pycnidium production by *Macrophomina phaseoli*. However, with *P. meliloti* pycnidia are formed on roots except in a few instances when they form on the stems at or near ground level. When pycnidia of *P. meliloti* are formed on roots at distances of five cm or more below ground level, light cannot be considered to have a direct effect in their production. On the other hand, when *P. meliloti* is cultured under laboratory conditions, the fungus does not form pycnidia in darkness, but they are produced when cultured in light. This is entirely opposite to what happens under natural conditions. Therefore the role of light in pycnidial formation is an indirect one and unlike that reported for other fungi which produce pycnidia only on above-ground plant parts.

Coons (1916), working on *Plenodomus fuscomaculans*, found that light was necessary for pycnidial formation, but he also noticed that mycelial growth was less in light than in darkness. When *P. meliloti* was grown in darkness using various amounts of synthetic medium, it was noted that pycnidial formation was induced when very little (<10 ml) medium was used (Table 2). Accompanying pycnidial formation there was also a decrease in the amount of aerial mycelium. In this respect the phenomenon of pycnid-

ial production associated with little aerial mycelial growth in the dark was similar to the observations made by Coons (1916) for pycnidial production by *P. fuscomaculans* and by Wu (1965) for pycnidial production by *P. meliloti* in light. It may be further noted from Table 2 that when the amounts of the culture medium were increased the growth of aerial mycelium was greater, and the number of pycnidia was reduced for cultures kept in darkness. It would appear that pycnidial formation is initiated when the nutritional requirements for vegetative growth are inadequate. These results support the hypothesis put forward by Henis *et al.* (1965), who suggested that the production of sclerotia by *Sclerotium rolfsii* is induced by nutritional and mechanical factors that bring about a partial retardation in the growth of the vegetative mycelium. This hypothesis was advanced when they found that the highest number of sclerotia occurred under sub-optimal growth conditions. They also found a correlation between abundant mycelial growth and a relatively poor sclerotial production. Chet and Henis (1968) proposed that a modification in -SH-containing Cu-linked protein entity, which acts as a repressor of sclerotial formation, would induce production of sclerotia. The modification in this protein could be either by an oxidation of the -SH group or an accumulation of internal natural chelates such as organic acids as a result of nutrient flow to the area where mechanical stimulus is present.

In the dark, when *P. meliloti* mycelium came into contact with the glass beads at the point where very little culture medium was present pycnidia were induced; but, when the glass beads were completely covered by the culture medium the pycnidia were absent. A direct contact with the glass beads would temporarily arrest growth, and result in the local increase of nutrients flowing from the center of the colony towards hyphal

tips. A similar explanation can be given for the presence of pycnidia in very old cultures incubated in darkness. It would appear that a certain threshold of a metabolite(s) or precursor(s) is necessary before pycnidial formation can be initiated. These levels may be reached much sooner in cultures incubated in light than in darkness.

Coons (1916) pointed out that light, in view of the experiments in which he was able to replace the effects of light by adding various oxidizing agents to the medium, acts as an oxidizing agent or a catalyst. On this basis he formulated a theory of 'opposed action of growth and fructification'. This theory considers that the competition for oxygen is the fundamental reason for the absence of fructification under conditions which allow abundant growth. In the present study, except for chromium trioxide and cobaltous nitrate, all the oxidizing agents tried were able to induce pycnidial formation in cultures kept in the dark. Potassium permanganate at low concentrations did not induce pycnidia formation in the dark. These results are similar to those of Coons (1916). However, with *P. meliloti*, pycnidia were produced in the presence of potassium permanganate at higher concentration. The absence of pycnidia in the presence of chromium trioxide and cobaltous nitrate could be explained on the basis that the anion and/or cation of these compounds, as such, were not suited for pycnidial production and mycelial growth. The use of these elements or compounds in other forms could add valuable information on their role in growth and pycnidial formation.

In these studies it was generally found that when pycnidia were induced due to the presence of an oxidizing agent the type of mycelial growth resembled more the controls incubated in light than those incubated in the darkness. This type of growth was quite similar to that already described in

the section dealing with the effects of different amounts of the synthetic medium on growth and pycnidial formation by *P. meliloti*.

Using excised wheat roots, Bjorn *et al.* (1963) reported that light had an inhibitory influence on both cell elongation and cell multiplication. Later on, Bjorn (1967), demonstrated that light was necessary for hydrogen peroxide production via the glycolic acid oxidase system. Weinhold and Hendrix (1963) also found that light inhibited mycelial growth of many fungi. Their evidence indicated that light influenced the production of peroxides which were responsible for the inhibition of mycelial growth in fungi. Somewhat similar deductions can be made from the results obtained with *P. meliloti*. On the basis of the findings that light retarded aerial mycelial growth and promoted pycnidia formation and that various oxidizing agents also had qualitively similar effects, it is tempting to hypothesize that: light induces a retardation in the mycelial growth of *P. meliloti*, which may result in a greater accumulation of certain metabolite(s), presumably strong oxidizing agents, since they have been found to replace the action of light to a certain extent. Whenever cultures incubated in darkness were supplemented with certain peroxides and other oxidants, and whenever conditions which would result in a temporary inhibition of mycelial growth was created, pycnidial production by *P. meliloti* in darkness was induced.

Pigment inhibition in *Aspergillus niger* by dimethyl sulfoxide (DMSO) was reported by Carley *et al.* (1967). They also noted that DMSO did not have any effect on the rate of growth of this fungus. However, in the present studies pigment inhibition was not observed in cultures of *P. meliloti* grown in light. It seems that pigments in *A. niger* and *P. meliloti* are synthesized through different metabolic pathways. With *P. meliloti* DMSO induced pycnidial formation at the lowest concentration, whereas at

the higher concentrations inhibition of mycelial growth was noted (Table 4). Sulfide and sulfhydryl compounds have generally been considered inhibitors of various metal-containing enzymes (White *et al.*, 1968). The inhibition of growth of *P. meliloti* at higher concentrations of DMSO may be due to inhibition of certain metal-containing enzymes, after DMSO reduction.

Phenol even at very low concentrations, and catechol only at high concentrations were found inhibitory to mycelial growth of *P. meliloti* (Table 5). Catechol (at the lowest concentration), salicin, tyrosine, and tryptophan induced pycnidial formation in the dark. Although no positive correlation between the presence of pycnidia and pigmentation was evident, there are indications that when there was an increase in pigmentation, for cultures incubated in the dark, the numbers of pycnidia produced were greater. Light has been found to increase quantitatively the level of synthesis of pigments in a number of fungi, e.g. *Phycomyces blakesleeanus* (Garton *et al.*, 1951), *Penicillium sclerotiorum* (Mase *et al.*, 1957), *Neurospora crassa* (Zalokar, 1955), and *Fusarium oxysporum* (Carlile, 1956). The presence of certain pigments has been reported to increase the oxidation of proteins by 50-fold (Harris, 1926). These effects of photosensitization were considered to result from the oxidation of cellular proteins and enzymes (Simon, 1967). One of the by-products of photooxidation of methionine and histidine has been found to be hydrogen peroxide (Weil *et al.*, 1951). In *P. meliloti* light induces pigmentation, and probably accelerates the oxidation of amino acids, resulting in a greater accumulation of a peroxide(s). Galston (1950), reported that an enzyme, containing heme protein, which had the properties of IAA-oxidase, showed a distinct light-stimulated IAA destruction. The role of this enzyme is in the production of hydrogen peroxide, which is

then utilized by an IAA-peroxidase. Wu (1965) found maximum pigmentation in cultures of *P. meliloti* which were incubated in blue light at a wavelength of about 4400 A. The flavoprotein mentioned by Galston (1950) also had a maximum activity at 4400 A. On the basis of these reports and the results presented in this thesis it can be postulated that induction of pigmentation in *P. meliloti* is in the form of flavoproteins which catalyze the production of hydrogen peroxide and probably other peroxides which would act as oxygen donors for IAA destruction by IAA-peroxidase. This could explain the presence of lesser aerial mycelial growth in the cultures grown in light than in the dark, and also the production of pycnidia in the light. Although some pycnidial induction was found in the dark when various organic compounds were incorporated in the medium, the results were nowhere comparable to the cultures kept in the light. It seems that a system which would oxidize these compounds is lacking in the dark; therefore, an effect similar to that observed due to photooxidation would not be found. It will be of interest to note, the effects on growth and fructification of the incorporation of oxidized forms of various organic compounds such as amino acids, phenols, growth substances, and glycosides in the culture medium.

When other amino acids were substituted for *l*-asparagine as the primary nitrogen source, mycelial growth was drastically reduced. *P. meliloti* was unable to utilize tryptophan and tyrosine and this may have been due to the presence of ring structure in these compounds.

Khan (1966) found that the growth substance IAA could be used to substitute for light in the asexual reproduction of *Sclerotinia fructigena*. However, when IAA was incorporated in the synthetic medium no pycnidial formation by *P. meliloti* was induced in darkness. Therefore,

IAA could not be used as a substitute for light for sporulation by *P. meliloti*.

Control cultures incubated in the dark often had several pycnidia only on the inoculum plug. These pycnidia may be accounted for when one considers that the inoculum was taken from the colonies incubated in the light. Mycelia of the inoculum would therefore have been influenced by light to give an effect similar to that of colonies grown in the light. However, this influence was not transferred to mycelium which formed in the dark. This phenomenon supports Leach's hypothesis (1962) that a photoactivated sporulation precursor moves only a short distance within irradiated mycelium to non-irradiated hyphae of *Ascochyta pisi*.

'Active' horseradish peroxidase in some way stimulated the rate of mycelial growth and pycnidial production over those cultured in the presence of denatured enzyme. According to Macko, Woodbury, and Stahmann (1968) the initial stimulation of germination of uredospores of *Puccinia graminis* f. sp. *tritici* by peroxidase and hydrogen peroxide is due to a change in the integrity of the cell membrane. In these studies horseradish peroxidase was found to have effects similar to those of light in the production of pycnidia for cultures incubated in darkness; this result may be due to a hastening of mycelial maturation resulting in pycnidia production. Increased permeability of hyphal cell walls to substrate could account for this accelerated growth of mycelium and production of pycnidia in the presence of peroxidase. An oxidizing enzyme, fungal tyrosinase, has been reported to increase cell wall permeability of mammalian tissue (Cory, 1967). Permeability was also found to be affected by peroxides (Siegel and Halpern, 1965).

The isolate P_{10}, isolated many years ago by Dearness and Sanford

(1930), no longer produces pycnidia in the presence or absence of light. The presence of some parenchymatous knot-like bodies in cultures of isolate P_{10}, grown on the culture medium plus 'active' horseradish peroxidase, and the accumulation of certain crystaline compounds in and around these bodies was quite unusual. These knot-like parenchymatous bodies may have resulted from increased nutrient uptake because of greater cell wall permeability which resulted from the action of horseradish peroxidase. It seems however, that further development of these knot-like bodies to form pycnidia is controlled through other processes which are now absent, inhibited, or have degenerated in P_{10}.

In the present studies hydrogen peroxide was not added to the peroxidase preparation to promote growth of mycelium of *P. meliloti* as was found necessary by Macko, Woodbury, and Stahmann (1968) for the germination of uredospores of *Puccinia graminis* f. sp. *tritici*. Various oxidants, without the use of enzymes, were found to substitute for light in stimulating germination of spores of common dwarf bunt of wheat (Niemann, 1954 as cited by Rubin and Artiskhovskaya, 1963). Appreciable amounts of peroxide were not found in the growing cultures of *P. meliloti*, and negative results for the presence of peroxides were obtained in cultures incubated in light or darkness when the procedure for the detection of peroxides in growing cultures (Whittenbury, 1964) was followed. However, this does not exclude the possibility of the presence of hydrogen peroxide in the growing cultures of *P. meliloti* since, in the presence of suitable catalysts, hydrogen peroxide and organic peroxides are extremely reactive substances. Because of their high reactivity, these substances are not easily isolated from, or measured in, biological systems (Bjorn, 1967). Therefore, their role must often be deduced by indirect means.

Probably one of the most significant findings of this thesis was that pycnidia matured much faster in those cultures which were grown on medium containing hematin. Normally cultures require about 40-60 days after inoculation before pycnospores are released from pycnidia. With hematin in the medium pycnospores were released 15 days after cultures were started. Although extremely important, this phenomenon was not given further attention since this was not the purpose of this thesis study. However, there is no doubt that further work using this and other compounds will provide much valuable information on maturation of pycnidia.

The behavior of fungal catalase was very much different from that noted above for horseradish peroxidase and beef liver catalase towards the mycelial growth and pycnidial production by *P. meliloti*. The *A. niger* catalase was found inhibitory to the growth of *P. meliloti*. Some comparative biochemical studies of commercial preparations of beef liver catalase and fungal catalases (*Penicillium* and *Aspergillus*) have been carried out by Scott and Hammer (1961), but the effects of these enzymes on growth and fructification of fungi have not been noted. In addition to the data presented by Scott and Hammer, the disc gel electrophoretic analyses showed that the protein patterns of the two catalases and the horseradish peroxidase differed considerably (Fig. 40). The significance of these protein patterns in relation to their effects on growth of mycelium and production of pycnidia is not at present understood.

When a solution containing horseradish peroxidase and fungal catalase was added to the culture medium the response of *P. meliloti* was intermediate to that when either of these enzymes was used alone. In

these cultures numerous pycnidia, although not as numerous as when horseradish peroxidase was used alone, were produced in the dark. In relation to controls i.e. cultures without added enzymes and those containing one of the above enzymes, horseradish peroxidase was only partially inhibited in action which resulted in a decrease in the colony diameter and in the formation of numerous pycnidia. On the other hand, the inhibitory action of fungal catalase to mycelial growth and pycnidia production was only partially suppressed by the activity of horseradish peroxidase. It would appear that a manipulation of amounts or rates of activity of either one or both of these enzymes could inhibit or stimulate mycelial growth and pycnidium formation of *P. meliloti* and possibly other fungi. Of a number of enzymes used by Macko, Woodbury, and Stahmann (1968), catalase was found to have no effect in 24 hr on germination of uredospores of *Puccinia graminis* f. sp. *tritici* and *Uromyces phaseoli*.

The inhibitory activity of the fungal catalase appears to be in the protohaematin portion, since when either protohaematin or protein fractions of commercial *A. niger* catalase, were added to the culture medium, pycnidia production was completely inhibited and the mycelial growth very much reduced. However, in the presence of protein fraction the pycnidia production was like controls, and the mycelial amounts and colony size were only slightly reduced. This slight reduction in the mycelial growth may have been due to trace amounts of protohaematin still associated with the protein fraction. Further evidence that the inhibitory properties attributed to protohaematin fraction is the inhibition of mycelial growth and pycnidia production when the fungus was grown in the soluble fraction of the autoclaved fungal catalase. The co-enzyme may be thermostable, although not determined, since Bingold and Lang (1955) found that the co-

enzyme of a bacterial catalase was thermostable while the apoenzyme was thermolabile. Further experiments involving the elucidation of the nature of co-enzyme inhibition and the nature of protein fraction should receive critical attention. The effects of co-enzymes of other enzymes on fungal growth and fructifications would also be of considerable interest.

In addition to the effect(s) that oxidizing enzymes may have on mycelial growth and fructification of fungi *in vitro*, the activity of each enzyme in separate reactions or the interaction of these enzymes, whether of host and/or pathogen origin may play a major role in pathogenesis. In host-parasite studies peroxidase is generally considered to be involved with the resistance of plants to pathogens (Clare *et al.*, 1966; Wood, 1967; Goodman, Kiraly, and Zaitlin, 1967; Lovrekovich *et al.*, 1968). Catalase (Le Tourneau, 1955) and polyphenol oxidase (Farkas and Kiraly, 1962; Cruickshank and Perrin, 1964; Wood, 1967) have been considered to play a role in disease resistance. However, the mode of action of the above enzymes in host-parasite relations is not known.

Under natural conditions, pycnidia of *P. meliloti* are produced on roots where light would not be the factor directly involved in their initiation; whereas, in cultural studies, light has been shown to promote pycnidium production (Wu, 1965). Therefore, a host metabolite(s) or host-parasite interaction product in the roots replaces the effects of light as observed in the *in vitro* studies. Sanford (1933) and Cormack (1934) found that the roots of legumes required a low temperature (below 10°C) treatment before successful infection with *P. meliloti* could occur. If peroxidase or other oxidizing enzymes are directly involved in promoting growth of the fungus then the enzyme(s) should be at a higher level or more active when plants are dormant under low temperature conditions

which occur during winter season. Sprouts of horseradish roots harvested in the spring or induced to sprout artificially by soaking them in cold running water have been found to give high yields of peroxidase (Maehly, 1955). Keilin and Hartree (1951) found that low temperatures intensify and sharpen peroxidase absorption bands of solutions and tissue slices of horseradish roots. Wounding of tap and large secondary roots or the breaking of root hairs by soil heaving as a result of frost action may increase peroxidase levels in roots and thus stimulate the fungus to grow rapidly, and also enhance pycnidium production. If *P. meliloti* can be used as an example, the growth and fructification of other facultative and saprophytic fungi could be promoted and/or inhibited in living and/or dead host tissue where peroxidase and other oxidizing enzymes are still biochemically and possibly biologically active.

The separation of soluble proteins extracted from fungal mycelium using starch (Clare, 1963; Clare and Zentmyer, 1966; Hall, 1967) and polyacrylamide gels (Durbin, 1966; Gottleib and Hepden, 1966) has been used principally for taxonomic purposes. Whitney *et al.* (1968) found that the protein patterns for species of *Verticillium albo-atrum, V. dahliae,* and *Fusarium oxysporum* respectively varied considerably in their composition with regard to the age of cultures. Sekhon (1969) reported that protein patterns of a low temperature basidiomycete also varied depending upon the incubation temperatures and age of cultures. However, information on the changes in proteins of fungus mycelium cultured in light and darkness have not been reported. Except for the variations in the staining intensities of individual protein bands and the presence of an additional band in the cultures incubated in the dark, the 10-day old cultures of *P. meliloti* kept in light or darkness showed a

similar protein pattern. As the cultures grew older, the changes in protein composition were more in light than in the dark-grown cultures. The relationship of these changes to pycnidial production in the light is not clear at this stage. However, more changes in the light-grown cultures may be used as an evidence to support the hypothesis given previously that photooxidation of certain amino acids will change the protein complement in mycelia cultured in the light.

Presence of more peroxidase isozyme bands in cultures incubated in the light may also be used as an evidence for the role of peroxidase in pycnidia production by *P. meliloti* in the cultures incubated in the light. It will be of interest to note the quantitative levels of various oxidizing enzymes in cultures grown in light or darkness. These studies will be very necessary to substantiate the findings of the role of oxidizing enzymes in growth and fructification in light or darkness. The limitation of such studies at the moment with *P. meliloti* is that the fungus cannot be grown to obtain a fungus mycelium of a similar physiological age. In shake cultures, a uniform growth of mycelium cannot be obtained, since *P. meliloti* always tends to form large clumps or pellets rather than filamentous mycelium. Therefore, development of a technique for obtaining a more uniform type of mycelial growth would be very beneficial.

It is very difficult to integrate, in this discussion, the results on the physiology and biochemistry of sweet clover varieties Arctic and Denta with the studies carried out on the root pathogen *P. meliloti*. Studies on the host, which followed chronologically the fungal studies, were carried out to determine whether the data resulting would support the data originating from the studies with the fungus. In these studies, a host-parasite relationship was not established. This was due in part

to the fact that infection studies have seldom been successful, and when successful, the results have been very erratic (Cormack, 1934; Netolitzky, 1965; Colotelo, 1969). These studies can in no way substitute for a host-parasite relationship; therefore, until a more reliable method for successful infections is obtained no conclusive evidence can be provided. However, a discussion to associate the results obtained on the fungus physiology and host physiology and biochemistry at low temperatures is still worthwhile.

The changes in the soluble protein levels have been associated with winter-hardy and non-hardy properties of many plants. Siminovitch and Briggs (1949) found that cold-hardiness of cambial cells of black locust tree increased with increasing water soluble protein contents. Similar associations have been reported in winter wheat (Pauli *et al.*, 1961; Zech and Pauli, 1960), alfalfa (Bula and Smith, 1954; Wilding *et al.*, 1960; Gerloff *et al.*, 1967), red clover and sweet clover (Bula and Smith, 1954; Hodgson and Bula, 1956). The higher soluble protein values for roots of winter-hardy sweet clover variety Arctic, as compared to non-hardy variety Denta, agree with the results of the above workers.

Appreciable differences in levels of proteins between roots harvested during fall, winter, and spring were not evident when calculations were based upon dry weights. However, on fresh weight basis the total soluble protein values for the winter months were appreciably higher than those obtained during fall and spring harvests. These results are quite in agreement with dry weight changes during these periods. An increase or a decrease in the dry weights would mask a respective increase or a decrease in protein values if the calculations were to be based upon dry weights. Considering dry weight changes during the overall period of harvesting, which were higher for winter harvests and very low for the

harvests during spring, a true presentation of protein changes would be better expressed if calculations were to be based upon fresh weights.

Because of the importance of various oxidizing enzymes in the regulation of disease resistance (Goodman *et al.*, 1967; Wood, 1967; Stahmann *et al.*, 1968) a study dealing with changes in the levels of these enzymes is of a particular interest. Gerloff *et al.* (1967) observed that peroxidase and catalase activities increased during hardening of alfalfa roots, but only small differences between hardy and non-hardy varieties were detectable. In sweet clover variety Denta, which is non-winter hardy and susceptible to *P. meliloti*, peroxidase activities were slightly higher than for variety Arctic which is winter-hardy and resistant to *P. meliloti*. However, a direct relationship was not found between soil temperatures and the peroxidase activities of the two varieties.

Peroxidase activities of plant tissues have been reported to increase due to the presence of IAA (Galston and Dalberg, 1954; Siegel and Galston, 1967). In addition several peroxidase isozymes have been shown to undergo quantitative alterations by gibberellic acid application to dwarf corn plants (McCune, 1960; Siegel, 1955). These reports can be used to explain the increases found in the peroxidase levels of roots of both sweet clover varieties which occurred at the same time the buds were undergoing morphological changes. General levels of hormones and auxins are quite high when the buds are growing rapidly. During this rapid growth phase these hormones may be involved in the increases in peroxidase activity. Conversely peroxidase has been found to destroy IAA (Goldcare, 1961). This mutual interaction between hormone and enzyme may be important in the regulation of growth. It is quite likely that enzyme

induction by endogenous substrates or by structurally-related molecules in the cells is a universal phenomenon whereby the adjustment of enzyme levels is normally accomplished (Cohn and Monod, 1953). The greater adaptive induction of IAA-oxidase (Galston and Dalberg, 1954) would inactivate IAA resulting in a decrease in peroxidase activity. Shifts in the balance of IAA-oxidase and peroxidase relationships could explain the increases which occurred above the generally prevailing levels during these studies.

Activities of catalase and polyphenol oxidase were higher in Arctic than in Denta. The levels of catalase, but not polyphenol oxidase activity, were higher during early and mid-winter as compared to fall and spring. These results agree with the findings of Gerloff *et al.* (1967) who found that catalase activity of alfalfa roots showed marked increases in December as compared to the earlier harvests; however, repeated analyses during the winter months and extending into spring were not carried out by them. These studies also support the earlier work of Protsenko and Polishchuck (1948, as cited by Vasil'yev, 1956), who found that higher catalase activity occurred in the most cold-resistant winter wheat variety, Minhardi, than in the least cold-resistant variety, Fulcaster. An increase in catalase, polyphenol oxidase and the second increase in the peroxidase activities occurred at the time buds were growing actively. A study relating activities for peroxidase, catalase, polyphenol oxidase, and growth substances would be very helpful to explain the role of these enzymes during the rapid growth of buds particularly at low temperatures.

From the quantitative studies on proteins, peroxidase, catalase, and polyphenol oxidase enzymes, it is evident that these macromolecules are always undergoing changes in their levels. It was earlier concluded

that a manipulation of amounts or rates of activities of many oxidizing enzymes could inhibit or stimulate fungal growth. Since the oxidizing enzymes and proteins are always changing in their amounts in sweet clover roots, the resistance or susceptibility of different varieties could depend upon ratios of these enzymes, unfavourable or favourable to the growth of *P. meliloti*. Therefore, these combinations and not the levels of single oxidizing enzymes will in part determine pathogenic or non-pathogenic host-parasite interaction.

Craker *et al.* (1969) demonstrated the electrophoretic protein band patterns changed at times when there were changes in hardiness in apple bark and arborvitae foliage. Coleman *et al.* (1966), and Gerloff *et al.* (1967) had also found that soluble proteins extracted from alfalfa roots had different electrophoretic protein patterns in winter-hardy and non-hardy varieties. In these studies differences in electrophoretic protein patterns were also observed between Arctic and Denta varieties during the period from October 8, 1968 to June 17, 1969. However, the conclusions of the above mentioned workers were based on a study during a very limited period, dealing with only a very few harvests during the early parts of winter (Coleman *et al.*, 1966; Gerloff *et al.*, 1967) or during spring (Craker *et al.*, 1969), unlike the more detailed studies reported in this thesis in which changes throughout hardening and dehardening were carried out.

The interpretation of these results on the basis of qualitative protein changes to hardiness is quite complicated. As noted by Craker *et al.* (1969) the complications could arise because of:

i. The protein bands observed on gels represent only those proteins present in sufficient amounts to be detected. The presence or absence

of certain very faint bands indicated qualitative changes insofar as the procedure is capable of revealing their presence.

ii. These protein changes may reflect physiological changes other than those involved in hardiness or non-hardiness. These changes could be a reflection of the ontogenetic changes occuring in the buds of sweet clover varieties Arctic and Denta throughout winter.

These protein changes were found to be more erratic in Denta than in Arctic during the cold acclimation. Also, the maximum number of protein bands in Denta was attained later than in Arctic. It is quite possible that in winter-hardy and non-hardy varieties similar physiological responses occurred due to the changes in the temperatures, but apparently the resulting changes in Arctic were attained sooner than in Denta. Most of the protein band changes occurred during the early part of the studies. Similar observations were made by Craker *et al.* (1969). Another striking characteristic of the electrophoretic protein pattern changes was a greater variation in the upper portions of the gels. This indicates that cathodic and/or relatively higher molecular weight proteins were more susceptible to changes in temperatures. Although the electrophoretic gels were divided into arbitrary sections A, B, C, and D, the relationship between the protein patterns in each section and changes in temperatures and ontogeny are not apparent.

The peroxidase isozyme patterns also showed changes throughout the period of harvesting of sweet clover roots. The changes in Denta were more common than noted for Arctic. However, no relationship between these changes and cold-hardiness was evident. Roberts (1969) did not find any major qualitative change in peroxidase isozyme between wheat plants grown at 6°C or 20°C. However, there was an increase in

one of the anionic peroxidase isozyme bands at the lower temperature. Gerloff *et al.* observed two more isozyme bands in alfalfa roots harvested in December as compared to those harvested earlier. No direct relationship between the number of bands and quantitative levels of peroxidase in sweet clover roots at a particular time was evident.

The intimate association of proteins, either as components of enzyme systems or in the structural organization of cellular organelles, with cellular metabolism implicates their important role in the physiological adaptation of plants to the environmental stresses. Therefore, in addition to quantitative levels of these substances, qualitative changes could also determine the adaptability of plants to their surroundings. Thus, both quantitative and qualitative changes in protein and enzymes of sweet clover roots would not only determine their cold hardiness but also the resistance or susceptibility to the attacks of pathogens.

Considerable attention has been focused on the relationship between changes in free amino acid contents and the development of cold-hardiness in plants. Wilding *et al.* (1960) reported a 20% increase in free amino acids in winter-hardy alfalfa variety Vernal; whereas, very little changes occurred in the non-hardy variety. In the present studies, with sweet clover roots, an increase in the amounts of total free amino acids was also noticed with a decrease in the soil temperatures. A 25% increase in the total free amino acid values was observed from October to December in roots of Arctic. The increases in Denta were very little at this stage. Buds of Denta variety seemed to be in a stage of growth whereas they were dormant in the variety Arctic at this time of the year. The possible explanation for the low amounts of free amino acids in the variety Denta as compared to the variety Arctic may be the trans-

location of amino acids from the roots to the buds to be utilized for growth. On the other hand in the roots of Arctic there may be a turnover of amino acids but no translocation.

The initial two- to three-fold increases in arginine, glutamic acid, proline, and isoleucine in the roots of Arctic indicate that the increases in the levels of these amino acids are important in determining winter-hardiness. Zech and Pauli (1962) also found that the amounts of these amino acids (except proline) increased greatly between November and January in the winter-hardy winter wheat variety. Wilding *et al.* (1960) found relatively higher amounts of proline in roots of winter-hardy alfalfa as compared to a non-hardy variety. Similar observations were made in these studies. The increases in the amounts of histidine and serine in Arctic and aspartic acid in Arctic and Denta occurred towards the end of winter. This suggests that increased quantities of these amino acids reflect changes other than those associated with cold-hardening. In these studies the presence of amide forms of aspartic acid and glutamic acid were not determined. In the analysis of free amino acids, any amides present would have been transformed into their respective amino acid due to acid hydrolysis. The determination of the presence and role of these amides in the root metabolism particularly with carbohydrates at low temperatures, and their possible relationship to the pathogenicity of *P. meliloti* would be of considerable interest.

Although both quantitative and qualitative changes in soluble proteins have been implicated in winter hardiness of many plant species (Siminovitch and Briggs, 1949; Zech and Pauli, 1960; Wilding *et al.*, 1960; Toman and Mitchell, 1968), changes in protein amino acids in

sweet clover roots, associated with the soluble protein changes during different seasons have not been reported. When analyses of protein amino acids in sweet clover roots at different times of the year were made, it was found that an increase in protein amino acids occurred from October to December. The total amounts of protein amino acids were much higher in Arctic than in Denta, which agrees with the higher soluble protein values in Arctic than in Denta. However, the percentage increase in the protein amino acids at various harvesting dates was not much higher in Arctic as compared to Denta. It appears that responses due to the onset of cold weather are similar in the two varieties, but it may be the relatively higher amounts of protein amino acids already present in Arctic which places it at more advantageous position to cope with adverse conditions than Denta. Denta has considerably lower soluble protein amino acid contents than Arctic. It is hard to say whether quantitative changes in free amino acids and/or protein amino acids determine cold-hardiness of plant species. Colemen *et al.* (1966) found that proteins which had antigenic properties were involved in the cold-hardening behaviour of alfalfa roots. They postulated that if these antigens were present in the protein complement of the non-hardened plant, they were present in insufficient quantities to produce a strong antibody reaction. Therefore, considering this postulation, it may be said that an increase in protein amino acids and a greater increase in particular free amino acids necessary for the synthesis of certain proteins, having antigenic properties, would determine winter-hardiness of any species.

Winding *et al.* (1960) found a great increase in sugars of both winter-hardy and non-hardy varieties of alfalfa from August to December.

The studies with sweet clover showed that no changes in sucrose occurred in both varieties due to changes in temperatures. Both glucose and fructose were present in higher amounts in Arctic than in Denta. However, over the period of harvesting the two sugars showed an entirely opposing tend. Associated with increases in glucose values from October to December was a decrease in the amounts of fructose, and when a decrease in the values of glucose occurred, the fructose values started to increase. Since sucrose values did not change appreciably, it seems that these two sugars are existing in an equilibrium, or the enzyme involved in their conversion is changing quantitatively with changes in temperatures. These results are contrary to those of Russian workers mentioned by Vasil'yev (1956). They observed a decrease in the temperature during winter caused a hydrolysis of sucrose, increasing the monosaccharide contents, and as the temperature rose the amounts of sucrose increased with a concomitant decrease in monosaccharides. He concluded that starch would be able to increase winter-hardiness only when converted to monosaccharides. In winter wheat the most important sugars were considered to be monosaccharides with sucrose acting simply as a reserve carbohydrate. However, Vasil'yev (1956) concluded that quantitative amounts of sugars alone would not necessarily make a particular variety winter-hardy.

In addition to the role these sugars might have in cold-hardiness, sugars have been shown to stimulate peroxidase synthesis (Parish, 1969). It is not clear whether this stimulatory effect is due to increased synthesis of the enzyme or due to an enhancement of peroxidase activity by sugars as reported by Siegel and Siegel (1960). They postulated that sugars provide a concentrating surface, hence increasing the probability

of reaction, and render adsorbed peroxidase more efficient as a catalyst. Whatever the interaction phenomenon is, the catalytic properties of peroxidase is influenced in part by carbohydrates.

Changes in carbohydrate and amino acids are likely to influence quantitatively and qualitatively peroxidase levels, perhaps other oxidizing enzymes, and certain proteins. These changes would not only determine cold-hardiness of sweet clover but also resistance to pathogens. It would appear that a qualitative and quantitative interrelationships between carbohydrates, nitrogenous substances which include, free amino acids, protein amino acids, enzymes and proteins, and growth substances are necessary before plants become winter-hardy and resistant to various plant pathogens.

In relating the effects of various oxidizing enzymes on the mycelial growth and pycnidia production by *P. meliloti* and the levels of these enzymes in the sweet clover varieties Arctic and Denta at various intervals of harvesting, it may be concluded that the higher catalase activities in the resistant variety Arctic support the evidence for the inhibition of mycelial growth and pycnidia production by *P. meliloti* by fungal catalase. The susceptible variety Denta had only a slightly higher peroxidase activity as compared to the variety Arctic; therefore, the role of host peroxidase has not been clearly shown. Of course it must be realized that the peroxidase and catalase of sweet clover varieties may be biologically and possibly biochemically different than the commercial preparations of horseradish peroxidase, *A. niger* catalase, and beef liver catalase used in the fungal studies.

SUMMARY

1. Suppression of aerial mycelial growth of *Plenodomus meliloti*, by reducing the quantities of nutrients or by the addition of various oxidizing compounds in the medium, was associated with pycnidia production in the dark.

2. In the presence of horseradish peroxidase, beef liver catalase, polyphenol oxidase numerous pycnidia were produced in light and darkness, and mycelial growth was accelerated.

3. *Aspergillus niger* catalase inhibited mycelial growth and pycnidia production of *P. meliloti*. The inhibitory factor was found to be present in the co-enzyme fraction of the enzyme.

4. In the presence of hematin the pycnidia released pycnospores much faster as compared to controls.

5. The protein and peroxidase isozyme patterns of mycelial extracts from cultures incubated in light and darkness were different for the two treatments.

6. A greater increase in protein, soluble amino acids, and protein amino acids occurred in the winter-hardy sweet clover variety Arctic as compared to the non-hardy variety Denta with a decrease in the soil temperature.

7. The levels of protein, catalase, polyphenol oxidase, soluble amino acids, protein amino acids, and carbohydrates were higher in Arctic than in Denta.

8. The changes in protein and peroxidase gel electrophoretic patterns were more erratic in Denta as compared to Arctic during the period of harvestings between October, 1968 and June, 1969.

9. Peroxidase levels in the variety Denta were only slightly higher than in Arctic.

10. The buds of variety Denta were undergoing morphological changes throughout winter; whereas, those of Arctic did not show such a change until April, 1969.

11. In the discussion attempts were made to correlate fungal studies with data obtained from separate biochemical studies made on the host roots.

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APPENDIX I

The composition of the basic synthetic medium used in the present investigation:

Major nutrients:

D- glucose (Fisher Sci.)	15.00 g
l- asparagine (K & K Labs.)	2.36 g
KH_2PO_4	1.00 g
$MgSO_4 \cdot 7H_2O$	0.50 g

Minor nutrients:

$Fe_2(SO_4)_3$	0.715 mg
$ZnSO_4 \cdot H_2O$	0.540 mg
$Co(NO_3)_2 \cdot 6H_2O$	0.245 mg
$MnCl_2 \cdot 4H_2O$	0.135 mg
$H_2MoO_4 \cdot H_2O$	0.085 mg
$CuSO_4 \cdot 5H_2O$	0.195 mg
$CaCl_2$	4.000 mg

The above constituents were dissolved in one liter of deionized distilled water to which was added 17.0 g agar agar (Difco). The pH of the medium was adjusted to 6.0 with 1 N KOH before the agar was added. The medium was autoclaved for 10 mins at 121°C.

Daily minimum and maximum soil temperatures at a depth of 13 cm from the ground level from August, 1968 to June, 1969. The temperature recorder was placed in the center of a sweet clover field at the University Parkland Farm.

Date	Min.	Max	Date	Min.	Max.
Aug. 30, 1968	9.5	15.0	Sept. 24, 1968	3.0	7.0
31	10.0	13.5	25	3.5	4.0
Sept. 1, 1968	9.5	12.5	26	3.5	7.5
2	7.5	9.5	27	3.5	7.5
3	7.0	9.0	28	4.0	8.0
4	7.0	11.0	29	5.0	9.5
5	8.0	10.5	30	5.0	8.5
6	9.0	12.0	Oct. 1, 1968	2.0	6.5
7	9.5	12.0	2	1.0	6.0
8	10.0	13.0	3	3.0	5.0
9	10.5	15.0	4	2.5	6.0
10	10.5	15.5	5	2.0	6.5
11	11.0	14.5	6	3.5	5.5
12	10.0	14.5	7		
13	8.0	11.0	8	1.0	3.5
14	9.0	13.5	9	1.5	2.0
15	8.0	10.5	10	1.5	2.5
16	8.0	12.5	11	2.0	3.0
17	5.5	11.0	12	0.5	4.0
18	5.5	0.5	13	0.5	4.0
19	2.5	5.5	14	0.0	2.5
20	3.5	5.0	15	0.0	2.0
21	3.5	4.0	16	0.0	3.0
22	3.5	5.5	17	2.0	3.0
23	3.0	6.5	18	0.5	4.0

Date	Min.	Max.	Date	Min.	Max.
Oct. 19, 1968	0.0	2.5	Nov. 19, 1968	-4.0	-3.0
20	0.0	2.5	20	-4.0	-3.0
21	0.0	2.0	21	-4.0	-4.0
22	-0.5	2.5	22	-4.0	-3.0
23	-1.0	0.0	23	-5.0	-3.0
24	0.0	2.0	24	-5.0	-4.0
25	1.0	3.0	25	-5.0	-5.0
26	0.0	3.0	26	-4.5	-4.0
27	-1.0	3.0	27	-5.0	-4.0
28	-1.0	1.0	28	-5.0	-3.5
29	1.0	2.5	29	-5.0	-3.5
30	0.5	3.0	30	-6.5	-4.5
31	-1.0	2.0	Dec. 1, 1968	-7.0	-6.0
Nov. 1, 1968	-1.0	0.5	2	-7.0	-6.0
2	-2.0	-1.0	3	-7.0	-5.5
3	-2.0	-1.0	4	-8.0	-7.0
4	-2.0	-1.0	5	-8.0	-7.0
5	-2.0	-1.5	6	-7.5	-7.0
6	-3.0	-2.0	7	-7.0	-6.5
7	-2.5	-2.0	8	-7.0	-6.0
8	-2.5	-2.5	9	-6.0	-6.0
9	-3.0	-2.5	10	-7.0	-6.0
10	-4.0	-2.5	11	-7.0	-6.5
11	-4.0	-3.0	12	-7.0	-6.5
12	-3.0	-2.0	13	-7.0	-7.0
13	-3.5	-2.5	14	-7.0	-6.0
14	-3.0	-2.0	15	-7.0	-6.0
15	-4.0	-3.0	16	-7.0	-6.0
16	-4.5	-3.5	17	-6.5	-6.0
17	-5.0	-3.5	18	-6.5	-6.0
18	-5.0	-3.5	19	-6.0	-6.0

Date	Min.	Max.	Date	Min.	Max.
Dec. 20, 1968	-7.0	-6.0	Jan. 21, 1969	-9.0	-7.0
21	-7.0	-6.0	22	-9.0	-7.5
22	-7.0	-7.0	23	-9.5	-8.5
23	-7.0	-7.0	24	-9.0	-8.5
24	-7.5	-7.0	25	-9.0	-8.5
25	-7.0	-7.0	26	-9.0	-9.0
26	-7.5	-7.0	27	-9.0	-8.5
27	-8.0	-7.5	28	-9.0	-8.0
28	-8.0	-8.0	29	-9.5	-9.0
29	-8.0	-8.0	30	-9.5	-9.0
30	-8.0	-7.0	31	-9.5	-9.0
31	-7.0	-7.0	Feb. 1, 1969	-9.0	-8.5
Jan. 1, 1969	-8.0	-7.0	2	-8.5	-8.0
2	-8.0	-7.5	3	-8.0	-7.5
3	-7.5	-6.5	4	-7.5	-7.0
4	-6.5	-6.0	5	-7.5	-7.0
5	-7.0	-6.0	6	-7.0	-7.0
6	-7.5	-7.0	7	-7.0	-7.0
7	-8.0	-7.0	8	-7.0	-6.5
8	-9.0	-8.0	9	-7.0	-6.5
9	-9.0	-8.5	10	-7.5	-6.5
10	-9.0	-8.5	11	-6.0	-6.0
11	-8.5	-8.0	12	-6.0	-6.0
12	-8.5	-8.0	13	-6.0	-6.0
13	-8.0	-7.5	14	-6.0	-5.5
14	-8.0	-7.5	15	-6.0	-6.0
15	-8.0	-7.5	16	-6.0	-6.0
16	-8.0	-8.0	17	-6.0	-6.0
17	-8.0	-7.5	18	-6.5	-6.0
18	-8.0	-8.0	19	-6.5	-6.0
19	-8.5	-8.0	20	-6.5	-6.0
20	-9.0	-8.5	21	-6.5	-6.0

Date	Min.	Max.	Date	Min.	Max.
Feb. 22, 1969	-7.0	-6.0	Mar. 26, 1969	-5.0	-4.0
23	-7.0	-6.5	27	-5.0	-4.0
24	-7.0	-7.0	28	-5.0	-4.0
25	-7.5	-7.5	29	-4.0	-4.0
26	-7.5	-7.0	30	-4.0	-3.5
27	-7.0	-6.5	31	-3.5	-3.0
28	-6.5	-6.0	Apr. 1, 1969	-3.5	-3.0
Mar. 1	-7.0	-6.5	2	-4.0	-2.5
2	-8.0	-7.0	3	-4.0	-2.5
3	-8.0	-8.0	4	-4.0	-3.0
4	-6.0	-6.0	5	-4.5	-3.0
5	-6.0	-5.5	6	-5.0	-3.0
6	-6.0	-5.0	7	-5.0	-4.0
7	-5.5	-5.0	8	-3.0	-2.0
8	-5.5	-5.0	9	-3.5	-1.5
9	-6.0	-5.0	10	-2.5	2.5
10	-6.0	-5.0	11	-2.0	4.0
11	-6.0	-5.0	12	0.0	4.5
12	-6.0	-5.0	13	0.0	6.0
13	-6.0	-5.0	14	0.0	4.0
14	-5.0	-5.0	15	-1.0	3.5
15	-5.0	-4.5	16	2.0	5.5
16	-5.0	-5.0	17	3.0	8.0
17	-5.0	-5.0	18	0.5	6.0
18	-4.5	-4.0	19	-0.5	4.0
19	-5.0	-4.0	20	-1.0	3.0
20	-4.5	-4.0	21	2.0	4.0
21	-4.5	-4.0	22	2.0	4.0
22	-4.5	-4.0	23	2.0	8.0
23	-4.5	-4.0	24	0.0	4.5
24	-5.0	-4.0	25	-2.0	0.0
25	-5.0	-4.0	26	-2.0	-1.0

		Min.	Max.			Min.	Max.
Apr.	27, 1969	1.0	4.0	May	23, 1969	12.0	18.0
	28	3.0	7.0		24	14.0	19.0
	29	2.0	7.5		25	14.0	19.5
	30	2.5	7.5		26	12.0	16.5
May	1, 1969	4.0	10.0		27	9.0	11.5
	2	5.0	11.0		28	9.5	14.5
	3	3.0	7.0		29	10.0	14.0
	4	3.0	9.0		30	9.5	13.0
	5	2.0	7.0		31	10.0	17.5
	6	3.0	9.0	June	1	12.5	17.0
	7	4.0	11.0		2	13.5	19.5
	8	7.0	12.0		3	15.0	19.0
	9	6.0	13.0		4	16.0	20.0
	10	9.0	15.0		5	15.5	18.5
	11	9.5	16.0		6	8.0	16.5
	12	9.5	16.0		7	9.0	16.5
	13	9.0	15.0		8	14.0	17.0
	14	9.0	13.0		9	15.0	21.0
	15	5.5	9.5		10	14.0	17.0
	16	5.5	8.5		11	12.0	15.0
	17	6.0	10.5		12	12.5	15.0
	18	7.0	11.5		13	13.5	16.0
	19	7.0	11.0		14	14.0	17.5
	20	9.0	13.0		15	15.0	18.0
	21	11.5	15.0		16	15.0	19.0
	22	11.5	16.5		17	14.0	20.0