THE USE OF HIGH-CO2 ATMOSPHERES TO STUDY DEFENSE METABOLISM OF *PHASEOLUS VULGARIS* IN RESPONSE TO PATHOGENESIS BY *COLLETOTRICHUM LINDEMUTHIANUM*.

by

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The use of high-CO_2 atmospheres to study defense metabolism of *Phaseolus vulgaris* in response to pathogenesis by *Colletotrichum lindemuthianum*.

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ABSTRACT.

Atmospheres containing a high concentration of CO_2 can prevent lesion development and coalescence in etiolated hypocotyls of the French bean, Phaseolus vulgaris, infected with a compatible race of the anthracnose fungus, Colletotrichum lindemuthianum. The degree of protection depended on the stage of development of the interaction at the time treatment with CO_2 was begun and on the duration of such treatment. The rate of colonization of host cells by infection hyphae of the fungus was reduced in 15% CO_2. Flecks, similar to those resulting from an incompatible interaction, developed when treatment with 15% CO_2 was begun before penetration by the pathogen had occurred, and when treatment was maintained until symptoms appeared. Hypocotyls showing this response were resistant to a second inoculation with a compatible race of the anthracnose fungus carried out in air. A resistant response was also obtained when CO_2 treatment was begun during the early stages of penetration. A susceptible response resulted, however, when infection hyphae had grown beyond the first cell colonized by the time CO_2 treatment was begun.

Treatment of cultures of C. lindemuthianum with 15% CO_2 resulted in delayed germination of conidia, a reduced rate of germ tube elongation, and delayed sporulation.

Treatment with 15% CO2 prevented the accumulation of phaseollin, an antifungal substance implicated in defense metabolism of beans, at sites of freezing injury on etiolated hypocotyls. The inhibitory effect was partially reversed, however, when injured plants were returned to air following CO2 treatment. In contrast, CO2 did not affect the accumulation of phaseollin at incompatible infection sites.

The results suggest that the protective effect of CO2 during a compatible interaction is exerted primarily via its action on the pathogen. They also indicate that the biotic and abiotic stimuli for phaseollin accumulation studied here differ in that the former is CO2-insensitive and the latter is CO2-sensitive.

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The investigation reported in this thesis was carried out under the guidance of Dr.J.E. Rahe for whose unfailing help and advice I am grateful. My thanks are also due to Drs. G.R. Lister and C.L. Kemp for valuable suggestions during the course of this work, and to Dr. Lister for the generous long-term loan of some of the equipment which made this research possible.

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INTRODUCTION TO THE THESIS

The host-parasite interaction between the green bean, Phaseolus vulgaris, and the anthracnose fungus, Colletotrichum lindemuthianum, has been studied extensively in recent years. Among the advantages this interaction has for study are the facts that the fungus is readily cultured on nutrient media and it exists as several fairly stable physiological races, each of which can parasitize only certain cultivars of P. vulgaris.

The interaction is very suitable for the study of the post-infectional changes in a plant that are brought about by contact with a pathogen. All available evidence indicates that the cellular environment of the host that is encountered by infection hyphae of the fungus during the early stages of penetration is equally favorable irrespective of whether the ultimate outcome of the interaction is one of susceptibility or resistance. That the cellular environment becomes progressively less favorable to cultivar-incompatible races of the fungus is apparent from the necrosis and plasmolysis of host cells, the production of fungitoxic compounds at infection sites, and the cessation of growth of infection hyphae, all of which are observed soon after penetration has occurred. In compatible interactions, however, the host environment remains favorable for growth of infection hyphae for a

sufficiently long period to enable the fungus to sporulate and thus successfully complete its life-cycle. The mechanism by which each cultivar of P. vulgaris differentiates between a compatible and an incompatible race of the anthracnose fungus, and stops the growth of the latter, is not known. Good circumstantial evidence exists that the host defense response in this system involves the production of fungitoxic metabolites such as the pterocarpan phaseollin and its structural analogues, though direct proof of such an involvement is lacking.

The overall objective of the research described in the thesis was to develop a system capable of providing information about defense metabolism of beans during parasitism by the anthracnose fungus. In all experiments etiolated seedlings of P. vulgaris, cv. Topcrop, were used. This cultivar is susceptible to the beta race and resistant to the gamma race of C. lindemuthianum.

The project owes its origin to work reported by Rahe (62) in which pods or etiolated hypocotyls of P. vulgaris inoculated with a race of C. lindemuthianum to which they were susceptible failed to develop symptoms when kept in sealed incubation chambers. Symptoms did appear on inoculated tissues, however, when the sealed chambers were provided with KOH solutions. It was suggested that an increase in the level of carbon dioxide might be the factor inhibiting symptom development in sealed chambers not

provided with alkaline solutions. All the work of the thesis has derived from the initial finding that the nature of the host-parasite interaction in this system can be changed from one of susceptibility to one of resistance in the presence of an atmosphere containing a high concentration of CO2.

CHAPTER I

EFFECTS OF 15% CO_2 ON THE SUSCEPTIBILITY OF *PHASEOLUS VULGARIS* TO *COLLETOTRICHUM LINDEMUTHIANUM*

ABSTRACT

Changes in the nature of the interaction between Phaseolus vulgaris and a compatible race of Colletotrichum lindemuthianum were obtained by treatment of inoculated plants with 15% CO2 in air. A shift towards resistance, manifested by the appearance of flecks similar to those which develop in a natural incompatible interaction, occurred on plants placed in CO2 during the early stages of fungal penetration and maintained there until symptoms appeared. Almost complete reversal of the interaction was obtained when the density of infection sites was sufficiently great to give coalescent necrosis in controls that were not CO2-treated. Increasing susceptibility occurred when CO2 treatment was begun at later stages of development of the interaction. Histological studies showed that the rate of colonization of host cells by hyphae of the fungus was reduced in the presence of 15% CO2. These results indicate that CO2 may change the nature of the interaction to one of resistance by slowing down the growth of the fungus while allowing a defense response of the host to develop to a degree where it can prevent further spread of fungal hyphae.

INTRODUCTION

Atmospheres containing high concentrations of carbon dioxide are often used during transport of highly perishable fruits such as strawberries and sweet cherries. A reduction in the rate of the ripening process and a delay in softening have been commonly observed under these conditions. In addition, a fungistatic effect of CO2 on postharvest pathogens has been described. The large volume of literature on this topic has been reviewed by Smith (76) and by Eckert and Sommer (30). Experiments by Rahe (62) showed that hypocotyls or pods of the bean (Phaseolus vulgaris L.) inoculated with a compatible race of the anthracnose fungus (Colletotrichum lindemuthianum (Sacc.& Magn.) Scribner) did not develop symptoms when maintained in sealed incubation chambers under conditions that were described as anaerobic. Symptoms developed more or less normally, however, if the chambers were provided with a wick soaked in KOH solution, suggesting that the absence of symptoms in sealed chambers not containing KOH was an effect of an increase in the concentration of CO2. The objectives of the present investigation were to determine whether atmospheres containing high concentrations of CO2 could influence the interaction between P. vulgaris and a compatible race of C. lindemuthianum and, if so, to investigate the mode of action of CO2.

MATERIALS AND METHODS

Growth of Plants

Bean seedlings were grown in vermiculite in plant pots (diameter 11.5 cm). The vermiculite was moistened to field capacity with Arnon - Hoagland nutrient solution and autoclaved before use. Pots were cleaned by immersion in aqueous sodium hypochlorite (containing approximately 1% available chlorine) and were rinsed with distilled water. Seeds of P. vulgaris, cv. Topcrop, of uniform weight (350±50 mg) were surface sterilized for three minutes in aqueous sodium hypochlorite (containing approximately 1% available chlorine) and washed with sterile water. Seeds with swollen or cracked testas were rejected. The remainder were planted aseptically onto the surface of 3 cm layers of vermiculite in the plant pots and covered with 4 cm layers of vermiculite. Pots containing growing plants were kept in plexiglass boxes (90x50x38 cm) covered with black polyethylene sheet plastic. The atmosphere inside each box was stirred continuously by means of a magnetically driven fan, and was maintained at high humidity by the evaporation of water from absorbent paper sheets which were hung from a frame within each box. Air, or 15% CO2 in air, was passed continuously through the boxes at a flow rate of 500 ml per minute. Mixtures containing 15% CO2 in air, were generated from individual cylinders of pure CO2 and compressed air

using a precision gas-mixing pump. (H. Wosthoff O.H.G., Bochum, W. Germany, type NA 18/3A). The effluent gas from the chambers was passed through Drierite and its CO2 content monitored using an infra-red gas analyzer (Beckman, model 215). The methods used to generate and monitor the CO2-containing atmospheres used are described more fully in the appendix to the thesis (p. 90).

Inoculation procedure

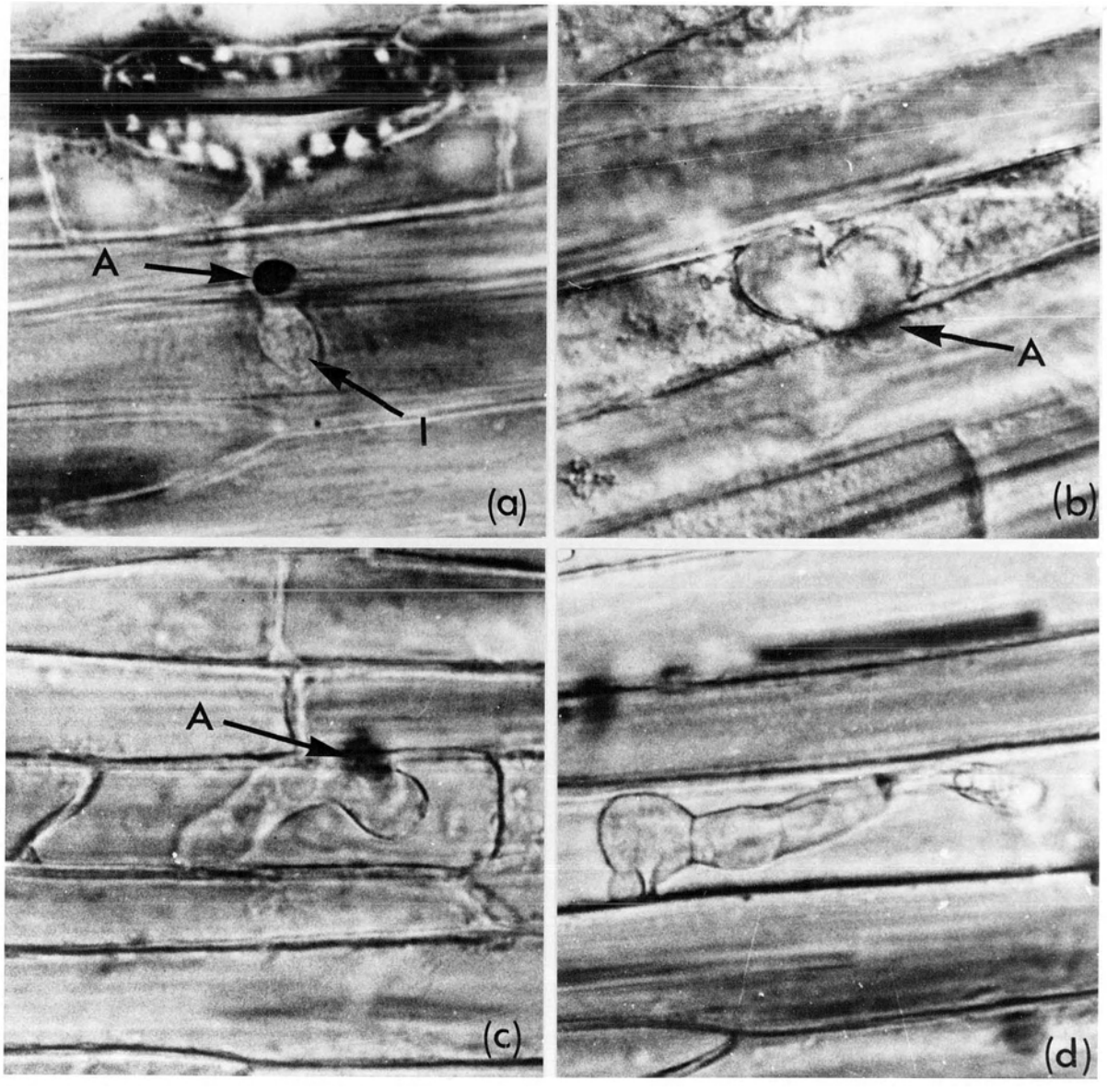
The beta race of C. lindemuthianum was grown at 24 C on agar containing 35% bean juice. For inoculation of seedlings a conidial suspension was prepared by flooding 10-day-old cultures with water and lightly rubbing the surface with a glass rod. The resulting suspension was filtered through cheesecloth to remove mycelial fragments and an estimate of the concentration of conidia in the filtrate was made using a hemocytometer. The volume of the suspension was adjusted by addition of water to give an inoculum containing about 2×10^6 conidia per ml. Plants were inoculated when the epicotyl hooks were beginning to open (about 10 days after planting). The inoculum was sprayed evenly as a fine mist onto all parts of the plants. After inoculation, plants were maintained in a high humidity environment for about 120 h. Some plants were transferred to a high humidity atmosphere of 15% CO2 in air at various times after inoculation.

Histology

Sample plants were removed from the incubation chambers at intervals so that microscope observations of the development of the interaction could be made. Where observations from several treatments had to be made at the same time, the appropriate hypocotyls were excised at the cotyledonary node and at the mid-point, and the excised segments were fixed in a solution of 3% glutaraldehyde in 0.05 M phosphate buffer, pH 7.2. Segments were stored in this solution for periods of up to 12 h and were then washed three times in 0.05 M phosphate buffer in preparation for microscopy. Epidermal strips were peeled from the upper half of the hypocotyl of each seedling. For each treatment estimates of the extent of fungal germination and penetration were derived from counts made in at least 70 random fields of view on two epidermal strips from each of three replicate hypocotyl segments. In addition, estimates of the extent of host cell colonization by infection hyphae of the fungus were made. For this purpose the grading system shown in Table 1.1 was devised. Epidermal strips (two from each of three replicate hypocotyls) were examined at 400x magnification and each penetration site that arose unambiguously from a single appressorium was graded.

Table 1.1. Grading system used for the description of stages of development of fungal hyphae in cells of Phaseolus vulgaris, cv. Topcrop, inoculated with the beta race of Colletotrichum lindemuthianum (compatible interaction).

Stage of development	Description
P	Early stage of penetration. Infection hypha just visible beneath appressorium (Fig. 1.1a) or in lobate form (Fig.1.1b)
1C	One cell colonized at infection site. Lateral growth of infection hypha apparent (Figs. 1.1c and 1.1d).
2C	Two cells colonized.
3C	Three cells colonized.
X	More than three cells colonized.


A
I
(a)
A
(b)
A
(c)
(d)

RESULTS

Development of the interaction in air.

Conidia of C. lindemuthianum germinated, by forming appressoria, over a period of several hours. Although there were large differences between experiments, in most cases germination had begun by 6 h after inoculation (Fig. 1.2) and continued for a further period of about 30 h. The timing of penetration by fungal hyphae was similarly subject to much variation among different experiments (Fig. 1.3). The mean values of per cent germination and penetration from Figs. 1.2 and 1.3 respectively suggest that a visible penetration hypha developed about 24 h after its parent appressorium had formed. Thus, penetration into host cells by infection hyphae was observed as early as 24 h after inoculation in some experiments, but more commonly was first seen at 30 - 36 h. Symptoms became visible on the upper 5 cm of hypocotyls between 70 and 84 h after inoculation.

In a typical experiment, epidermal peels from hypocotyls of plants were prepared at intervals between 16 and 84 h after inoculation. The development of fungal hyphae in host cells at each of these intervals is described in Fig. 1.4. No penetration had occurred by 16 h after inoculation. By 40 h, 30% of the conidia with appressoria had formed visible penetration sites, all of which were at the P stage. Thereafter, colonization of host cells was

Fig. 1.2 Germination of conidia of Colletotrichum lindemuthianum (beta race) on hypocotyls of Phaseolus vulgaris, cv. Topcrop, (compatible interaction).

Means and standard errors were derived from eight separate experiments. In each experiment estimates of numbers of germinated conidia were obtained as described in Materials and Methods.

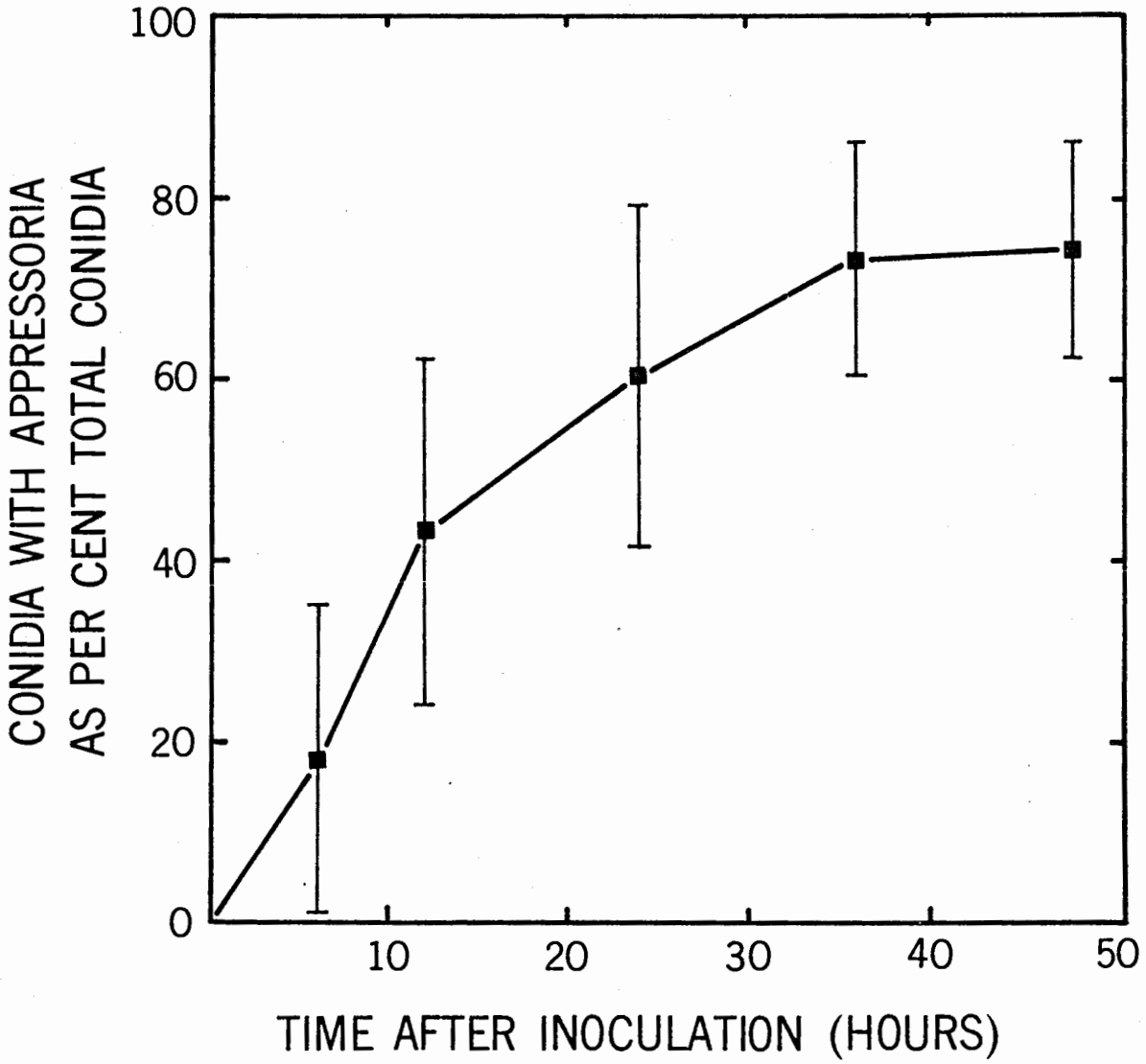

CONIDIA WITH APPRESSORIA
AS PER CENT TOTAL CONIDIA
100
80
60
40
20
0
10
20
30
40
50
TIME AFTER INOCULATION (HOURS)

Fig. 1.3 Penetration from appressoria of Colletotrichum lindemuthianum (beta race) into hypocotyls of Phaseolus vulgaris, cv. Topcrop, (compatible interaction).

Means and standard errors were derived from eight separate experiments. In each experiment, estimates of numbers of penetration sites were obtained as described in Materials and Methods.

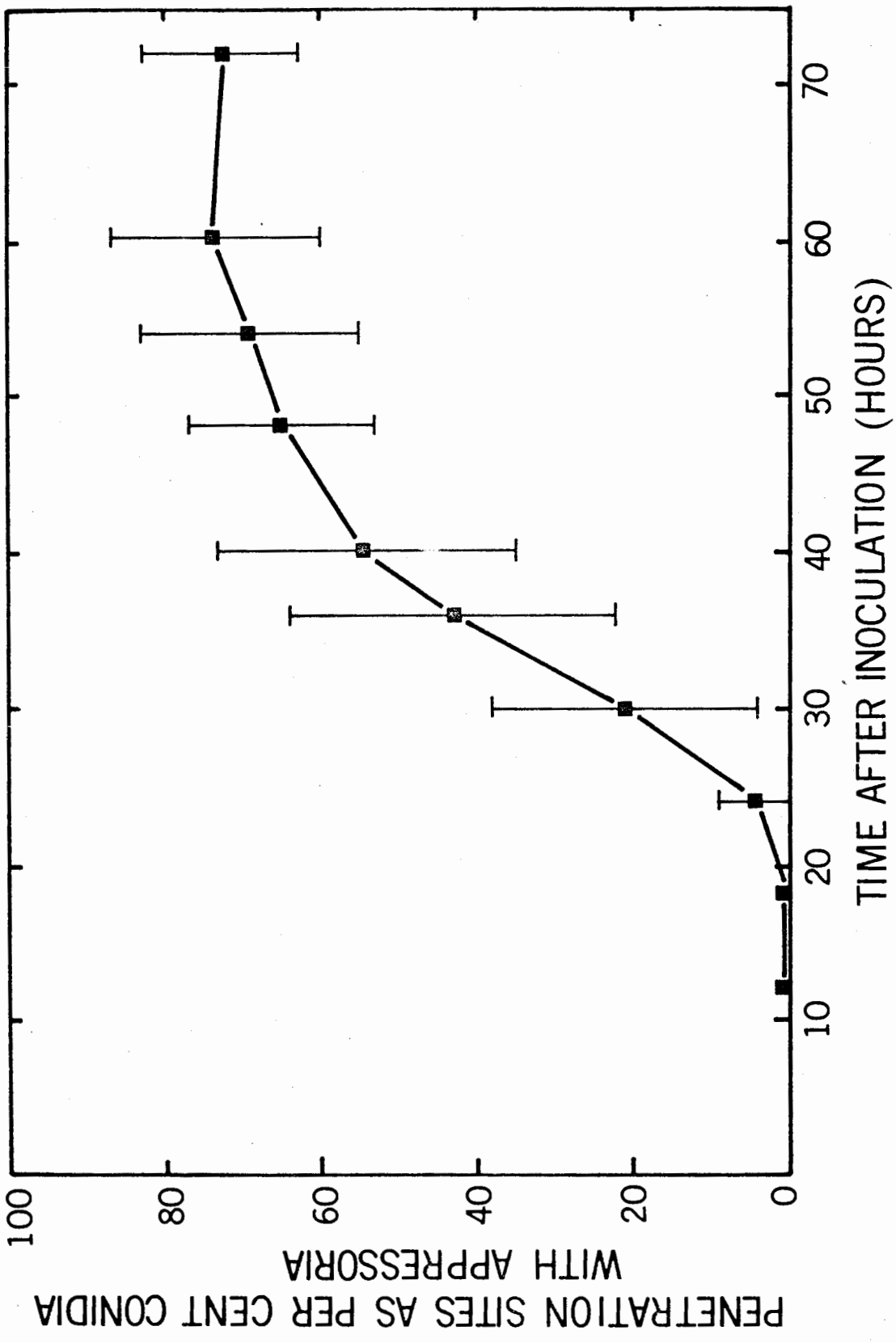
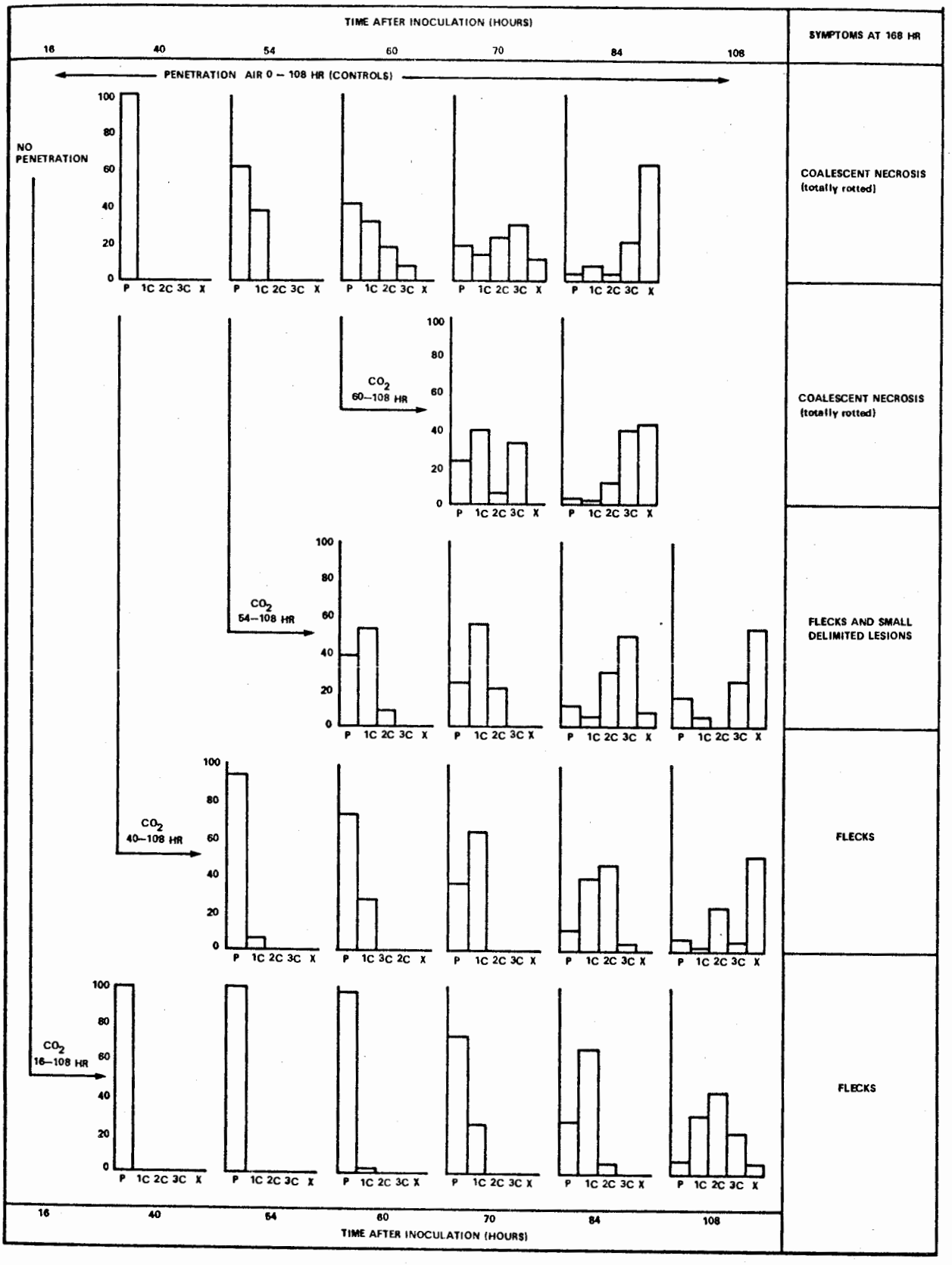

PENETRATION SITES AS PER CENT CONIDIA WITH APPRESSORIA
0
20
40
60
80
100
10
20
30
40
50
60
70
TIME AFTER INOCULATION (HOURS)

Fig. 1.4 Nature of symptoms occurring on hypocotyls of Phaseolus vulgaris, cv. Topcrop, as affected by treatment with 15% CO_2 begun at progressively later stages of colonization of host cells in a slowly developing infection by the beta race of Colletotrichum lindemuthianum (compatible interaction).

Abscissae:- stages of development of fungal hyphae in host cells. (For explanation of symbols, see Table 1.1).

Ordinates:- the number of infection sites at stages P, 1C, 2C, etc., each expressed as a per cent of the total number of infection sites at all stages.


TIME AFTER INOCULATION (HOURS)
16
40
54
60
70
84
108
SYMPTOMS AT 168 HR
PENETRATION AIR 0 – 108 HR (CONTROLS)
NO PENETRATION
CO2 60–108 HR
CO2 54–108 HR
CO2 40–108 HR
CO2 16–108 HR
P 1C 2C 3C X
P 1C 3C 2C X
COALESCENT NECROSIS (totally rotted)
COALESCENT NECROSIS (totally rotted)
FLECKS AND SMALL DELIMITED LESIONS
FLECKS
FLECKS
TIME AFTER INOCULATION (HOURS)

rapid and by 84 h (the time when symptoms were first visible in this experiment) over 60% of the penetration sites had developed to the X stage. The initially discrete lesions coalesced by 120 h after inoculation, and the plants became totally rotted (Fig. 1.5).

Development of the interaction in 15% CO2

Seedlings were transferred to 15% CO2 at 16, 40, 54, and 60 h after inoculation. Earlier experiments showed that reduced germination of conidia occurred on seedlings placed in 15% CO2 immediately after inoculation. By contrast to those results, conidia that had not germinated by 16 h in the present experiment did germinate in 15% CO2 at a rate similar to that of the controls. Penetration however was delayed in CO2 although the final proportion of conidia with appressoria that formed penetration sites was similar in CO2 and in air (Fig. 1.6).

The rate of host cell colonization by the fungus in hypocotyls from each CO2 treatment was estimated from histology of epidermal peels. The results are presented in Fig. 1.4. Growth of fungal hyphae between 40 and 108 h was strongly inhibited in seedlings placed in CO2 at 16 h after inoculation. Symptoms, in the form of brown flecks, were first visible near the cotyledonary nodes of these seedlings at about 100 h. By 120 h flecks had more or less ceased development and were distributed fairly evenly along the

Fig. 1.5 Symptoms at 136 h after inoculation of hypocotyls of Phaseolus vulgaris, cv. Topcrop, with the beta race of Colletotrichum lindemuthianum (compatible interaction) as affected by time of beginning treatment with 15% CO2.

Plants were placed in CO2 at 16, 40, and 54 h after inoculation. Those which remained in air served as controls.

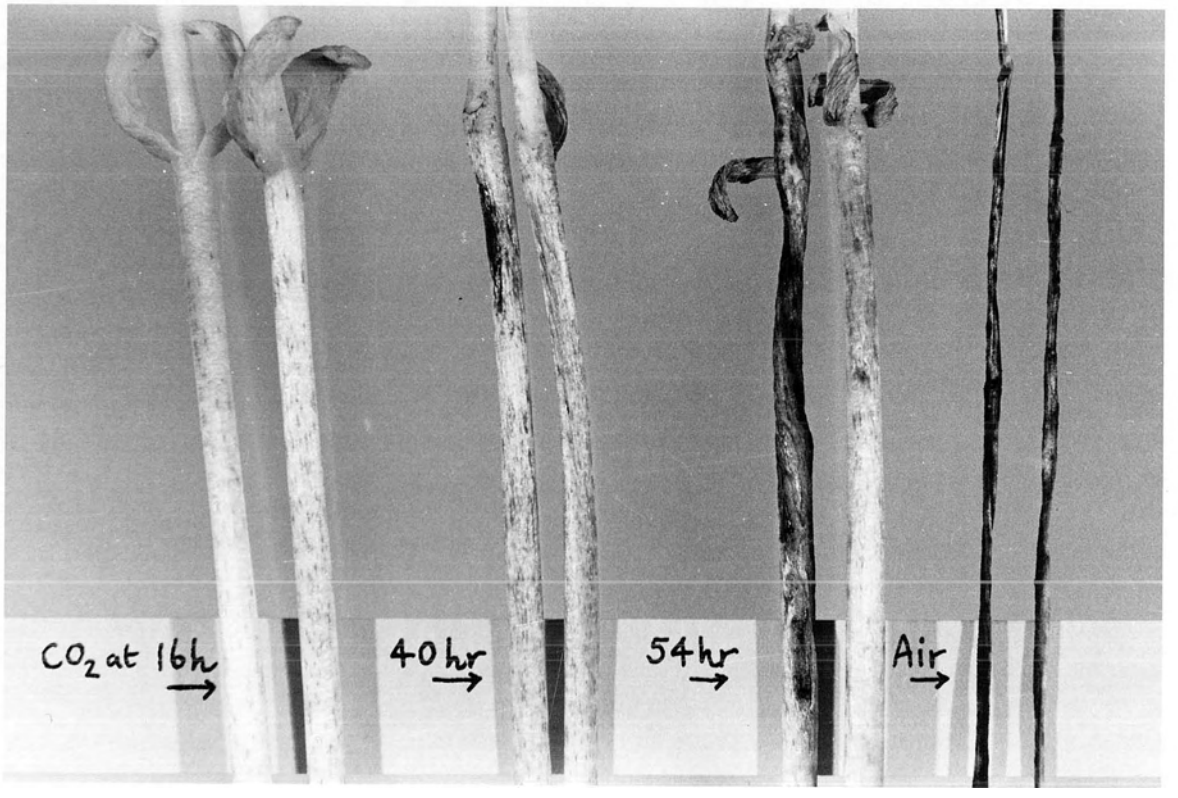

CO_2 at 16h
40hr
54hr
Air

Fig. 1.6 Effect of 15% CO2 on penetration from appressoria of Colletotrichum lindemuthianum (beta race) into hypocotyls of Phaseolus vulgaris, cv. Topcrop (compatible interaction). Means and standard errors were derived from three separate experiments. In each experiment, estimates of the numbers of penetration sites were obtained as described in Materials and Methods.

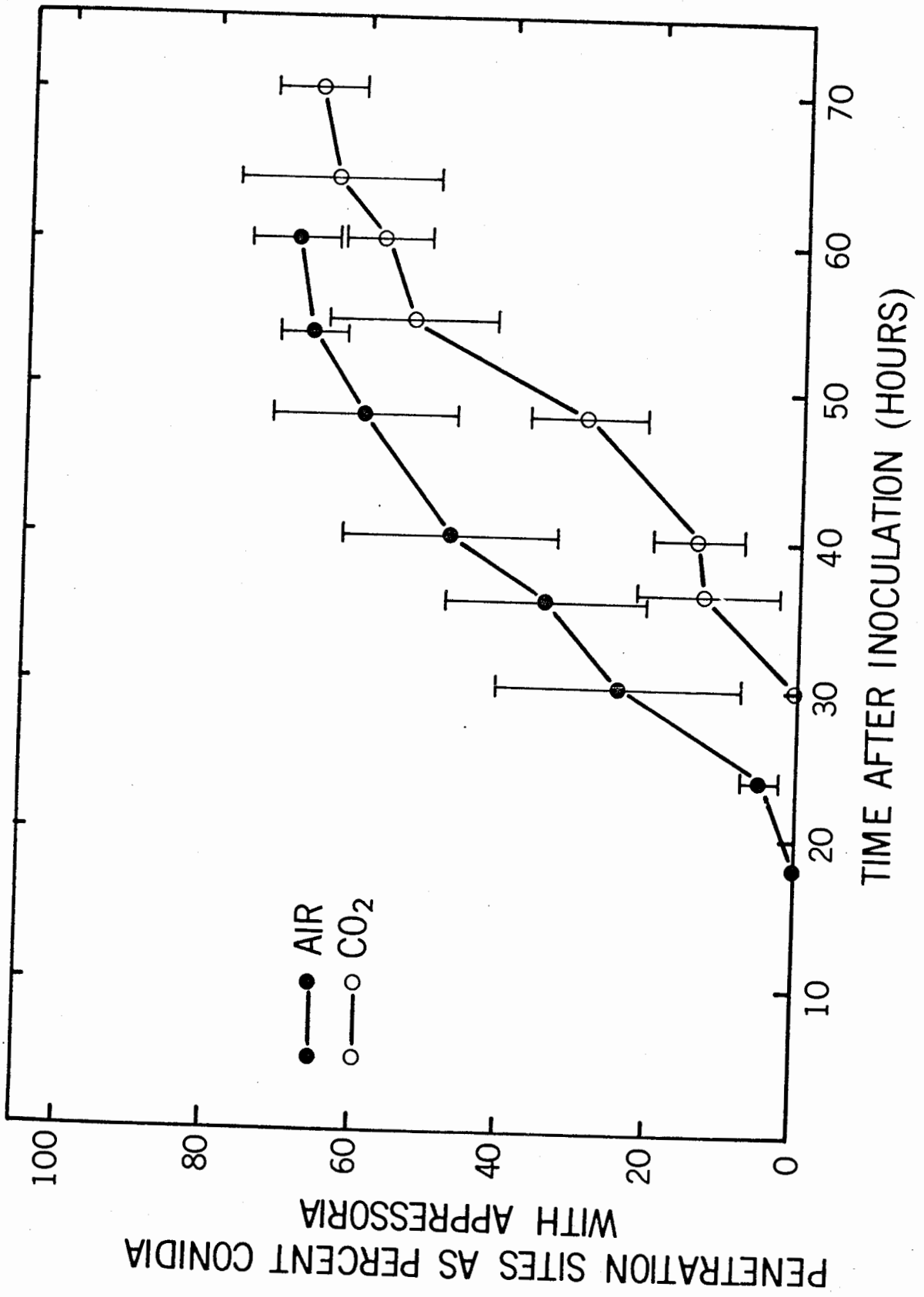
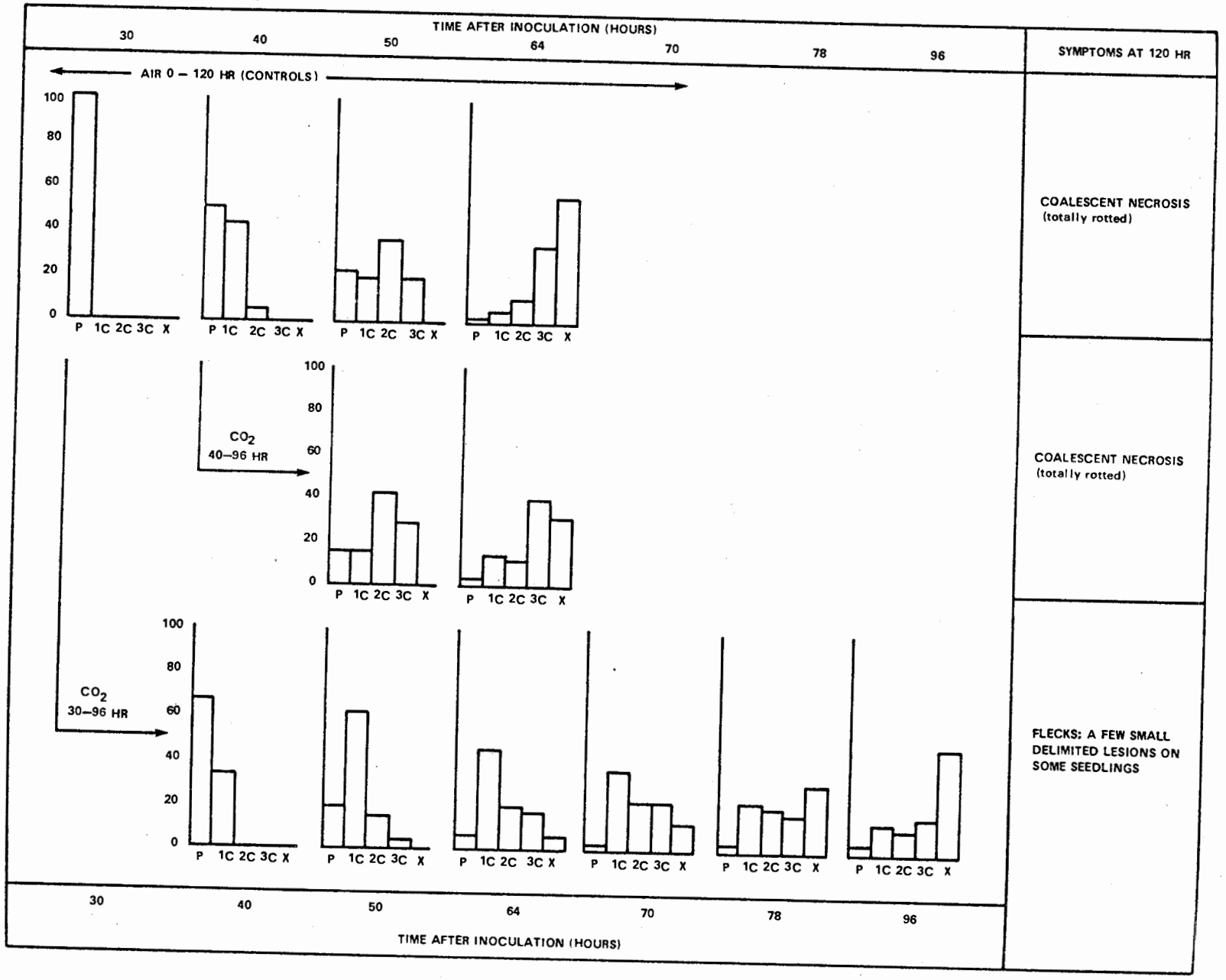

PENETRATION SITES AS PERCENT CONIDIA WITH APPRESSORIA
0
20
40
60
80
100
10
20
30
40
50
60
70
TIME AFTER INOCULATION (HOURS)
AIR
CO_2

Fig. 1.7 Nature of symptoms occurring on hypocotyls of Phaseolus vulgaris, cv. Topcrop, as affected by treatment with 15% CO2 begun at progressively later stages of colonization of host cells in a rapidly developing infection by the beta race of Colletotrichum lindemuthianum (compatible interaction).

Abscissae:- stages of development of fungal hyphae in host cells. (For explanation of symbols, see Materials and Methods).

Ordinates:- the number of infection sites at stages P, 1C, 2C, etc., each expressed as a per cent of the total number of infection sites at all stages.


TIME AFTER INOCULATION (HOURS)
30
40
50
64
70
78
96
SYMPTOMS AT 120 HR
AIR 0 – 120 HR (CONTROLS)
100
80
60
40
20
0
P 1C 2C 3C X
COALESCENT NECROSIS
(totally rotted)
CO_2
40–96 HR
COALESCENT NECROSIS
(totally rotted)
CO_2
30–96 HR
FLECKS: A FEW SMALL
DELIMITED LESIONS ON
SOME SEEDLINGS
TIME AFTER INOCULATION (HOURS)

length of the hypocotyls (Fig. 1.5). Fully developed flecks were approximately 0.5-1.5 mm in length and about 0.1 mm in width, and thus were somewhat larger than those which develop during the infection of bean hypocotyls with an incompatible race of the anthracnose fungus. However, in common with an incompatible interaction, infection hyphae of the fungus were contained within the cells constituting the fleck, and hypocotyls showing flecks were resistant to a challenge inoculation with a compatible race of the anthracnose fungus carried out in air. In addition, extracts from these hypocotyls were shown to contain a high level of the phytoalexin phaseollin. This compound accumulates in natural resistant interactions between P. vulgaris and C. lindemuthianum (see Chapter 3). Hence the appearance of flecks resulting from CO2 treatment of seedlings will be called a "resistant reaction"

When CO2 treatment was begun after penetration by fungal hyphae had occurred, a resistant reaction was obtained provided all penetration sites were still at the P stage (40 h in Figs. 1.4 and 1.5). If some infection sites had developed to a IC stage when CO2 treatment was begun (54 h in Figs. 1.4 and 1.5) then although most of the infection sites remained as flecks, some enlarged and formed small delimited lesions (Fig. 1.5). Finally, when seedlings were maintained in air until growth to a 2C stage had occurred at some penetration sites (60 h in Fig. 1.4) the

outcome was a susceptible reaction indistinguishable from that of the controls.

Experiments in which a faster rate of colonization of host cells by the fungus occurred gave somewhat different results. An example is shown in Fig. 1.7 in which most penetration sites had developed to a 3C or X stage by 64 h. Due to the difficulty of separating individual penetration sites from each other when extensive hyphal growth had occurred, no further measurements of controls were made after 64 h. Symptoms, in the form of coalescing lesions, were visible in the upper one-third of control hypocotyls by 70 h. CO2 treatment of plants in this experiment, begun when each penetration site had developed only to a P stage (30 h in Fig. 1.7), again slowed down the rate of growth of fungal hyphae. Delimited flecks, indicative of a resistant reaction, were seen in the upper halves of hypocotyls at 78 h and had spread to the lower halves by 92 h. However, by contrast to the experiment described in Fig. 1.4, a susceptible reaction, indistinguishable from that of the controls, occurred when CO2 treatment was begun when about half the penetration sites had developed to 1C stages (40 h in Fig. 1.7). CO2 treatment begun at a similar stage of development in the previous experiment (54 h in Fig. 1.4) gave a resistant reaction at the large majority of penetration sites.

In experiments designed to determine the minimum duration of exposure to 15% CO2 required to convert the interaction to one of resistance, inoculated seedlings were placed in CO2 when penetration of the fungus was first observed and some were removed at ten hour intervals until symptoms appeared. It was found that in order to give more or less complete protection it was necessary to maintain seedlings in CO2 at least until flecks began to appear. When seedlings were removed from CO2 18 and 28 h before flecks appeared on those kept in CO2, lesions developed in the upper 1/3 - 1/4 of the hypocotyls although flecks predominated below. Seedlings removed from CO2 earlier than this gave essentially susceptible reactions.

In experiments in which the concentration of conidia in the inoculum was reduced to a level which gave discrete lesions rather than coalescent necrosis in controls, CO2 had no effect on the outcome of the interaction.

DISCUSSION

Three main conditions determine whether the compatible interaction studied here can be converted to one of resistance by treatment with 15% CO2. Firstly the distance between individual infection sites must be sufficiently small so that coalescent necrosis occurs in controls that are maintained in air. Secondly, CO2 treatment should begin either before penetration of fungal hyphae has occurred or while the penetration hypha at each infection site is still in the lobate form characteristic of the early stages of penetration. Thirdly, plants must be maintained in CO2 until flecks are apparent.

The finding that a resistant reaction in 15% CO2 was obtained only at high infection site densities is consistent with observations from experiments in which etiolated bean hypocotyls were inoculated with a dilute suspension of conidia of an incompatible race of the anthracnose fungus The flecks formed were noticeably larger than those obtained at high infection site densities and some approached the size range of the delimited lesions that occur in a compatible interaction at low infection site densities (unpublished observations). These observations suggest that there may be short-range interactions between infection sites that are necessary for the expression of the higher degree of resistance observed with high density

inoculations. Such interactions could be mediated by the diffusion of a fungitoxic substance from infection sites into the immediately adjacent healthy tissue. At high infection site densities the concentration of such a substance in the healthy tissue surrounding the infection sites could become sufficiently large to ensure that fungal hyphae do not grow out of the few cells first colonized. Phaseollin and its phytoalexin analogues are ideally fitted to such a role. Phaseollin is fungitoxic to a wide variety of fungi in _in vitro_ assays (17) and accumulates in the immediate vicinity of individual infection sites in both compatible and incompatible interactions (64).

The rate of colonization of host cells by infection hyphae of the fungus was reduced in CO2. The simplest explanation for this finding is that CO2 acts directly on the fungus to inhibit its rate of growth rather than indirectly through its action on a metabolic process in the host. Support for this hypothesis has been obtained in experiments concerning the effect of 15% CO2 on _C. lindemuthianum_ growing in pure culture. An inhibitory effect on germination, germ tube elongation, and spore production has been found. These results are described in Chapter 2. The data reported here also suggest that infection hyphae of the fungus are sensitive to CO2 only in the early stages of growth. Once infection hyphae have grown beyond the first cell penetrated by the time CO2

treatment is begun their subsequent growth rate is rapid and CO2 exerts a smaller inhibitory effect.

A similar interruption of pathogenesis has been reported to be the result of heat-treatment of compatible interactions of cotton infected with Verticillium albo-atrum (6,7) and in the bean anthracnose interaction (63). Treatment of infected bean hypocotyls for 15 h at 36 C was sufficient to stop pathogenesis if applied when fungal hyphae had colonized 1-10 cells at individual infection sites. In comparison with the results reported above for CO2 it is apparent that heat treatment is more effective in changing the nature of the interaction to resistance. Cultures of C. lindemuthianum on bean juice agar medium cease growth when heated at 36 C for 15 h. By contrast 15% CO2 slows down, but does not stop, germ tube elongation of the fungus growing on bean juice agar. This differential sensitivity probably explains why CO2 treatment must begin earlier and be continued for longer periods than is needed for heat treatment in order to bring about a similar degree of protection.

Thus it seems likely that CO2 retards development of the fungus until the defense response of the host has developed to an extent where it can contain the fungus. It seems improbable to this author that the fungus, under conditions where its growth is inhibited, would actively induce a resistant reaction. Rather, the results presented

here tend to support the hypothesis that a pathogen normally blocks a non-specifically triggered defense response that is initiated by the act of penetration or by the introduction of foreign metabolites into the host tissue. CO2, by slowing down the growth of the fungus, could inhibit its ability to block the defense response of the plant.

CHAPTER 2

EFFECTS OF 15% CO2 ON GERMINATION, GERM TUBE ELONGATION, AND SPORULATION IN CULTURES OF *COLLETOTRICHUM LINDEMUTHIANUM*

ABSTRACT

Germination of conidia in cultures of the beta and gamma races of Colletotrichum lindemuthianum growing on bean juice agar was delayed in 15% CO2, though the proportion of conidia that finally germinated under these conditions was similar to the proportion that germinated in cultures maintained in air. Germ tube elongation in CO2 was inhibited during the early stages of development, but vegetative growth was ultimately stimulated under these conditions. Sporulation of cultures maintained in CO2 was delayed and fewer conidia were formed than in cultures maintained in air.

The results suggest that there is an interrelationship between vegetative growth and spore production. The implications of these results to previous work on the effects of 15% CO2 on the host-parasite interaction between C. lindemuthianum and Phaseolus vulgaris are also discussed.

INTRODUCTION

Fungi have often been described as aerobic organisms with respect to their requirements for growth and development (1,13,32). However, many fungi can grow in low concentrations of oxygen and a few have been shown to be facultative anaerobes under conditions where suitable growth factors are provided (5,33). The very extensive literature concerning the effects of different gaseous environments on the growth and metabolism of fungi has been reviewed by Tabak and Cooke (81). These authors concluded that, although there are large variations between different species, in general fungi are sensitive to changes in carbon dioxide and relatively insensitive to changes in oxygen concentration over a wide range.

The effect of treatment with 15% CO2 in air on the compatible interaction between Phaseolus vulgaris, cv. Topcrop, and the beta race of the anthracnose fungus, Colletotrichum lindemuthianum, has been described in Chapter 1. The rate of colonization of host cells by hyphae of the fungus was reduced in the presence of CO2, and under certain conditions the nature of the interaction was changed to one of resistance. The simplest explanation for these findings seems to be that CO2 acts directly on the fungus to reduce its rate of growth, thus allowing the plant to undergo an effective defense response which would otherwise be

counteracted by the activities of the pathogen. The investigation described here was carried out in order to determine whether the growth of C. lindemuthianum in pure culture is sensitive to 15% CO2.

MATERIALS AND METHODS

Small Petri plates (60x15 mm, "Integrid" type) containing bean juice agar were inoculated with an aqueous suspension of conidia of C. lindemuthianum (beta or gamma race). Suspensions of conidia were prepared from 10-day-old cultures, unless otherwise stated. Each plate was inoculated with about 5×10^3 conidia contained in 0.05 ml of suspension. Some of the plates were transferred to a glass cylinder, capacity 1.2 liters, through which 15% CO2 in air was passed at a flow rate of 80 ml per minute.

Other plates were transferred to a similar cylinder through which air was passed at the same flow rate. Some of these latter plates were transferred to the CO2-containing cylinder at various times after inoculation. Those remaining in air served as controls. At intervals after inoculation germ tube lengths of germinating conidia within marked squares of each integrid plate were measured. This technique made it possible to measure the lengths of germ tubes belonging to the same conidia at each time of sampling. It was considered necessary to do this, because in random samples the delaying effect of CO2 on spore germination would have caused an increment of short germ tubes from newly germinated conidia to be included in calculations of mean germ tube lengths in the CO2 treatments. Measurements were made at 125x magnification

using a compound microscope fitted with a calibrated eyepiece micrometer. All operations were carried out in a laminar flow chamber to avoid contamination of the cultures during the experiments.

Sporulation studies were carried out using plates inoculated and treated as above. Plates were flooded with water and conidia were brought into suspension by lightly rubbing the agar surface with a glass rod. This procedure was repeated four times to ensure as complete a removal of conidia from each plate as possible. The resulting suspensions were made up to a known volume with water. The number of conidia per ml of suspension was calculated from the mean of the number in each of twenty 10 µl drops of the suspension, counted using a hemocytometer. The value obtained was then multiplied by the total volume of the suspension to give an estimate of the number of conidia per culture.

RESULTS

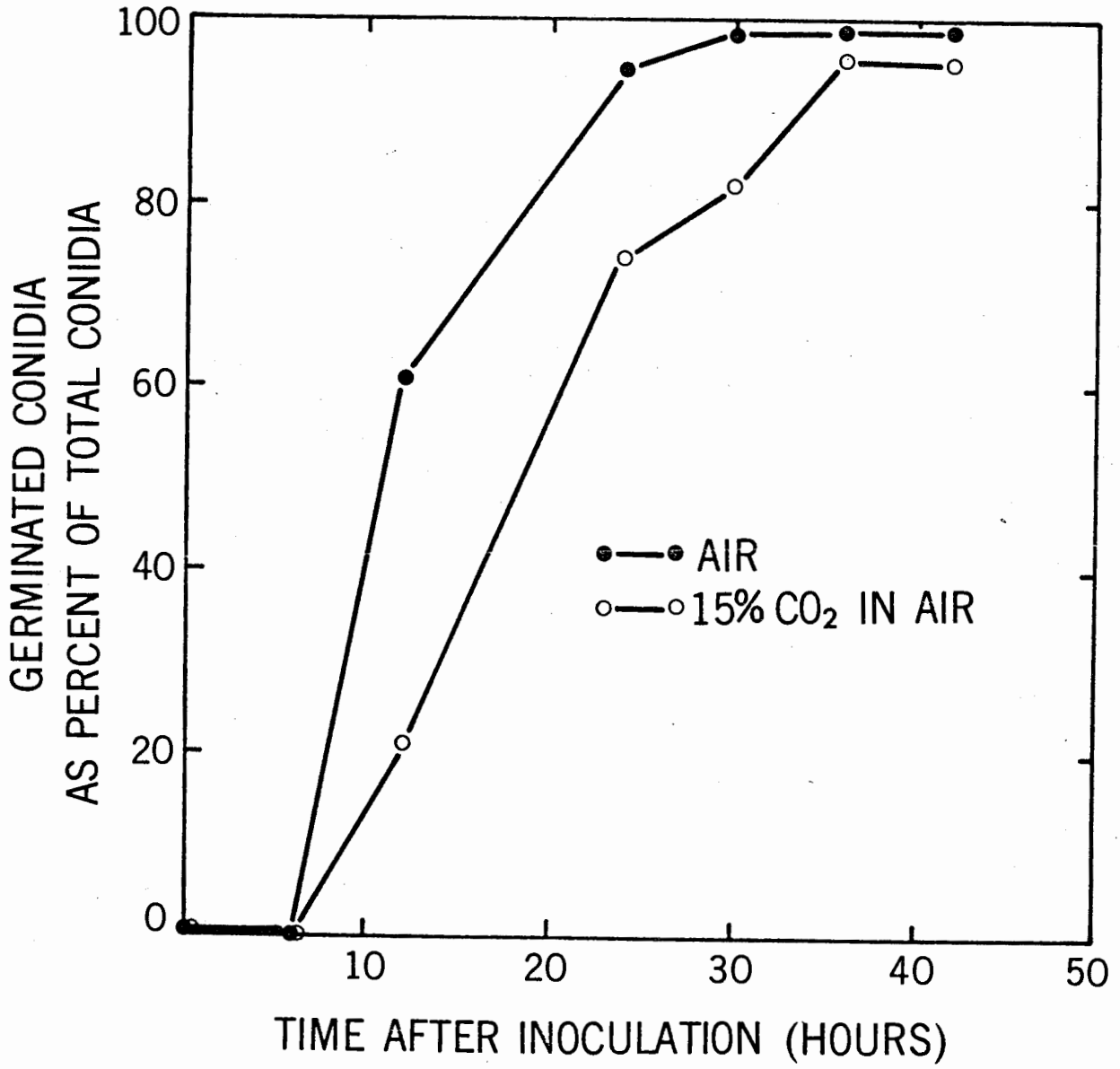
Effect of 15% CO2 on germination of conidia.

Germination of conidia of both the beta and gamma races of C. lindemuthianum was delayed in the presence of 15% CO2. Table 2.1 shows the results of three separate experiments carried out with the beta race and one with the gamma race of C. lindemuthianum. In all cases, CO2 delayed the germination of conidia, though the degree of inhibition was variable. Observations made at times later than 12 h after inoculation showed that the proportion of conidia that germinated reached 90-95% in both CO2 and air. Experiment 2, in which the greatest degree of inhibition was observed, differed from the other three in that the suspension with which the plates were inoculated was prepared from a 21-day-old culture. In this experiment the inhibiting effect of CO2 was greatest within the first 12 h after inoculation (Fig. 2.1). At times after 12 h the proportion of conidia that had germinated increased to reach a maximum value, comparable to that of cultures maintained in air, at 38 h after inoculation.

Table 2.1. Effect of 15% CO2 on the germination of conidia of Colletotrichum lindemuthianum (beta and gamma races) on bean juice agar at 12 hours after inoculation.

Race of fungus	Expt. number	Number of germinated conidia		Total number of conidia		Per cent germination	
		Air	CO2	Air	CO2	Air	CO2
beta	1	65	49	78	62	83	79
	2	56	18	92	84	61	21
	3	98	73	148	129	66	57
gamma	4	103	95	130	142	79	67

Fig. 2.1 Effect of 15% CO2 on the germination of conidia in cultures of the beta race of Colletotrichum lindemuthianum growing on bean juice agar. The data derives from experiment 2, Table 2.1.


100
80
60
40
20
0
10
20
30
40
50
GERMINATED CONIDIA
AS PERCENT OF TOTAL CONIDIA
TIME AFTER INOCULATION (HOURS)
AIR
15% CO_2 IN AIR

Effect of 15% CO2 on germ tube elongation of germinating conidia

A reduction in the rate of germ tube elongation in cultures of the beta and gamma races during the early stages of growth in CO2 was observed. However the isolates of the two races were not equally sensitive to CO2. Results are presented in Table 2.2. Germ tube elongation in cultures of the beta race of the fungus was unaffected by CO2 during the first 12 h after inoculation. The rate of germ tube elongation after 12 h was reduced by CO2 when treatment was begun immediately after inoculation of the plates, or following a 12 h period in air. An apparent stimulation in the rate of germ tube elongation occurred when cultures were placed in 15% CO2 at 24 h after inoculation. After about 32 h the germ tubes had become so highly branched that further measurements were not possible. However, visual comparison of air- and CO2-treated cultures during the subsequent 10 days showed that considerably denser hyphal mats were produced in CO2 than in air, even in those cultures that had been placed in CO2 immediately after inoculation or at 12 h.

Germ tube elongation in cultures of the gamma race of C. lindemuthianum showed a similar sensitivity to CO2 applied following a 12 h period in air. However, in contrast to beta, the gamma race was also sensitive to CO2 during the first 12 h after inoculation (Table 2.2). Because the isolate of the gamma race grew faster than that

of the beta race, measurements of germ tube lengths could only be made up to 24 hours after inoculation. However, cultures kept in CO2 produced denser hyphal mats in the period from 4 to 10 days after inoculation than did controls.

Thus, CO2 seems to inhibit germ tube elongation only during the early stages of development, and apparently stimulates later stages of vegetative growth. To the author's knowledge a differential effect of CO2 on germ tube elongation of fungi dependent on the stage of development, like that described above, has not been reported elsewhere.

Effect of 15% CO2 on sporulation.

Sporulation of cultures of the beta and gamma races of the fungus was delayed in CO2, and the maximum number of conidia per culture produced in CO2 was less than in controls. Results obtained using cultures of the beta race of C. lindemuthianum are presented in Fig. 2.2. Microscope observations of the cultures maintained in air showed that no sporulation had occurred at one and two days after inoculation, although small aggregates of hyphae were visible at two days. Sporulation in cultures of the beta race began between 2 and 3 days after inoculation and continued for a further 7 days, after which the number of conidia recovered per culture remained essentially constant.

Sporulation by cultures of the beta race that were maintained in 15% CO2 did not occur until 9-10 days after inoculation. Instead, the cultures developed a relatively profuse aerial mycelium (Fig. 2.3). Collapse of this aerial growth at 9-10 days coincided with sporulation. Once the process of sporulation had been initiated, however, the number of conidia per culture did not continue to increase, but remained essentially constant in the period from 11 - 15 days, (Fig. 2.2).

Sporulation in cultures of the gamma race of the fungus also began at 2-3 days after inoculation. The number of conidia per culture had reached a maximum of $3.5 \pm 0.26 \times 10^7$ at about 9 days after inoculation. Sporulation in cultures of the gamma race that were maintained in 15% CO2 began at 6-7 days after inoculation. The number of conidia per culture had reached a maximum of $5.3 \pm 0.9 \times 10^6$ by about 9 days after inoculation.

Table 2.2. Effect of 15% CO2 on the elongation of germ tubes from conidia of Colletotrichum lindemuthianum on bean juice agar.

Race of fungus	Length of treatment (hours)		Mean germ tube length (µm)			Conidia scored
	Air	CO2	12 h	24 h	32 h	
beta	0 - 32		10 ± 3*	86 ± 39*	346 ± 201	207
		0 - 32	9 ± 4*	36 ± 14	147 ± 53	153
	0 - 12	12 - 32	9 ± 3*	55 ± 22	206 ± 93	139
	0 - 24	24 - 32	11 ± 4*	79 ± 28*	502 ± 211	84
gamma	0 - 24		25 ± 10*	121 ± 59		75
		0 - 24	14 ± 5	50 ± 17*		75
	0 - 12	12 - 24	27 ± 12*	64 ± 33*		60

*difference between indicated values within individual vertical columns insignificant ($p > 0.05$).

Fig. 2.2 Effect of 15% CO2 on sporulation in cultures of Colletotrichum lindemuthianum (beta race) growing on bean juice agar.

Vertical bars represent the standard deviations of the means, obtained as described in Materials and Methods.

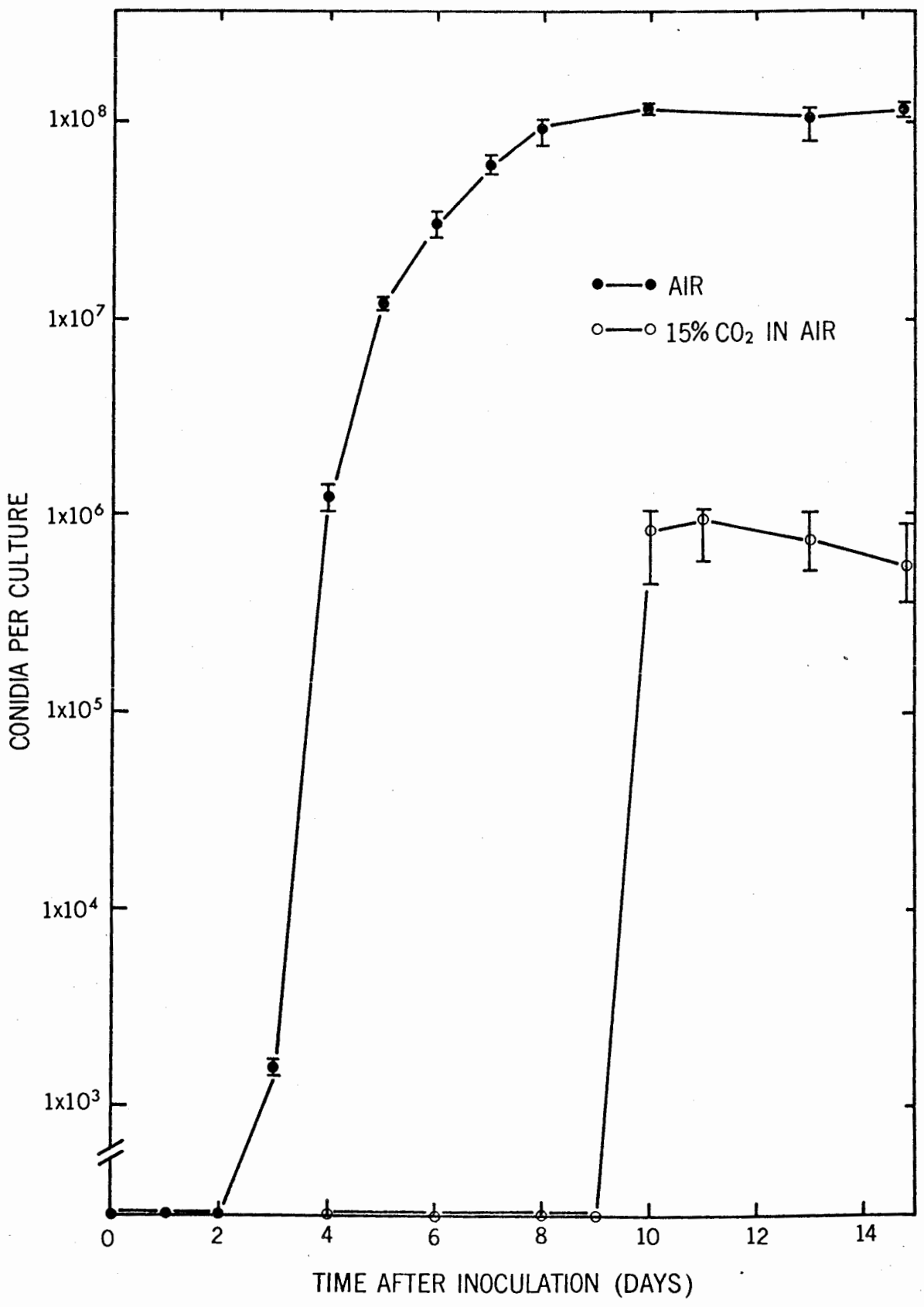
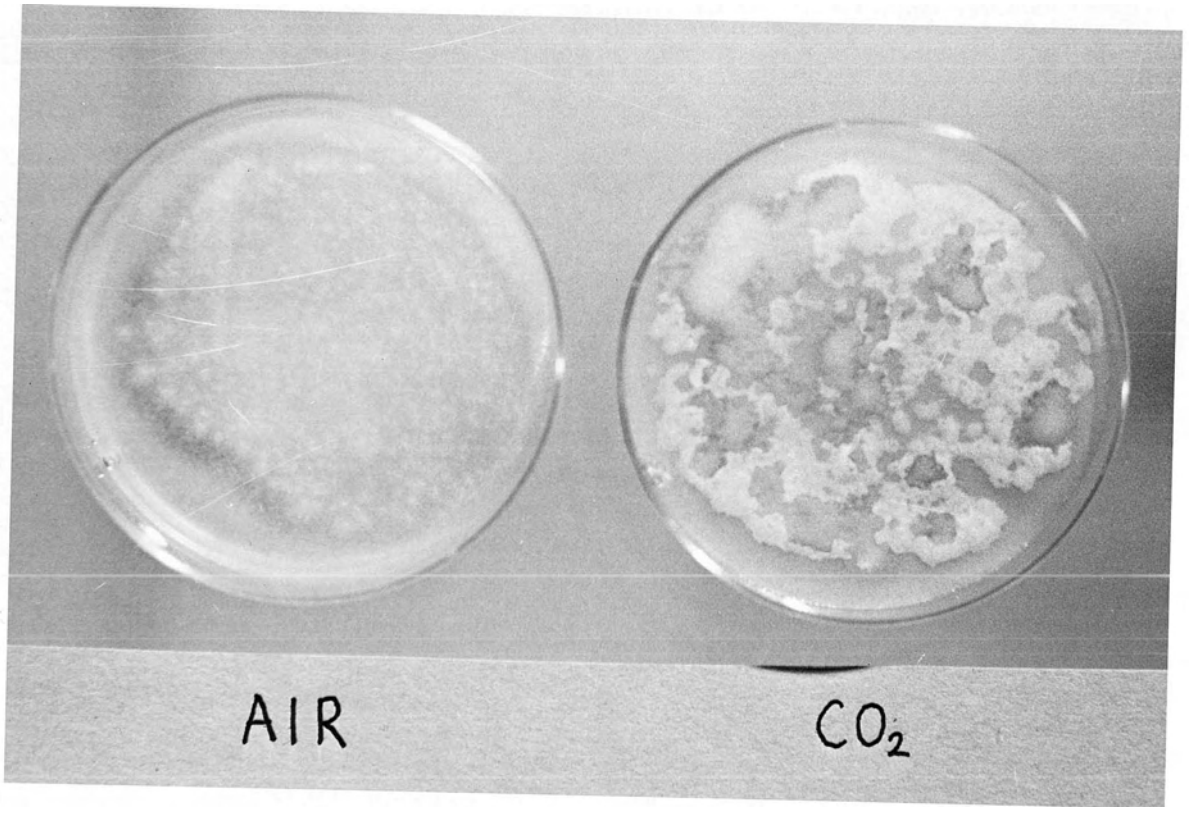

1x10^8
1x10^7
1x10^6
1x10^5
1x10^4
1x10^3
CONIDIA PER CULTURE
AIR
15% CO2 IN AIR
0
2
4
6
8
10
12
14
TIME AFTER INOCULATION (DAYS)

Fig. 2.3 Effect of 15% CO2 on the morphology of cultures of Colletotrichum lindemuthianum (beta race) growing on bean juice agar.

Cultures were photographed at 9 days after inoculation.


AIR
CO_2

DISCUSSION

The processes of growth and development of fungi are affected by a large number of factors in addition to the concentrations of O2 and CO2. These include temperature, composition of the growth medium, light, and pH. There are also large differences in sensitivity to changes in O2 and CO2 levels between different species. Because of these factors, comparisons of the findings reported here with those of other workers probably have restricted value and will be drawn only when differences appear to be marked. The rates of growth and spore production by C. lindemuthianum growing on bean juice agar were reduced in 15% CO2. Conidia began germination later in CO2 than in air, but the subsequent rates of germination were comparable and the same proportion eventually germinated in both environments. The findings with respect to the effects of CO2 on germination seem to be in contrast to most existing reports which describe its effects on the process in other species of fungi (81). Most commonly, CO2 concentrations above the range 1-5% not only delay germination but reduce the proportion of spores that finally do germinate.

The rate of germ tube elongation in cultures maintained in 15% CO2 was reduced relative to controls during the first 32 h after inoculation, although vegetative growth continued in CO2 for several days after the process had ceased in cultures maintained in air. The data suggest that there is

an interrelationship between vegetative growth and spore production. For example, cultures of the beta race that were maintained in air began to produce conidia at 2-3 days after inoculation and vegetative growth of the mycelium apparently ceased soon after this. Similarly the onset of sporulation at 9-10 days in cultures maintained in 15% CO2 was accompanied by collapse of the aerial mycelium and an apparent cessation of vegetative growth. These results indicate that the profuse vegetative growth that ultimately occurred in CO2 was a consequence of the inhibition of sporulation.

A reduction in the rate of colonization of cells of P. vulgaris by infection hyphae of C. lindemuthianum in the presence of 15% CO2 has been described in Chapter I. The magnitude of the reduction was dependent on the stage of development of infection hyphae in host cells, and was greater when CO2 treatment was begun before or soon after penetration had occurred. Once infection hyphae had grown beyond the first cell colonized at most infection sites their subsequent rates of growth in CO2 were similar to those in control plants that were maintained in air. The similarities between the effects of CO2 on the fungus growing in culture and in cells of P. vulgaris lend support to the hypothesis that the outcome of the host-parasite interaction in the presence of CO2 is a consequence of an inhibitory effect of CO2 on the fungus.

CHAPTER 3

EFFECTS OF 15% CO2 ON THE ACCUMULATION OF THE PHYTOALEXIN PHASEOLLIN IN *PHASEOLUS VULGARIS* IN RESPONSE TO MECHANICAL INJURY AND TO INFECTION BY *COLLETOTRICHUM LINDEMUTHIANUM*

ABSTRACT

Accumulation of phaseollin at sites of point-freezing injury on etiolated hypocotyls of Phaseolus vulgaris occurred in air but was prevented when seedlings were placed in air containing 15% CO2 immediately after injury. The inhibitory effect was partially overcome when CO2-treated seedlings were returned to air. Phaseollin accumulation in 15% CO2 did occur, however, when injured seedlings were maintained in air for 3-9 h before being transferred to CO2 indicating that the sensitivity to CO2 lies at an early stage of the process leading to phaseollin production. In contrast, phaseollin accumulation at sites of infection of P. vulgaris by an incompatible race of Colletotrichum lindemuthianum was not inhibited by 15% CO2. These results indicate that the processes leading to the accumulation of phaseollin at incompatible infection sites and at injury sites may be regulated differently.

INTRODUCTION

The effects of elevated concentrations of carbon dioxide on a compatible interaction between *Phaseolus vulgaris* and *Colletotrichum lindemuthianum* have been described in Chapter 1. The rate of colonization of host cells by infection hyphae of the fungus was reduced in 15% CO_2, an effect accompanied by a change in the nature of the host-parasite interaction in favour of increased resistance. The growth and development of *C. lindemuthianum* in culture was also inhibited by 15% CO_2 (see Chapter 2). These results suggest that the protective effect of CO_2 during a compatible interaction may be due primarily to its effect on the pathogen. However, there can be little doubt that there are changes in host metabolism resulting from prolonged treatment with 15% CO_2. Such changes could affect the potential of the host for undergoing a defense response and may in turn influence the outcome of a host-parasite interaction.

The present chapter describes the effect of 15% CO_2 on one such plant metabolic process, namely the accumulation of the phytoalexin phaseollin in etiolated hypocotyls of *P. vulgaris* in response to mechanical injury and to infection by an incompatible race of *C. lindemuthianum*.

MATERIALS AND METHODS

Accumulation of phaseollin at incompatible infection sites.

The procedures described in Chapter I were used for growing and inoculating seedlings and for producing high-CO2 atmospheres. Seedlings of P. vulgaris, cv. Topcrop, were inoculated with a suspension of conidia of the gamma race of C. lindemuthianum when the epicotyl hooks were beginning to open (about 10 days after planting). Inoculated seedlings were maintained in air at high humidity. At 16 h after inoculation some seedlings were transferred to a high humidity atmosphere of 15% CO2 in air and maintained under these conditions for a further 120 h. The remaining seedlings (controls) were maintained in air at high humidity for the same period. At intervals, four replicate seedlings were removed from each of the air- and CO2-containing incubation chambers. These seedlings were used for histological studies of the development of the interaction and for ethanol extractions.

Quantitative determinations of phaseollin were carried out on two 3 cm segments, cut at 4-7 and 9-12 cm from the cotyledonary node of the hypocotyl of each of the four replicate seedlings that comprised the sample from each treatment. The eight segments belonging to each treatment were extracted three times with boiling 95% ethanol. The

three ethanol fractions were combined and evaporated to dryness under vacuum at 40 C. The residue was extracted with four successive 2 ml volumes of ethyl acetate. The combined ethyl acetate fractions were evaporated to dryness under vacuum at 24 C. The residue was dissolved in 150 µl of n-propanol and stored in a microvial (250-300 µl volume) which was closed with parafilm and capped with a serum stopper. Quantitative analyses of phaseollin in the propanol solutions were carried out as described by Rahe (64). The density of penetration sites in the hypocotyl segments extracted for phaseollin analysis was estimated from microscope observations of epidermal strips from the intervening 2 cm segments (7-9 cm from the cotyledonary nodes). Three 2 cm strips were taken from each of four 2 cm segments. The density of penetration sites on each segment was calculated from 30 random fields of view (0.25 mm^2) on the three strips obtained from that segment. Thus the density of penetration sites in the tissue extracted for phaseollin analysis was estimated from the mean of the density on four 2 cm segments immediately adjacent to the tissue extracted.

Accumulation of phaseollin at injury sites.

Seedlings of P. vulgaris, cv. Topcrop, were grown as described above. Injuries were made by touching small pieces of dry ice to the hypocotyls for about 0.5 sec. The injuries were spaced about 1.5 cm apart in a single vertical line down each hypocotyl. After injury, seedlings were kept in an atmosphere of air or 15% CO2 as described above. Injury sites were subsequently excised so as to include at least 1 mm of healthy tissue around each injury and all the tissue beneath the injury to the mid-point of the pith cavity. Excised injury sites were extracted with three separate 15 ml volumes of boiling 95% ethanol. Extraction with ethyl acetate and analysis of phaseollin in the final n-propanol solutions were carried out as described above.

RESULTS

Accumulation of phaseollin at incompatible infection sites.

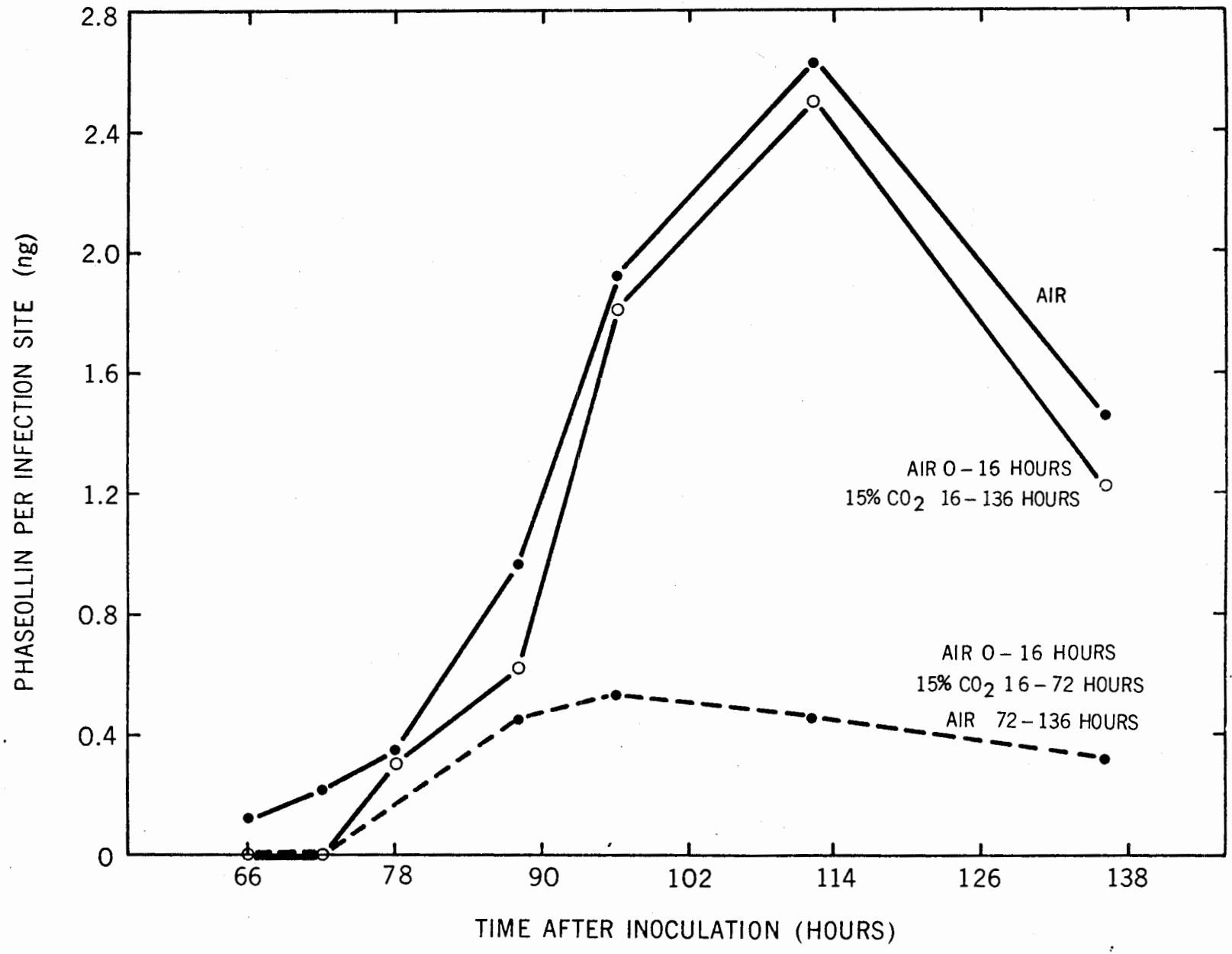
Treatment of inoculated seedlings with 15% CO2 was begun 16 h after inoculation. By this time approximately 50% of the conidia visible on epidermal peels taken from selected seedlings had germinated and formed appressoria. However, no penetration by infection hyphae had occurred. Penetration was first seen in controls at 30 h after inoculation. The number of penetration sites per mm^2 increased from 2.6 at 36 h to 6.9 at 48 h and thereafter remained essentially constant (Table 3.1). Penetration in CO2 occurred a few hours later than in air and did not reach a maximum until 72-78 h after inoculation (Table 3.1).

Phaseollin was first detected in extracts from seedlings that were maintained in air (controls) at 66 h after inoculation, and in extracts from CO2-treated seedlings at 78 h. The detection limit for the determinations at 66 and 72 h in the samples from CO2-treated plants was about 0.6×10^{-4} µg per infection site. The appearance of phaseollin coincided with development of the first hypersensitive flecks in both control- and CO2-treated seedlings. Thereafter phaseollin accumulated at a similar rate in both air and CO2, reached a maximum level at about 112 h after inoculation and

Table 3.1. Effect of treatment with 15% CO2, begun at 16 h after inoculation, on penetration of infection hyphae in the incompatible interaction between *Colletotrichum lindemuthianum* (gamma race) and *Phaseolus vulgaris*, cv. Topcrop.

Time after inoculation (hours)	Penetration sites per mm^2	
	Air	CO2
36	2.6 ± 0.6	0.4 ± 0.5
48	6.9 ± 0.9	4.2 ± 1.2
66	6.8 ± 1.5	5.6 ± 0.7
72	7.9 ± 2.5	6.7 ± 2.4
78	7.6 ± 1.9	7.2 ± 2.6

Fig. 3.1 Effect of 15% CO2 on the accumulation of phaseollin at infection sites in the incompatible interaction between Colletotrichum lindemuthianum (gamma race) and Phaseolus vulgaris, cv. Topcrop.


2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
PHASEOLLIN PER INFECTION SITE (ng)
66
78
90
102
114
126
138
TIME AFTER INOCULATION (HOURS)
AIR
AIR 0 – 16 HOURS
15% CO2 16 – 136 HOURS
AIR 0 – 16 HOURS
15% CO2 16 – 72 HOURS
AIR 72 – 136 HOURS

subsequently declined (Fig. 3.1). A lower level of phaseollin accumulated in hypocotyls of infected seedlings that were transferred from CO2 to air at 72 h. In addition, thin-layer chromatograms of extracts from these seedlings showed at least quantitative differences in other components when the extracts were compared with those from seedlings that were maintained throughout in air or 15% CO2 (Fig. 3.2). However, the seedlings that were transferred from CO2 to air at 72 h closely resembled the controls in both size and density of infection sites (Fig. 3.3). Hypocotyls of seedlings that remained in CO2 for 120 h developed a golden-yellow discoloration in addition to otherwise normal flecks, especially in the areas where there was a high density of flecks. In contrast to the brown colored materials of the hypersensitive flecks, this coloration was not removed by boiling 95% ethanol. Transverse sections cut with a razor blade and examined with a microscope showed deposits of brown-colored substances in the walls of cortical cells in these hypocotyls (Fig. 3.4b). Control hypocotyls, in which a golden-yellow coloration was absent, lacked a comparable deposit of this material (Fig. 3.4a).

Fig. 3.2 Occurrence of fluorescing substances in extracts from air- and CO2-treated hypocotyls of Phaseolus vulgaris, cv. Topcrop, infected with the gamma race of Colletotrichum lindemuthianum (incompatible interaction).

Extracts were separated on silica gel thin layers using benzene : ethyl formate : formic acid (75 : 24 : 1, v/v/v) as developing solvent. Components were visualized with ultra-violet radiation (340 nm) and photographed immediately after development.

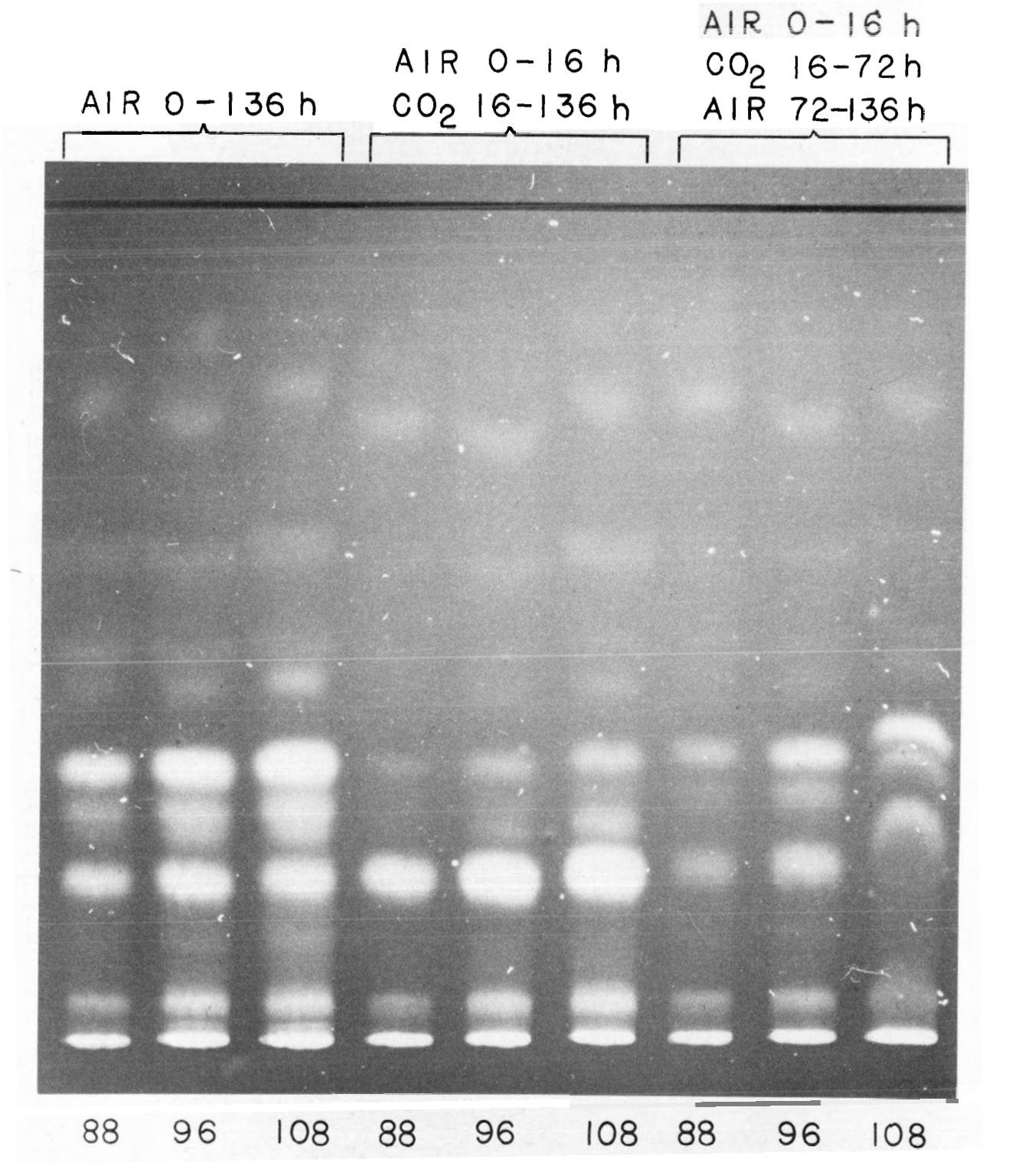

AIR 0-136 h
AIR 0-16 h
CO2 16-136 h
AIR 0-16 h
CO2 16-72 h
AIR 72-136 h
88
96
108
88
96
108
88
96
108
TIME AFTER INOCULATION (HOURS)

Fig. 3.3 Appearance of hypocotyls of Phaseolus vulgaris, cv. Topcrop at 160 h after inoculation with the gamma race of Colletotrichum lindemuthianum (incompatible interaction). Seedlings were given the following treatments:

(a) air 0-160 h.

(b) air 0-16 h, 15% CO2 16-72 h, air 72-160 h.

(c) air 0-16 h, 15% CO2 16-160 h.

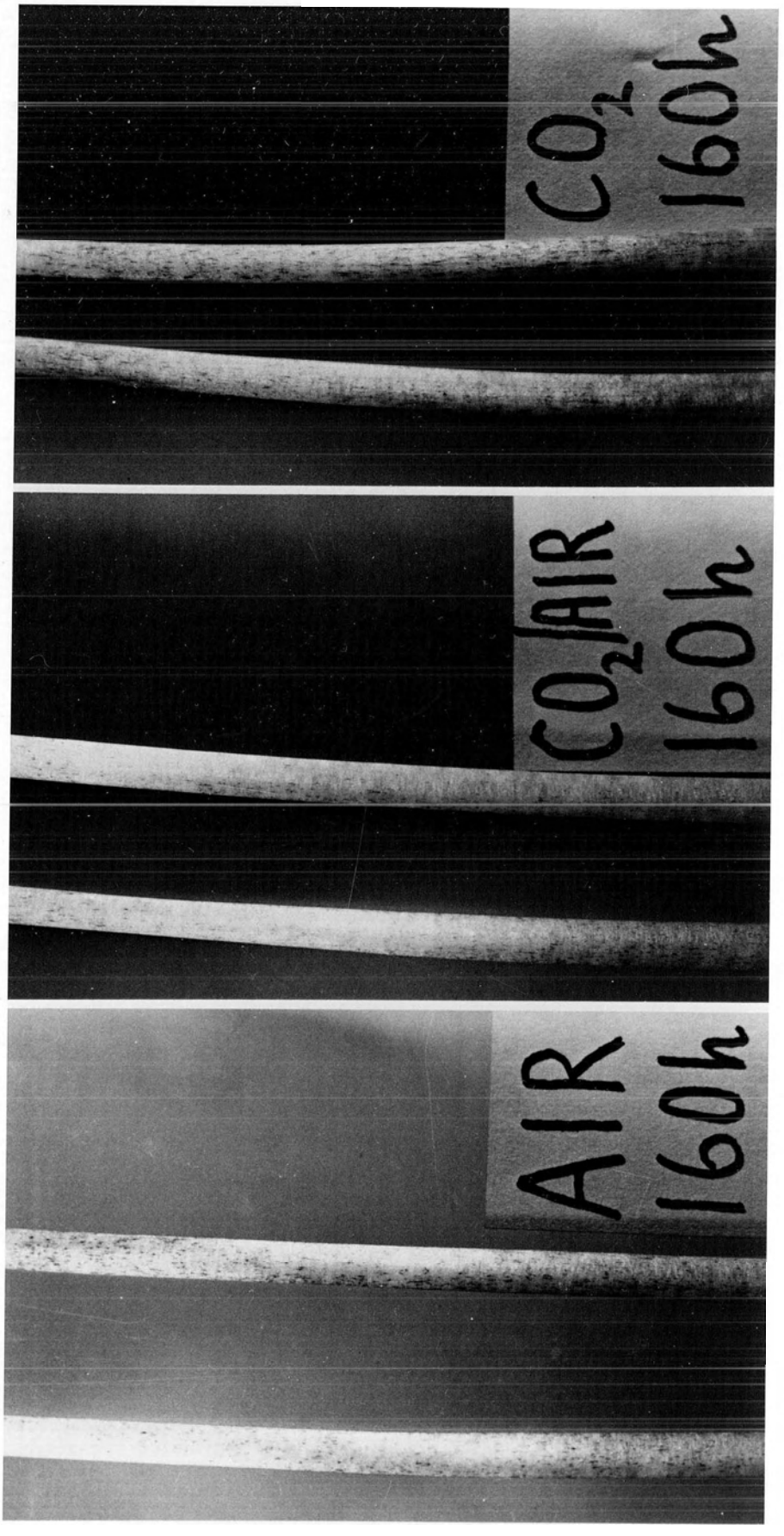
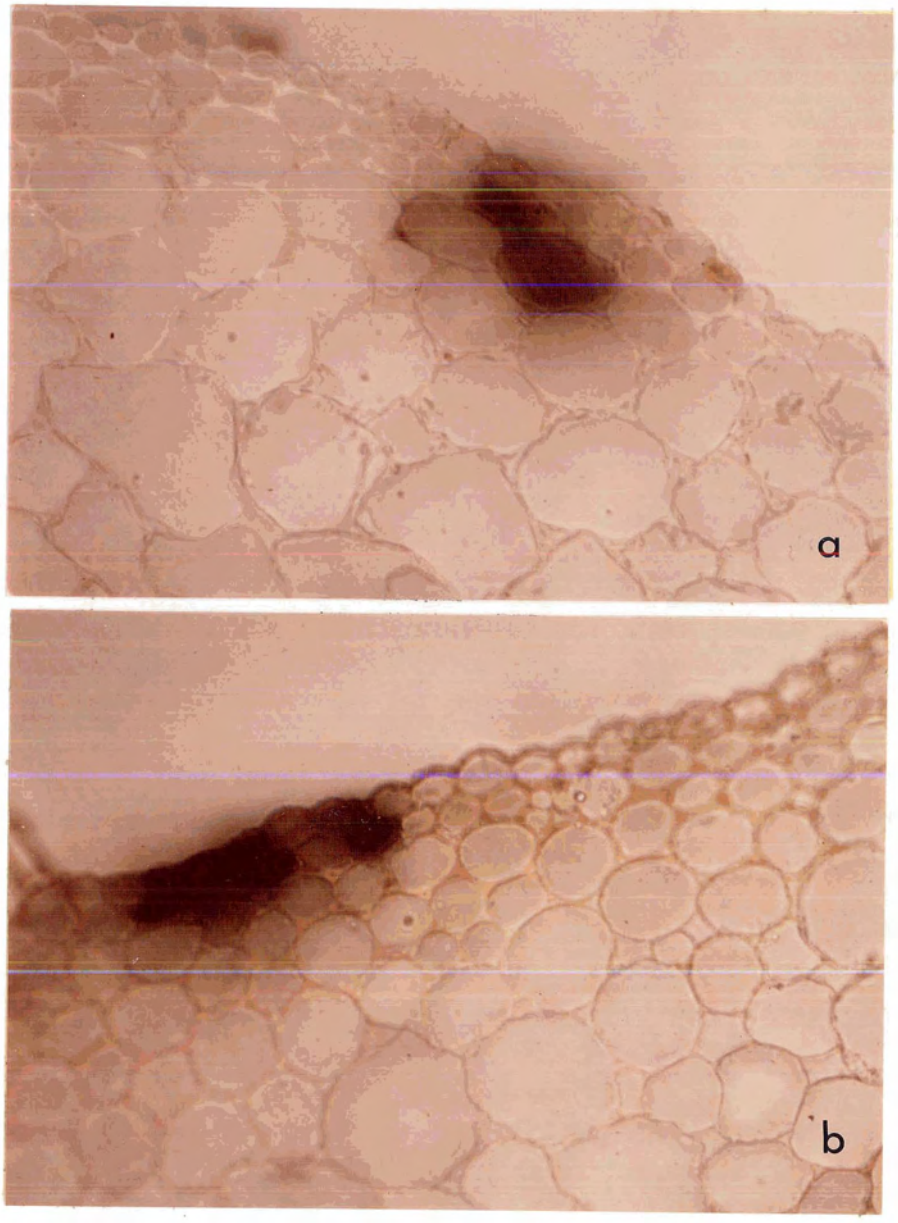

AIR
160h
CO2/AIR
160h
CO2
160h

Fig. 3.4 Transverse sections of hypocotyls of *Phaseolus vulgaris*, cv. Topcrop at 160 h after inoculation with the gamma race of *Colletotrichum lindemuthianum* (incompatible interaction). Seedlings were treated in (a) air 0-160 h (b) air 0-16 h, CO_2 16-160 h.


a
b

Accumulation of phaseollin at injury sites.

No phaseollin was detected when injury sites were excised and extracted immediately after the injuries were made. The detection limit for this determination was about 1×10^{-3} µg per injury site. Increasing amounts of phaseollin per injury site were found when the extraction was carried out at later times. The amount reached a maximum at 24-30 h after injury and decreased during the period from about 48-72 h (Fig. 3.5). 15% CO2, applied immediately after injury or following a 3 h lag period in air inhibited the accumulation of phaseollin at injury sites on the upper halves of hypocotyls for periods up to 72 h after injury (Figs. 3.5 and 3.6). These injury sites remained white in color throughout the experiment in contrast to the light brown discoloration which develops at about 12 h after injury in air. When the length of the lag period in air was increased to 6 or 9 h some phaseollin (6 and 21% respectively of the control values) was present at 24 h after injury (Fig. 3.6). No inhibition of phaseollin accumulation occurred when seedlings were kept in air for 12 h preceding treatment with 15% CO2.

In contrast, phaseollin accumulation at injury sites on the bottom halves of hypocotyls was less sensitive to CO2. Some phaseollin had accumulated by 24 h at these sites when seedlings were placed in 15% CO2 immediately after injury (Fig. 3.6). Also, these sites developed a brown

Fig. 3.5 Effect of 15% CO2 on the accumulation of phaseollin at injury sites on the upper halves of hypocotyls of Phaseolus vulgaris, cv. Topcrop.

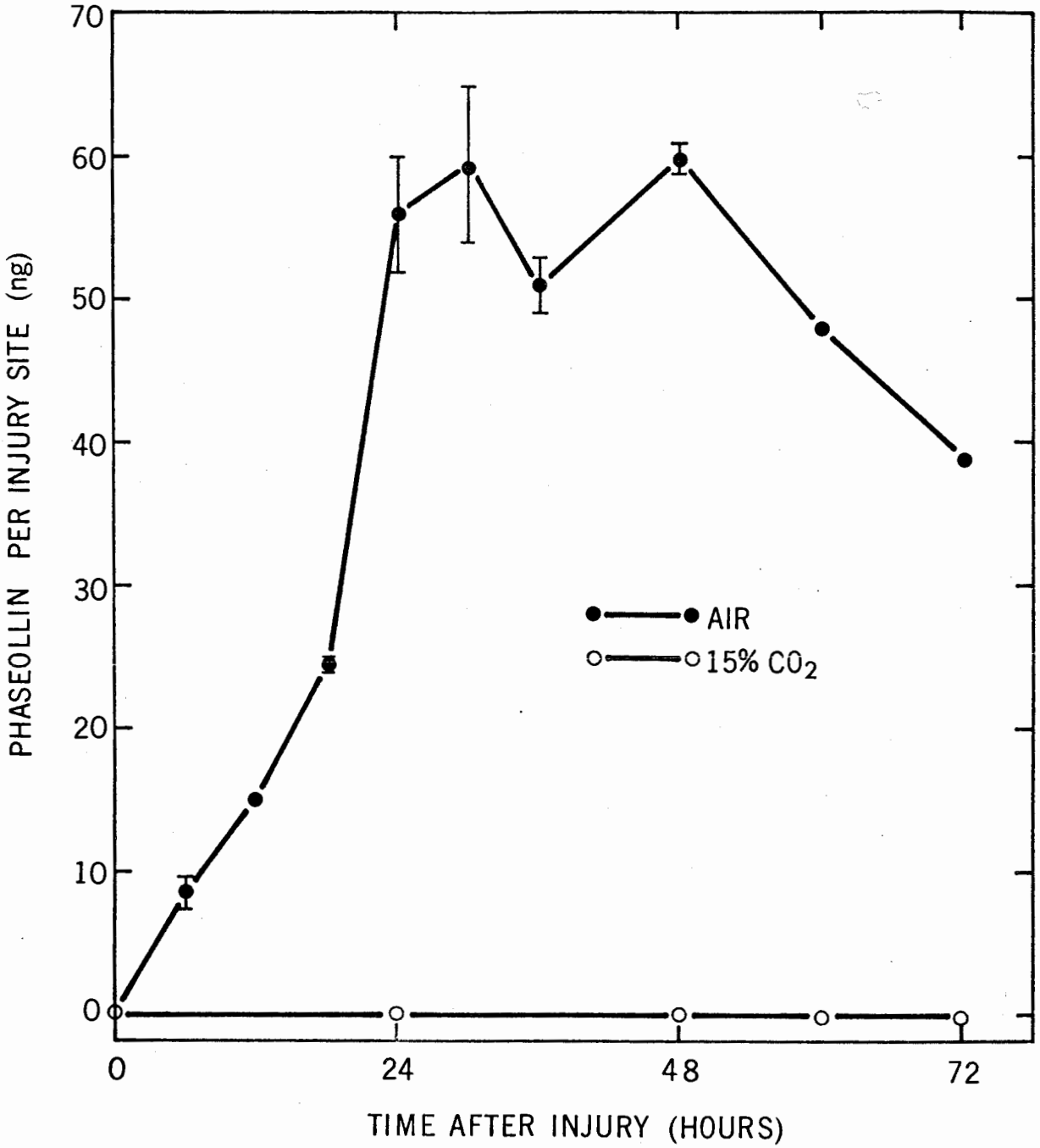

70
60
50
40
30
20
10
0
PHASEOLLIN PER INJURY SITE (ng)
0
24
48
72
TIME AFTER INJURY (HOURS)
AIR
15% CO_2

Fig. 3.6 Occurrence and levels of phaseollin at injury sites on hypocotyls of Phaseolus vulgaris, cv. Topcrop, in relation to time of beginning treatment with 15% CO2.

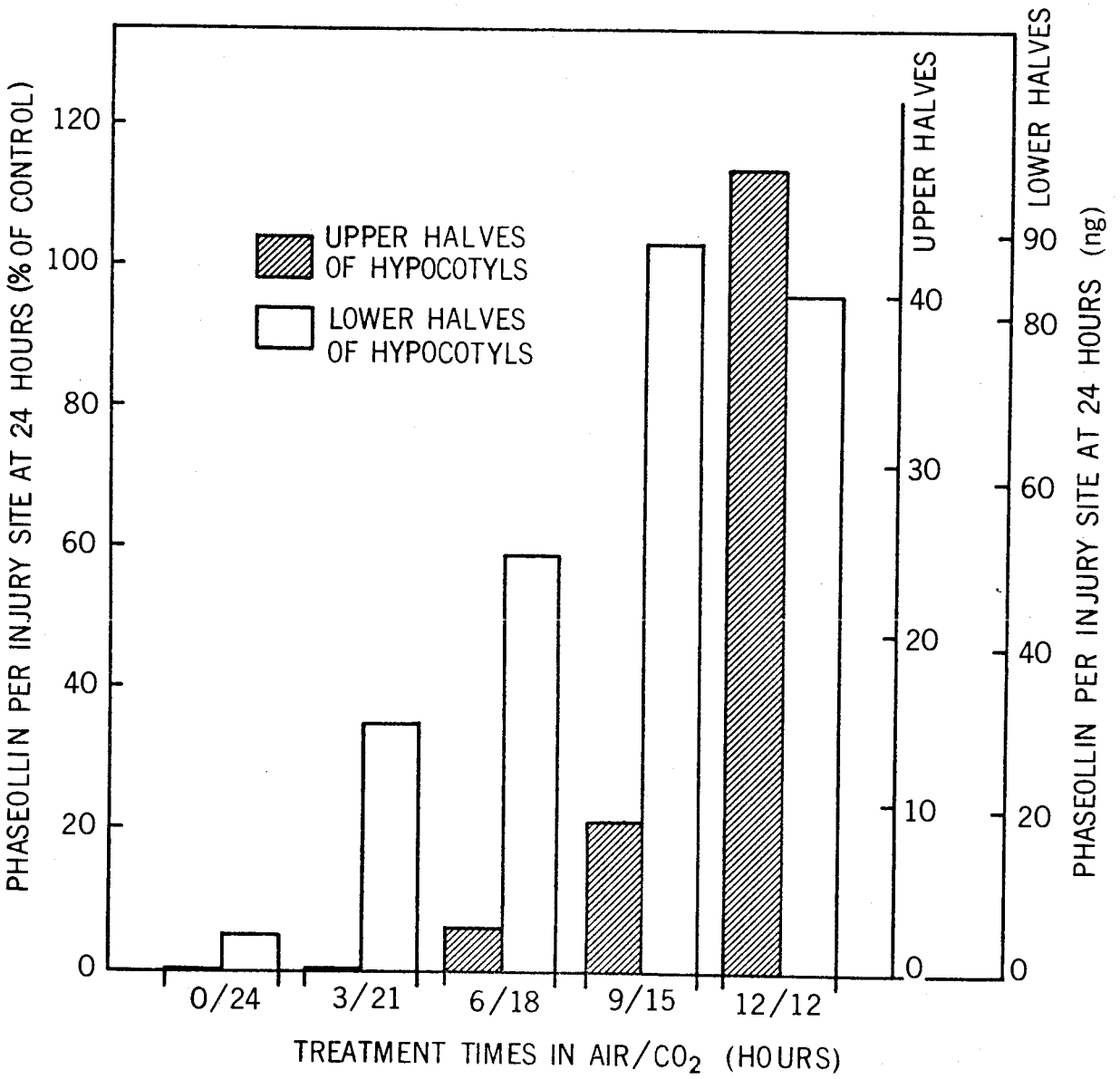
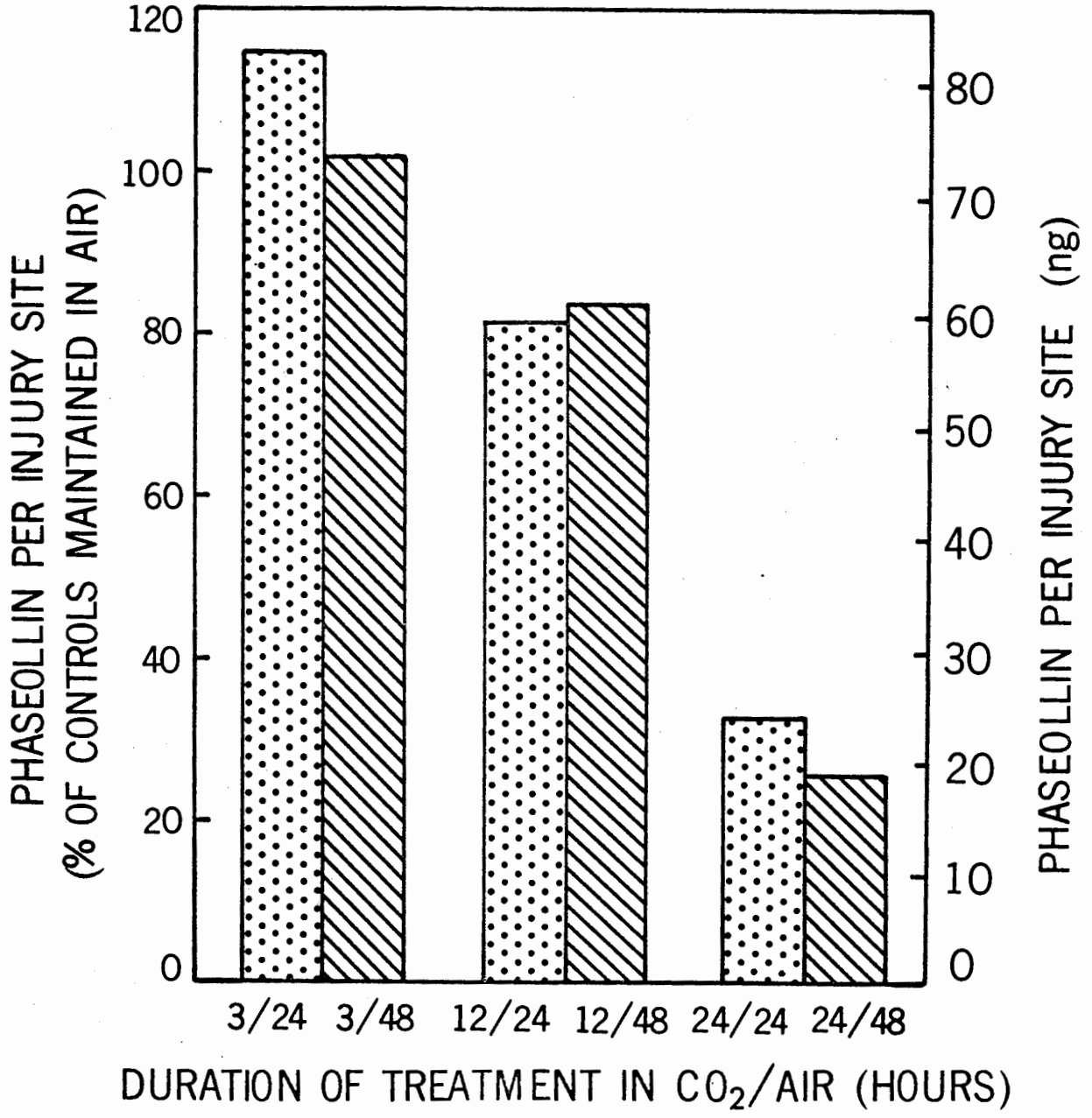

PHASEOLLIN PER INJURY SITE AT 24 HOURS (% OF CONTROL)
120
100
80
60
40
20
0
UPPER HALVES OF HYPOCOTYLS
LOWER HALVES OF HYPOCOTYLS
UPPER HALVES
40
30
20
10
0
LOWER HALVES
90
80
60
40
20
0
PHASEOLLIN PER INJURY SITE AT 24 HOURS (ng)
0/24
3/21
6/18
9/15
12/12
TREATMENT TIMES IN AIR/CO_2 (HOURS)

Fig. 3.7 Occurrence and levels of phaseollin at injury sites on hypocotyls of Phaseolus vulgaris, cv. Topcrop, following increasing lengths of treatment with 15% CO2.


PHASEOLLIN PER INJURY SITE
(% OF CONTROLS MAINTAINED IN AIR)
120
100
80
60
40
20
0
PHASEOLLIN PER INJURY SITE (ng)
80
70
60
50
40
30
20
10
0
3/24
3/48
12/24
12/48
24/24
24/48
DURATION OF TREATMENT IN CO_2/AIR (HOURS)

discoloration similar to that of the controls. However, a 9 h period in air before beginning CO2 treatment was necessary before the amount of phaseollin that accumulated in 24 h equalled that of the controls.

In order to determine whether the inhibitory effect of CO2 is reversible or not, seedlings were placed in 15% CO2 immediately after injury, kept there for 3, 12, or 24 h, and then returned to air. The amounts of phaseollin that accumulated at injury sites on the upper halves of hypocotyls in the 24 and 48 h periods following CO2 treatment were measured for each of the three treatments. A separate control was set up for each CO2 treatment. Injuries were made on each set of control seedlings at the time the appropriate CO2-treated seedlings were returned to air, and the amounts of phaseollin that accumulated in controls in the 24 and 48 h periods after injury were measured. Results are presented in Fig. 3.7. A 3 h exposure to CO2 after injury had no effect on the subsequent accumulation of phaseollin. The 12 and 24 h CO2 treatments, however, caused a progressive reduction in the amount of phaseollin that accumulated in the subsequent 24 and 48 h periods.

DISCUSSION

Despite the large amount of work that has been done on the phytoalexin phaseollin, the nature of the initial stimulus which triggers its accumulation is not known, nor have the details of its biosynthetic pathway been worked out. There exists good circumstantial evidence for an important role of phaseollin in defense metabolism of P. vulgaris (17). Rahe and Arnold (manuscript in preparation) have studied the accumulation of phaseollin at or around mechanical injury sites on etiolated bean hypocotyls. It was suggested that the accumulation of phaseollin at sites of infection could be a non-specific response to the injury a fungus must make when it penetrates the cell walls and membranes of its hosts. If phaseollin is in fact a functional component of the defense response in beans, it follows that a successful bean pathogen would have to subvert or block a defense response that it non-specifically induces.

The most significant finding in this chapter is that, although phaseollin accumulation at injury sites can be inhibited by CO2, its accumulation at incompatible infection sites is CO2-insensitive. The degree of inhibition at injury sites depends on the location of the injuries, those in the upper halves of the hypocotyls being more sensitive to CO2, and on the length of time the seedlings remain in

air after injury. The results indicate that CO2 treatment must begin soon after injury in order to give a high degree of inhibition of phaseollin accumulation. In the incompatible interaction, CO2 treatment was begun about 14 h before penetration of fungal infection hyphae into the plant was first observed, and thus probably before the stimulus for phaseollin accumulation had occurred. Support for the belief that the stimulus for phaseollin accumulation is an event connected with penetration has been obtained from experiments in which bean hypocotyls were inoculated with the corn pathogen Helminthosporium carbonum (66). This fungus causes a hypersensitive response in beans. However, it does not apppear to penetrate the epidermal cells of the host, nor does it cause the accumulation of phaseollin. The results presented here suggest that the stimulus for phaseollin accumulation in an incompatible interaction is probably more complex than a direct response to cell damage associated with penetration, though such a response may still be an important part of the whole stimulus.

Considerably less phaseollin accumulated at incompatible infection sites on seedlings removed from CO2 at 72 h than on seedlings that were maintained in CO2 or in air throughout the experiment. The reason for this difference is not known. Some questions can be raised as to whether the phaseollin concentration in and around the necrotic cells in this treatment would be sufficient to

inhibit the fungus in an *in vitro* assay. Since these infection sites remained as discrete flecks in which the fungus was contained, this must be considered an important question needing clarification in future work.

The data do not permit more than speculation about the mechanism of the inhibitory effect of CO2 on phaseollin accumulation at injury sites. Of interest in this regard is the observation that CO2 does not inhibit phaseollin accumulation at injury sites on the upper halves of hypocotyls during a 12 h period of treatment following 12 h in air (Fig. 3.6). The data in Fig. 3.5 indicate that 75% of the amount of phaseollin present at 24 h under these conditions must have accumulated during the 12 h period in CO2. Hence it can be concluded that the inhibitory effect of CO2 is exerted at a relatively early stage in the process leading to phaseollin accumulation. A more thorough interpretation of these results must await the elucidation of the nature of the stimulus that causes phaseollin accumulation in a host-parasite interaction and of the pathway of its synthesis.

CHAPTER 4

LITERATURE SURVEY AND CONCLUSIONS

This chapter will give an account of the recent literature that is relevant to the findings described in the previous chapters of this thesis, and will attempt to interrelate those findings with the work of others in the field.

During compatible interactions between Phaseolus vulgaris and Colletotrichum lindemuthianum, host cells and fungal infection hyphae apparently coexist for 2-3 days after inoculation. After this time colonized host cells become necrotic and the characteristic anthracnose lesions are produced. In contrast, incompatible interactions are characterized by death and necrosis of host cells soon after penetration has occurred. The means by which interaction type is determined in this system is not known, though it has been the subject of a considerable amount of research and speculation. All available evidence indicates that the initial environment encountered when an infection hypha penetrates from an appressorium is equally favorable irrespective of whether the ultimate outcome of the interaction is one of resistance or of susceptibility. Hence, emphasis has been placed on post-infectional changes, and particularly on the accumulation of the fungitoxic metabolite phaseollin after infection hyphae have become established in host cells.

The concept that post-infectional fungitoxic substances might be the basis of disease resistance was formally stated by Müller and Börger (59) who called such substances

"phytoalexins". They defined a phytoalexin as a compound produced only when living cells of the host undergo necrosis resulting from invasion by a pathogen. In recent years it has been shown that some phytoalexins may accumulate in trace quantities in apparently healthy tissues or in larger quantities in response to abiotic stimuli. Hence Kuć has proposed the concept that a substance believed to contribute to disease resistance should be classified as a phytoalexin irrespective of the nature of the stimulus leading to its accumulation (55). An ever-increasing list of compounds from many different families of plants fits this latter definition and has been the subject of several reviews in recent years (22,23,49,50,55).

The phytoalexin concept, at least in the more restrictive sense defined by Müller, is very much bound up with the concept of hypersensitivity. Ward (88) and Stakman (79) described a rapid necrosis and death of cells of wheat during infection with incompatible races of the rust fungi Puccinia graminis and P. recondita. Their work led to the formulation of the concept that such a hypersensitive response is the cause of the resistance exhibited by plants to incompatible races of pathogenic fungi. Obligate parasites, by definition, can only grow and reproduce in living host tissues, hence it is not difficult to understand how the hypersensitive death of cells came to be regarded as an effective resistance mechanism. However the

hypersensitive response is also characteristic of incompatible interactions between facultative parasites and their hosts. Here the role of hypersenstivity *per se* in limiting spread of a pathogen is less obvious since these pathogens can grow readily on tissues killed in other ways (92). The finding that fungitoxic "phytoalexins" accumulated in tissues undergoing a hypersensitive response has provided a means of explaining how such a response might be capable of inhibiting the growth of facultative parasites in resistant hosts.

In many cases the accumulation of phytoalexins is associated temporally with hypersensitive necrosis in tissues expressing resistance to a pathogen. Bailey and Deverall (3) showed a progressive increase in the amounts of phaseollin extracted from bean hypocotyl tissue at times after the onset of necrosis in response to incompatible races of *Colletotrichum lindemuthianum*. The amount reached a maximum when all penetrated cells had become brown. Histologial studies of this interaction by Skipp and Deverall (75) showed that very little growth of infection hyphae occurred in brown cells, and that hyphae did not grow out of cells which had become brown. Rahe (64) reported that the occurrence of phaseollin coincided with the appearance of necrosis in etiolated hypocotyls of *Phaseolus vulgaris* infected with an incompatible race of *C. lindemuthianum*. Similarly, accumulation of rishitin in potato varieties

inoculated with an incompatible race of Phytophthora infestans has been associated with cell death and browning. Sato, Kitazawa and Tomiyama (72) reported that rishitin was produced earlier in the cut surface of potato tubers inoculated 24 h after cutting than in those inoculated immediately after cutting. They further showed that the time that elapsed between the death of about 20% of infected cells and initiation of rishitin formation was the same (about 8-9 h) in both treatments, and suggested that cell death might be a trigger for the synthesis of rishitin. The rates of growth of intercellular hyphae of compatible and incompatible races of P. infestans were similar until rishitin began to appear, suggesting that hypersensitive cell death per se did not inhibit hyphal development, but that rishitin may play an important role in the resistant response.

Whether a temporal association between the hypersensitive response and cessation of growth of a pathogen is also a cause and effect relationship as suggested by Tomiyama's group (72) is a subject of debate. Király, Barna, and Érsek (53) applied chloramphenicol to potato tuber tissue before or 1-2 h after inoculation with a compatible race of P. infestans. A typical hypersensitive response was obtained and rishitin was produced. Hypersensitive necrosis and rishitin accumulation did not occur in response to the fungus or to chloramphenicol alone,

but only when the growth of the fungus was inhibited by chloramphenicol. They interpreted these results to indicate that in an incompatible interaction some unknown factor stops the growth of the pathogen, and that the hypersensitive response is no more than an inevitable result of this inhibition.

Other investigators have suggested that phytoalexin accumulation may be separate from the changes associated with hypersensitivity. Rathmell and Bendall (68) showed that treatment of hypocotyls of P. vulgaris with mercuric chloride solution or with culture filtrates of Penicillium expansum led to the formation of phaseollin and to the structurally related isoflavonoid coumestrol. Only small changes in the levels of flavonols and hydroxycinnamic acids were detected, however. Later work by Rathmell (67) in which hypocotyls of P. vulgaris were infected with a compatible race of C. lindemuthianum gave similar results. The authors concluded (68,67) that phaseollin production formed part of a specific stimulation of isoflavonoid metabolism distinct from, and regulated differently from, flavonoid metabolism or the increases in phenolic compounds associated with cell necrosis. Stholasuta et al (80) reached similar conclusions from the results of experiments in which P. vulgaris was inoculated with avirulent races of Pseudomonas spp. Ps. phaseolicola caused both a hypersensitive response and accumulation of phaseollin.

Ps. mors-prunorum, however, caused a hypersensitive response, but no phaseollin was detected. The authors concluded that the formation of phaseollin is controlled by factors that are associated with the type of pathogenic organism involved, and is independent of factors that cause a hypersensitive response. Work of Cruickshank and Perrin (16) also supports this hypothesis. They found that monilicolin A, a polypeptide isolated from Monilinia fructicola, induced phaseollin formation without necrosis in bean endocarp tissue.

What factors determine the specificity of an interaction is clearly a question of fundamental importance. In some of the systems where phytoalexins have been studied, it has been found possible to make a good case, based on circumstantial evidence, for an important role of these compounds in defense metabolism (17,72). Based on investigations of pisatin production in Pisum sativum, Hadwiger and Schwochau (37) proposed an induction hypothesis in which they stated their belief that a resistant response is the result of the activation of a genetic sequence specifying pisatin synthesis in response to metabolites produced by non-pathogens of peas. According to this hypothesis, virulence in a pathogen of P. sativum would result from a failure to produce the appropriate inducer, or from the production of a defective inducer. Hadwiger and co-workers obtained support for this hypothesis by showing

that pisatin and phaseollin could be induced, in pea and bean tissue respectively, by microbial metabolites and by compounds known to complex with DNA or to interfere with protein synthesis (34,35,38,39,41,73,74). For example, Schwochau and Hadwiger (74) reported that increases in RNA and protein synthesis were observed in pea pods during the early stages of pisatin induction by low concentrations of actinomycin D, (10 µg per ml). They also found that pisatin induction in response to low levels of the antibiotic could be inhibited by applying higher levels of the same antibiotic (450 µg per ml) early in the induction period, although the inhibitory effect was less pronounced when the antibiotic at the higher concentration was applied at later times. The authors inferred that pisatin induction was dependent on de novo RNA synthesis and that the induction mechanism involved gene activation. Hadwiger and Schwochau (37) also proposed that the suggested mechanism for induction of a defense response is not limited to those systems in which phytoalexins can be implicated as components of defense metabolism, but that it applies generally to "every type of resistance response". In contrast to the results of Scwochau and Hadwiger (74), Biggs (8) found no evidence of an increased formation of any specific RNA fraction in bean endocarp tissue treated with actinomycin D (25-30 µg per ml), although the antibiotic at this concentration did stimulate phaseollin production. These results can not readily be interpreted in terms of

a mechanism for phytoalexin production involving gene activation.

A somewhat different hypothesis based on induced resistance has been proposed by Van Dijkman and Sijpesteijn (86). These authors, in a study of compatible and incompatible interactions in tomato leaves infected with different races of the leaf mould fungus, Cladosporium fulvum, showed a good correlation between the occurrence of a hypersensitive reaction in vivo and leakage of ^{32}P-labelled compounds from infected leaf disks. In all the cases tested, leakage occurred when the plant was resistant to the race of C. fulvum used and did not occur in susceptible combinations. The authors proposed that each physiological race of C. fulvum produces one or more metabolites, each of which is toxic only to tomato varieties which carry the appropriate resistance gene(s). Thus, in this system, it is proposed that resistance is induced by specific toxins which change the permeability properties of host cell membranes. Until these toxins are isolated and their properties studied, it is not possible to say whether they might also have the properties of the inducer molecules proposed by Hadwiger and co-workers.

In contrast to these results, a similar leakage study by Luke et al (57) suggests an 'induced susceptibility' mechanism. These authors treated oats with victorin, the toxin isolated from culture filtrates of Helminthosporium victoriae, and found that leakage of phosphorylated

sugars occurred only from oat varieties that are susceptible to H. victoriae. It will be of interest to see whether other host-parasite combinations can be placed in one or the other of these two groups on the basis of similar studies.

There is, in the minds of some investigators, a fundamental objection to the induction hypothesis. Does each microorganism have, coded in its genome, the information to direct the synthesis of the specific inducers which activate the defense responses of all the plants to which that microorganism is a non-pathogen? As resistance is the rule in nature, that would seem to be a tall order. Proof of the hypothesis would require the isolation of an inducer substance that is unambiguously the product of a gene for avirulence in a pathogen, and the characterization of a gene for resistance in the host which interacts with, or forms a product which interacts with, the inducer (21). Neither of these has yet been achieved.

An alternative hypothesis, for which there is some evidence, proposes that when a microorganism penetrates, or attempts to penetrate the cells of a plant, it non-specifically triggers a defense response, either by the introduction of foreign metabolites and (or) by physical damage associated with penetration. Only those microorganisms capable of specifically blocking the defense response of a given plant will be able to parasitize that

plant. Tomiyama (82) inoculated potato petiole tissue with a compatible race of Phytophthora infestans and found that host and parasite apparently did not react to each other for a period of 2 days. After this time, cell death and necrosis was observed. In contrast, inoculation of petiole tissue with an incompatible race of P. infestans resulted in the necrosis and disorganization of some host cells within 10 min of penetration. Most penetrated cells had become necrotic by 40-60 minutes (83). In a double infection experiment, Tomiyama (82) inoculated potato petiole tissue with a compatible race of P. infestans and then, after a delay of several hours, he reinoculated with an incompatible race. He found that the potato cells reacted in a hypersensitive manner to the second inoculation when the time interval between the two inoculations was 5-6 h. When this interval was increased to 15 h, however, the host cells did not respond in a hypersensitive manner to the incompatible race, although penetration by this race did occur. However a hypersensitive reaction to an incompatible race of the fungus was observed when the tubers had been inoculated with the same incompatible race 15-20 h earlier. These results suggest that the defense response against the incompatible race of the fungus was being prevented by the presence of the compatible race. They will be discussed again later in connection with the induced susceptibility hypothesis suggested by Daly (18).

Similarly, Varns and Kuć (87) found that when potato tuber tissue had been inoculated with a compatible race of P. infestans, reinoculation with an incompatible race of the fungus 12 h later did not cause the accumulation of the phytoalexins rishitin and phytuberin. The hypersensitive and phytoalexin responses could also be elicited by treatment of tuber tissue with sonicated homogenates of either race of the fungus. Preinoculation of the tissue with a compatible race of P. infestans suppressed the responses to this stimulus also, sugggesting that susceptibility may be the result of suppression, by compatible races, of the host response to a factor possessed by both compatible and incompatible races of the fungus.

Other evidence which is difficult to reconcile with an induction hypothesis has resulted from experiments with several different host-parasite systems. Bailey and Ingham (4) reported the accumulation of phaseollin in leaves and etiolated hypocotyls of Phaseolus vulgaris in response to treatment with a suspension of tobacco necrosis virus (TNV). It is most unlikely that the formation of phaseollin results from the release of a specific inducing compound by the virus. Bailey and Ingham suggest that its formation may be closely linked with general changes in metabolism that accompany cell necrosis. More recently, Bailey and Burden (2) identified four compounds with fungitoxic properties in etiolated bean hypocotyls infected with TNV. These were

phaseollin, phaseollidin, phaseollinisoflavan, and kievetone. The authors proposed that the production of these compounds might explain reports that viral infections that result in necrotic local lesions may protect plants from subsequent fungal attack (40,90). Phytoalexin accumulation can be stimulated by other factors which cannot be classified as inducers within the terms of reference of the induction hypothesis. Thus, pisatin formation can be stimulated by ethylene (12), phaseollin by point-freezing injury (65) and hydroxyphaseollin, the phytoalexin from soybeans (Glycine max), by mechanical injury (52,54). Studies on the accumulation of phaseollin in P. vulgaris in response to C. lindemuthianum by Bailey and Deverall (3) and by Rahe (64) have indicated that the timing of production of phaseollin, rather than differences in the amounts of the phytoalexin that are produced in resistant and susceptible interactions, may be important in determining the outcome of an interaction.

If phytoalexin production is an important factor in a defense response then such a response will not stop the growth of a microorganism that is capable of degrading or detoxifying the phytoalexin. Thus, specificity of interaction type could be mediated via the differential abilities of pathogens and non-pathogens to degrade a phytoalexin that they non-specifically induced earlier in the interaction. Deverall and Vessey (24) have investigated

phytoalexin production in broad beans (Vicia faba) in response to parasitism by Botrytis fabae and B. cinerea. The former causes spreading lesions and widespread necrosis whereas the latter causes small limited lesions. The authors have attributed this difference in pathogenicity to the ability of B. fabae to degrade the phytoalexin wyerone acid. Similarly, Higgins and Millar (42,43), working with the phytoalexin medicarpin, isolated from alfalfa, showed that Stemphylium botryosum (pathogenic) degraded medicarpin to two phenolic compounds that were not inhibitory to germ tube elongation by the fungus, whereas Helminthosporium turcicum (non-pathogenic) did not interfere with the accumulation of the phytoalexin. Some pathogens of peas can degrade pisatin in a process that is subject to catabolite repression by high concentrations of glucose or sucrose (25). Phaseollin can be detoxified by Fusarium solani f.sp. phaseoli in an oxidative process believed to involve an inducible enzyme system (85).

Despite the good circumstantial case that can be made for a role of some phytoalexins in disease resistance, in no single case is there proof that a given substance plays a role in host defense metabolism against pathogenic attack. Such proof continues to be elusive. Daly (18) has described the basic difficulties. These may be summarized as follows:-

1. It has not been found possible to determine the concentrations of phytoalexins at the sites in plant tissues where they are presumed to act. Estimates of the concentration of a phytoalexin in a tissue are usually derived from extractions of whole tissue samples with organic solvents and quantitation of the amounts in some convenient unit per gram fresh weight or dry weight of tissue. Then on the basis of assumptions about the localization of the compound concerned, calculations of the amounts that might be present at infection sites are made.

2. Many of the compounds implicated in defense metabolism are sparingly soluble in water so that it is necessary to employ special solubilization procedures in order to experimentally determine ED50 values in in vitro assays (14). Because of their solubility characteristics, it is possible that phytoalexins might be formed and retained within specific cell organelles or membranes and thus remain separated from a pathogen growing in the aqueous cytoplasm and vacuoles of the host cells.

The antibiotic properties of these compounds and the fact that fungitoxic levels are reached in host cells especially during hypersensitive resistant reactions, are not in dispute. But whether these compounds are primary determinants of interaction type is not clear, and some of

the evidence is contradictory. For example, work by Tomiyama (51,72,82,83) has shown that the phytoalexin rishitin cannot be detected in potato tuber tissue inoculated with an incompatible race of Phytophthora infestans until at least 12 h after inoculation, and does not reach concentrations that are toxic in in vitro assays until 48-72 h after inoculation. Yet cell necrosis, commonly taken to be a sign of a resistant reaction, occurred between 30 min and 3 h after inoculation. Similarly Rahe (64) has suggested that phaseollin is probably not the primary determinant of interaction type in the anthracnose disease of beans, but that it may be a product of the changes that do determine the outcome of an interaction, and thus may still play an important role in defense metabolism in this interaction.

Proposals for alternative mechanisms of resistance in plants have come from studies on the possible roles of ethylene and of the enzyme peroxidase in host-parasite interactions. Ethylene is known to exert an influence on many different plant processes and to be produced by almost all plants and plant tissues. The literature on the distribution of ethylene in plants and on the effects of ethylene on plant growth has been reviewed by Pratt and Goeschl (61). It has been suggested that ethylene may be involved in the more "abnormal" growth processes that are associated with parasitism by microorganisms. There are a

number of reports that ethylene stimulates the activities of enzymes, including phenylalanine ammonia-lyase (58,70) tyrosine ammonia-lyase (58) peroxidase (31,69,78) and polyphenoloxidase (78). Since these enzymes are often regarded as being of fundamental importance in disease resistance, a possible role of ethylene as a determinant of interaction type can be proposed. In addition, there are reports that ethylene production is greater in infected or injured tissue than in healthy tissue (10,47,48) and that a large number of bacteria and fungi produce relatively large quantities of this gas in culture (10,46).

During studies on cross-protection in sweet potato root tissue infected with compatible and incompatible races of Ceratocystis fimbriata, Stahmann et al (78) showed that prior exposure of root tissue to low concentrations (8-150 ppm) of ethylene induced resistance to infection by a normally compatible race of the fungus. In addition, increases in the activities of peroxidase and polyphenoloxidase were described. Higher levels of ethylene were produced by root tissue inoculated with a non-pathogenic race of C. fimbriata which induces resistance to pathogenic races than were produced by tissue inoculated with the non-pathogenic C. minor which does not induce resistance to pathogenic races of C. fimbriata. Most of the ethylene produced appeared to be of host origin, though the fungus does produce the gas in culture (10,47). Stahmann

and co-workers suggested that ethylene might be one of the stimuli which diffuse from infected areas of tissue to adjacent areas where the initiation of metabolic changes that lead to resistance to further penetration by the pathogen occur and that this process could explain the induction of resistance to black rot in susceptible tissues inoculated with non-pathogenic races of C. fimbriata.

These results have been the subject of some controversy. It is clear that ethylene production is a characteristic of many host-parasite interactions, but the relationship of biochemical and enzymatic changes induced by ethylene to disease resistance is not clear. Chalutz and DeVay (10) found that, in contrast to data by Stahmann et al (78), black rot development in sweet potato root tissue was not affected by ethylene applied before or after infection. However they noted that inoculation with several different pathogenic races of C. fimbriata produced widely different rates and amounts of ethylene production, so that adequate controls were difficult to devise.

Since ethylene stimulates the activity of phenylalanine ammonia-lyase it is perhaps not surprising that increases in some phytoalexins have been detected following ethylene treatment. Thus, prolonged exposure of carrot root and pea pod tissue to low concentrations of ethylene stimulated the production of an isocoumarin and pisatin, respectively (11,12). Attempts to implicate ethylene as a triggering

agent for phytoalexin production are hampered, however, by the fact that many chemical and physical agents stimulate production of phytoalexins. In addition, ethylene is comparatively ineffective as a phytoalexin-inducer.

A role for ethylene in disease resistance has also been suggested on the basis of the increases in the activities of peroxidase that frequently occur following treatment of plant tissue with ethylene. The findings of Stahmann et al (78), described above, suggested to them that peroxidase might be involved in resistance mechanisms. However, stimulation of peroxidase activity by ethylene is not a universal phenomenon. No such stimulation was found when barley seedlings were incubated in an ethylene-containing atmosphere, and although increases in the activity of peroxidase in leaves inoculated with Erysiphe graminis were detected, the amounts were not correlated with the outcome of the interaction in terms of its resistance or susceptibility (44).

Critical evidence against a relationship between ethylene, peroxidase and disease resistance has come from the work of Daly and co-workers (20) who have studied resistance to stem rust of near-isogenic lines of wheat carrying the Sr6 and sr6 alleles. This gene is temperature-sensitive, such that plants possessing the dominant allele are resistant to race 56 of the stem rust fungus, Puccinia graminis tritici, at 20 C, but susceptible

at 26 C (56). Increases in the activity of peroxidase were found in resistant lines of wheat infected with race 56 of the fungus or treated with ethylene. However, when leaves from a line susceptible to race 56 were treated with ethylene, peroxidase activity increased to levels higher than were found in leaves showing a resistant reaction. In addition, wheat leaves carrying the resistance gene Sr6 became susceptible to stem rust at 20 C after treatment with ethylene in spite of the fact that high peroxidase levels were induced. Daly (20) *et al* concluded that any relationship between ethylene and peroxidase activity was not related to disease resistance in this system.

The induction, by ethylene, of susceptibility in plants carrying the Sr6 allele suggested to Daly and his co-workers that perhaps it is susceptibility, rather than resistance, that is normally induced in plant hosts. This induction might take the form of a series of metabolic adjustments to the presence of the pathogen while, conversely, a failure to make the necessary adjustments might cause death of the pathogen, an event followed by the metabolic reactions commonly interpreted as "defense reactions". Day (21) has suggested that susceptibility might result from the induction of degradative enzymes, such as proteases, in host cells. There is evidence for the existance of phenylalanine ammonia-lyase-inactivating systems in pea seedlings (45) and in potato tubers and *Xanthium* leaves (94).

Susceptibility could be "induced" by a factor which shifts the equilibrium between synthesis and inactivation of key enzymes controlling defense reactions in favour of inactivation. A precisely opposite role for hydrolytic enzymes has been claimed by Pitt and Coombes (60). These authors found particles similar to lysosomes in potato tuber tissue and described the release of hydrolytic enzymes (acid phosphatases, ribonucleases, and proteases) from these particles in response to infection by pathogenic tuber-rotting fungi. The presence of cytoplasmic particles in potato petiole cells, observable within minutes of injury or infection by an incompatible race of Phytophthora infestans, has been described by Tomiyama (84). It has been suggested (21) that the avirulent pathogen might trigger the release of hydrolytic enzymes from lysosomes. These enzymes would then kill the cell and perhaps stimulate the synthesis of phytoalexins in the adjacent healthy tissue. A summary of the evidence for a role of lysosomes in host-parasite interactions is available in a recent review by Wilson (89).

The data of Tomiyama (82,83), as described above, could be interpreted to mean that both induced resistance and induced susceptibility could operate in the same system. It will be recalled that prior inoculation of potato tuber tissue with an avirulent race of Phytophthora infestans produced an incompatible interaction that was not reversed

by a second inoculation with a virulent race carried out 15-20 h later, ("induced resistance"). If the tubers received an inoculation with a virulent race first, a compatible reaction occurred which was similarly not reversed by a second inoculation with an avirulent race given 15-20 h later ("induced susceptibility"). However, as described above, it is possible to interpret both these findings in terms of a non-specific triggering of defense metabolism by both the virulent and avirulent races, followed by specific blockage of defense metabolism by the virulent race.

Finally, mention should be made of a hypothesis which predicts that the primary factor determining the outcome of a host-parasite interaction is the degree of antigenic disparity between host and pathogen (26,27). In general, it is believed that the greater the antigenic disparity, the greater will be the degree of resistance of the host to the pathogen. Doubly et al (28) showed that susceptibility of flax to flax rust (Melampsora lini) was correlated with a relatively high titer of rust antiserum with flax antigens, indicating that rust antigens were present in susceptible lines of flax. Wimalajeewa and DeVay (91) found common antigens between corn and all tested lines of the smut fungus Ustilago maydis. Evidence was presented that the antigens are to be found in the protein components of the

ribosomes of both the host and the fungus. Antigenic similarities between two such phylogenetically distinct organisms as a higher plant and a fungus are very surprising. How such similarities arise is not at all clear, though the evidence of incorporation of segments of DNA of the crown gall bacterium Agrobacterium tumefaciens into tobacco cells at gall tumor sites (for example,77) suggests one possible mechanism.

OBSERVATIONS CONCERNING DEFENSE METABOLISM OF BEANS IN THE PRESENCE OF 15% CO2.

A conversion of susceptibility to resistance when hypocotyls of Phaseolus vulgaris were inoculated with a compatible race of Colletotrichum lindemuthianum and maintained in 15% CO2 was reported in Chapter 1. Evidence from this study, and from the effect of 15% CO2 on the fungus in culture as described in Chapter 2, suggest that the protective effect of CO2 during a compatible interaction is exerted primarily via its action on the fungus. Rahe (64) reported that the most obvious difference, with respect to phaseollin production, between a susceptible and a resistant reaction was in the timing of appearance of the phytoalexin. Phaseollin was first detected at the time of appearance of necrotic flecks in an incompatible interaction, but was not detected in a compatible interacton (of low infection site

density) until the individual infection sites became delimited. Király et al (53) have argued that a hypersensitive response may be no more than an inevitable result of a process which inhibits the growth of a pathogen in an incompatible interaction. Perhaps the conversion to a resistant reaction described in Chapter I is likewise an inevitable consequence of the inhibition of fungal growth by CO2.

As described in the discussion section of Chapter I, it seems unlikely that the presence of CO2 could cause the fungus in a compatible interaction to actively induce a defense response. To that extent the data may be said to support the idea that the conversion to resistance is a result of the action of CO2 in slowing down the growth and, presumably, the metabolism of the fungus and thus interfering with its ability to block a defense response of the host that it non-specifically triggered by an event associated with the process of penetration. Data presented in Chapter 3 show that the presence of 15% CO2 does not interfere with the production of phaseollin at incompatible infection sites. Hence, if phaseollin has an important role in defense metabolism of beans, it can be concluded that CO2 provides the conditions in which growth of the pathogen is inhibited, whereas host defense metabolism is unaffected, thus shifting the nature of the interaction in favour of increased resistance.

The sensitivity to CO2 of the process leading to phaseollin production at sites of point-freezing injury is of interest, especially when compared with the lack of such sensitivity in the case of the process at incompatible infection sites. Clearly there are major differences in the nature of the initial stimulus in the two cases. The biotic stimulus probably arises from a process of slow penetration of host cells accompanied perhaps by the release of metabolites capable of diffusing to other cells and eliciting a response in advance of the growing infection hyphae. By this means an inhibitory zone of cells around the fungus at each infection site may arise and prevent further spread of hyphae. In contrast, the abiotic stimulus probably occurs in cells adjacent to the large number of cells at each injury site that are killed more or less instantaneously by contact with the dry ice. It would be interesting to investigate whether ethylene might play a role in the process of phaseollin accumulation, especially as CO2 has been found to be a competitive inhibitor of ethylene action in many processes in which the latter is implicated as a plant hormone (9). Ethylene has been shown to promote phytoalexin production in peas (12) and in carrots (11). In addition, ethylene is itself produced in many host-parasite interactions and in response to injury of plant tissues (10,47,48,70,71,78). It is possible that the stimulus for phaseollin production at sites of

point-freezing injury may be mediated via the release of ethylene from cells adjacent to those killed by the injury. This would explain why CO2 applied soon after injury inhibits phaseollin accumulation, and why the process is no longer sensitive to CO2 if injured plants are maintained in air for about 12 h after injury and then transferred to CO2. In other words, the initial stimulus for phaseollin accumulation (perhaps ethylene production) is sensitive to CO2 whereas the actual metabolic reactions leading to phaseollin production are not affected by CO2. However, the lack of sensitivity to CO2 of the process at incompatible infection sites indicates that ethylene is not essential to the process which triggers phaseollin accumulation in a host-parasite interaction.

SUMMARY OF THE THESIS

1. The compatible interaction between Phaseolus vulgaris, cv. Topcrop, and the beta race of Colletotrichum lindemuthianum can be converted to one of resistance by treatment with 15% CO2. In the experiments described, treatment was most effective

(a) if it was begun before penetration by hyphae of the fungus had occurred, or while the hypha at each infection site was in the lobate form characteristic of the early stages of penetration;

(b) when infection sites were sufficiently close to each other so that coalescent necrosis developed in plants that were not treated with CO2;

(c) when plants were maintained in 15% CO2 at least until necrotic flecks, characteristic of a resistant reaction, began to appear.

2. 15% CO2 delayed germination, germ tube elongation and sporulation in cultures of C. lindemuthianum (beta and gamma races). The similarities between the effects of 15% CO2 on the beta race of the fungus in culture and in the compatible interaction with P. vulgaris (described in 1. above) lend support to the hypothesis that the outcome of the host-parasite interaction in the presence of CO2 is a consequence of an inhibitory effect of CO2 on the fungus.

3. It seems improbable to this author that the fungus, under conditions where its growth is inhibited, could actively induce the resistant reaction described above. To that extent, the data support the hypothesis that a pathogen normally blocks a defense response that it initiates non-specifically by a stimulus associated with the process of penetration. CO2, by slowing down the growth of the fungus, could inhibit its ability to block the defense response of the plant.

4. 15% CO2 inhibited accumulation of the phytoalexin phaseollin at sites of point-freezing injury on hypocotyls of P. vulgaris, cv. Topcrop, but not at sites of infection by the gamma race of C. lindemuthianum (incompatible interaction). The data suggest that the processes leading to phaseollin accumulation at injury sites and at incompatible infection sites are regulated differently, and that its accumulation in host-parasite interactions is not simply a response to an injury stimulus associated with penetration.

SUGGESTIONS FOR FUTURE WORK.

1. Chapter 3 describes a reduced accumulation of phaseollin at incompatible infection sites on hypocotyls of Phaseolus vulgaris that were transferred from 15% CO2 to air about 6 hours before necrotic flecks began to appear on plants which remained in CO2. It would be most interesting

(a) to investigate the physiological and biochemical basis for this unexpected result, and

(b) to repeat the experiment and calculate the amount of phaseollin per unit cell volume on the basis of assumptions about the localization of the phytoalexin. The calculated value could then be compared with published ED50 values for phaseollin against Colletotrichum lindemuthianum that have been derived from in vitro assays.

2. Determine whether ethylene affects

(a) the nature of the compatible and incompatible interactions between P. vulgaris and C. lindemuthianum, and

b) the accumulation of phaseollin at injury sites and incompatible infection sites in the presence and absence of different concentrations of CO2 (a competitive inhibitor of ethylene action).

APPENDIX

Methods used for the production and measurement of high-CO2 atmospheres.

Treatment of plants with atmospheres containing 15% CO2, 85% air, was carried out in a plexiglass growth chamber (internal dimensions, 90 x 50 x 38 cm) through which the mixed gas was passed at a flow rate of 500 ml per minute. This was found to be the minimum flow rate which would prevent an increase in the concentration of CO2 due to respiration by plants maintained inside the growth chamber. The atmosphere inside the chamber was stirred continuously by means of a magnetically driven fan.

Mixtures of CO2 and air were generated from individual cylinders of compressed air and pure CO2 using a high-precision gas mixing pump (H. Wosthoff O.H.G., Bochum, W. Germany. Type NA 18/3a) fitted with the appropriate gears. The manufacturer's specifications indicate that the pump, when operated with gear ratios of 15:85 (as used here), maintains a constant output of 15 ± 0.01% CO2. The concentration of CO2 in the atmosphere in the growth chamber was monitored by passing the effluent from the chamber through an infra-red gas analyzer (Beckman Model 215) fitted with a short-path cell (basic range 0-5% CO2). Dry nitrogen, as a zero-CO2 reference gas, was passed

continuously through the reference cells of the analyzer. The apparatus used is shown in diagrammatic form in Fig. A.1.

The gas analyzer was calibrated over the range 10-25% CO2 (balance air) using gas mixtures generated by the mixing pump (Fig. A.2). Assuming the error in reading the scale on the analyzer to be at the most ± 0.5 microamps, the limit of accuracy in the measurement of CO2 concentrations in the range 10-20% was about ± 0.5%.

At the start of each experiment, plants were placed inside the growth chamber and the concentration of CO2 in the atmosphere inside the chamber was increased as quickly as possible from its atmospheric level by introducing pure CO2 at a flow rate of 500 ml per min into the chamber until the concentration of CO2 in the effluent reached 15 ± 0.5% (about 35 min). The atmosphere inside the chamber was then maintained at a constant composition (15 ± 0.01% CO2, balance air) using the gas mixing pump as described above. Each time the growth chamber was opened for the removal of sample plants, the CO2 content of the atmosphere inside the chamber was restored to 15% using pure CO2 as described above.

Fig. A.1 Diagram of the apparatus used for treatment of plants with 15% CO2 (balance, air).

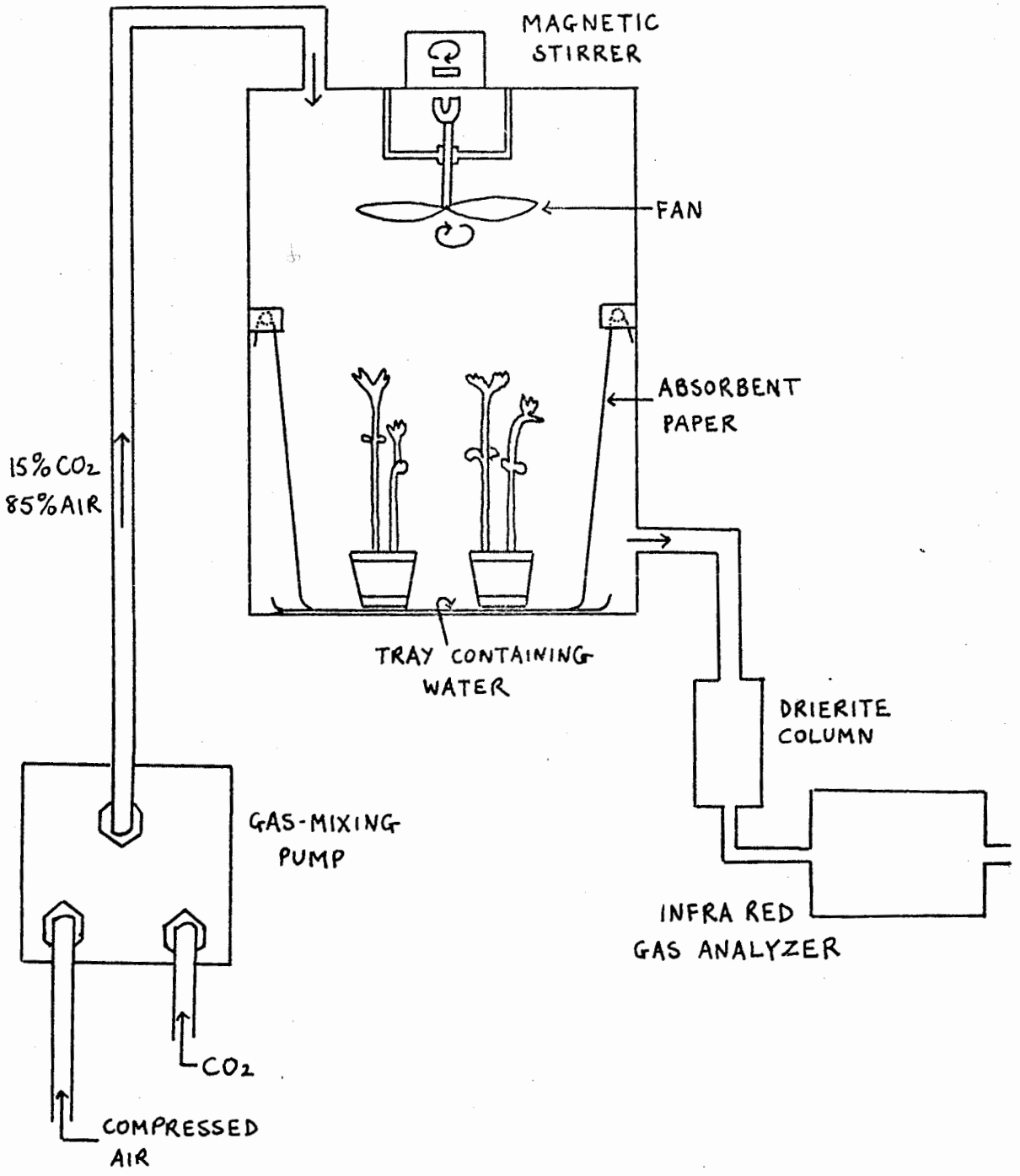
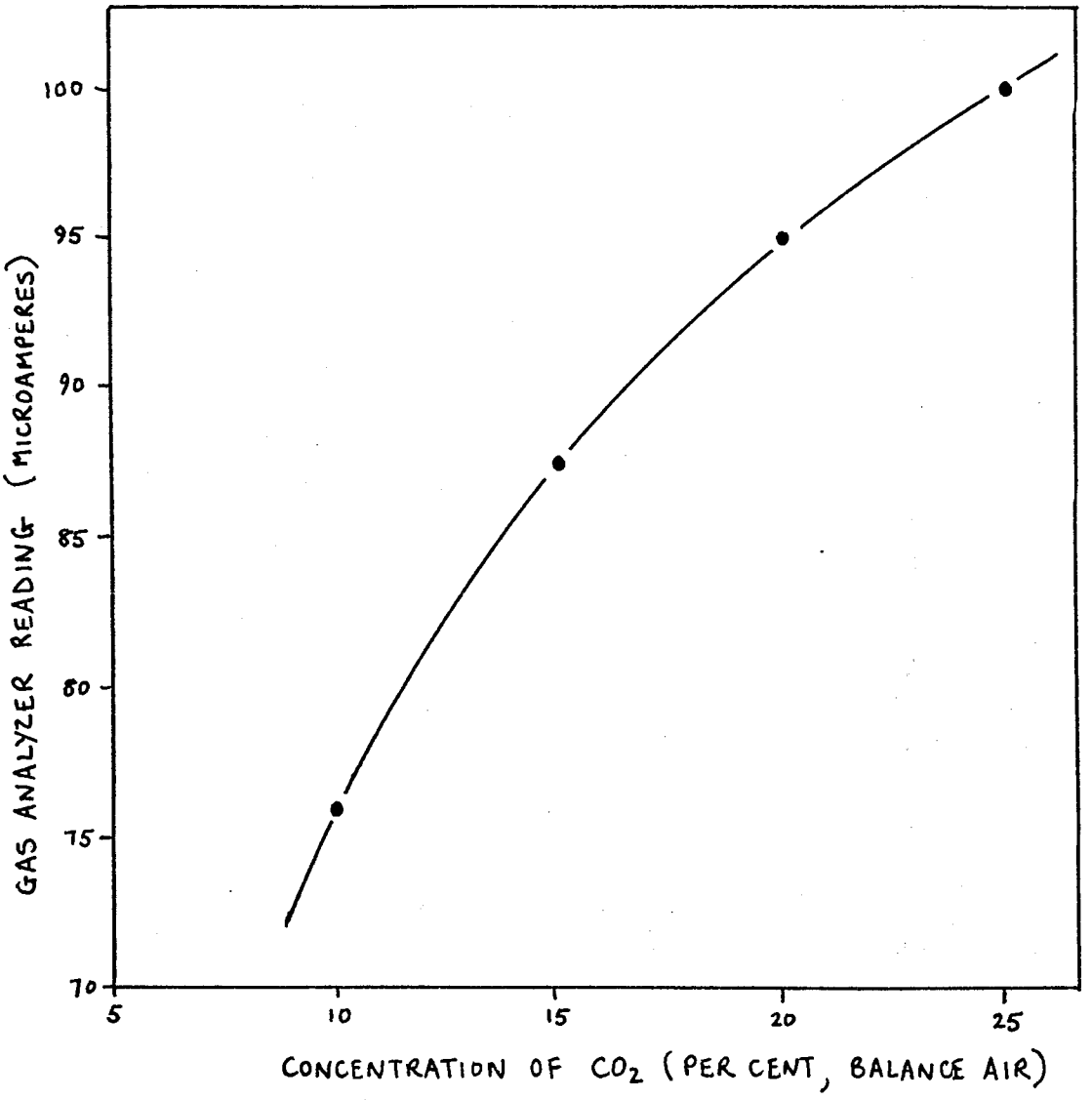

MAGNETIC STIRRER
FAN
ABSORBENT PAPER
15% CO_2
85% AIR
TRAY CONTAINING WATER
DRIERITE COLUMN
GAS-MIXING PUMP
INFRA RED GAS ANALYZER
CO_2
COMPRESSED AIR

Fig. A.2 Calibration curve for infra-red gas analyzer in the range 10-25% CO2 (balance, air).


100
95
90
85
80
75
70
GAS ANALYZER READING (MICROAMPERES)
5
10
15
20
25
CONCENTRATION OF CO_2 (PER CENT, BALANCE AIR)

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