INFLUENCE OF *Penicillium simplicissimum* (Oud.) Thom
AND *Penicillium citrinum* Thom ON GROWTH,
CHEMICAL COMPOSITION AND ROOT EXUDATION
OF AXENIC MARIGOLD

by

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INTRODUCTION

Plant root-soil system interactions form extremely complex relationships. The soil system is characterized by a dynamic equilibrium created by the associations and interactions among elements of highly diverse microbial populations, which differ both in species number and population level. The rhizosphere effect is recognized by qualitative and quantitative changes in the soil microbial population, which are directly or indirectly influenced by plant roots. These changes may in turn have profound effects on the welfare of the plant and on its root exudation.

Research on root diseases incited by biotic entities has been concerned primarily with soil microorganisms capable of functioning as primary parasites. Design of these investigations has centered around the search for a primary root invader and then determining the factors that enhance its parasitic and pathogenic potential.

While this approach has resulted in the identification and control of many important root diseases, it is becoming increasingly evident that there are a large number of instances in which the degradation of the root system is actually the outgrowth of a succession of interactions among a group of soil microorganisms, the soil physico-chemical environment, and the root. Furthermore, it has been very difficult to simulate the natural microflora in successive trials for root disease studies. Therefore, studies with germ-free plants versus known combinations of plants and soil-borne organisms are
required to compile information on the role of saprophytic root-associated microflora in the economy of the plant and its root exudation patterns.

This technique, plant gnotobiology, consists of growing germ-free plants in sterile isolator chambers, introducing propagules of known species of microorganisms into the rooting medium at the appropriate time, then identifying and studying the magnitude of interactions among these known biotic combinations. By designing experiments with known models, the investigator is able to proceed from simple gnotogiotic systems to more complex combinations, and thereby progressively analyze the contribution of each element of the biological environment to the development of a given root disease.

*Penicillium* species are suitable as test organisms in a model system. They are common rhizosphere- and soil-inhabiting fungi and have great potential in the production of exocellular metabolites (159). Also, some *Penicillium* species dominate the rhizosphere after certain treatments of the plant foliage (9, 156, 157).

Many plant species have been grown under gnotobiotic conditions (75, 107, 119, 194). However, different species required special growing techniques. Earlier work with marigold (*Tagetes erecta* L.) plants demonstrated their suitability for experimental work under gnotobiotic conditions (75).

The present investigations were undertaken to study the effects of *Penicillium simplicissimum* (Oud.) Thom and *Penicillium citrinum* Thom and their metabolites on marigold plants under gnotobiotic conditions. Specifically, the studies included their effects on: (a) plant
growth and development, (b) accumulation of certain metabolites in the
plant, and (c) root exudation patterns in relation to the plant stage
of growth.
LITERATURE REVIEW

Plant-Soil System

The soil environment is governed by the biological equilibrium created by the association and interactions of all individual components found in the soil. This equilibrium, however, is in a continual state of change, and this dynamic state is maintained at a level characteristic of the flora. Environmental changes may temporarily upset the equilibrium, but it is reestablished, possibly in a modified form, as the microbial populations shift to become acclimated to the new circumstances (3, 34).

As plant roots grow through the soil, they increase the potential for changes of the existing state of equilibrium. Hiltner in 1904 observed that there were higher microbial populations close to the plant roots than there were at a distance from the root. He called the area under the influence of the plant root the "rhizosphere" (181, 238). Further investigations have revealed that soil microflora (42, 89, 90, 91, 92, 101, 186, 203), nematodes (79, 91, 150, 226) and protozoa (50, 51, 89, 91, 226) in the root region are qualitatively and quantitatively different from these at a distance from the plant roots (91, 110, 178, 180). These observations were based on direct observations of plant-soil systems (52, 76, 111, 187, 203, 209, 226) and the isolation on artificial media and counting (111, 142, 145).

Plant roots influence the soil environment in numerous ways. Germ-free roots, for example, have been shown to be surrounded with a coarse granular and rather electron-dense material contained within
a membranous layer (31, 53, 86). Roots also contribute to the chemical components of the microenvironment by releasing diffusible materials (42, 54, 66, 132, 133, 143, 172, 173, 181, 182, 188, 199) and non-diffusible materials, i.e., sloughed off cells (132, 133, 143, 181, 182). It has been shown that plant roots increase the CO_2 content of the surrounding soil (163, 205), induce moisture stresses in their growth zone (42, 66), lower the oxygen tension (226) and alter the pH and redox potential of soil in their immediate vicinity (168, 237).

These phenomena have been assessed on a microenvironmental scale rather than on total content of the soil habitat. The rhizosphere effect and microbial activities are localized potentials which at first appear insignificant when diluted on a gross scale (138, 148). When considering the dynamics of plant-soil interrelationships, however, plants are strongly influenced by their microbial associates (42, 101, 161, 162).

Sterile versus non-sterile (13, 14, 107, 119), and monoculture (75, 108, 141, 205, 208) systems have been utilized to gain insight into plant-soil interactions. Techniques for re-infestation of sterilized soil to duplicate the exact pre-sterilization microbial status and simulation of the natural rhizosphere population have not been perfected (49).

Germ-free techniques and gnotobiology (75, 107, 108, 119, 205) provide an opportunity to construct model systems to investigate the plant-soil microorganism interactions at different stages of plant growth and development. Attempts to achieve this gnotobiotic combination, though artificial, provide fundamental information that will
be compiled and interpreted. Studies under uncontrolled conditions, are difficult to interpret because of the unidentified biotic conditions (119, 138).

Plant Root Exudation

It has long been postulated that organic materials are released from plant roots. DeCandolle in 1832 ascribed an important role in the soil sickness problem to excretion of materials from plant roots (54). In 1912, Lipman (239) reported the excretion of nitrogen from roots of some leguminous plants. Wilson, in 1937, showed that though this phenomenon was not universal, it varied according to environment and plant species (239). The release of thiamin and biotin from plant roots was demonstrated by West in 1939 from seedlings of two flax cultivars (234).

Since then, several workers have shown that an extensive number of compounds are liberated from plant roots (23, 181). This phenomenon has been demonstrated with roots of young seedlings (172, 173, 212, 223) at plant "fruiting and fructification" (224) and with mature tree roots (126, 199, 200, 201). Compounds released from germinated seeds, however, have often been confused with root originated materials (2, 74, 131, 220). Foliarly applied materials have also been observed to be exuded from the roots of treated plants (154, 160).

Particulate materials (non-diffusable) released from plant roots include individual cells (66, 100, 131, 133, 142, 185, 203), root cap cells (74) and sloughed off tissue (142, 203). This source of organic nutrients has recently been considered in the definition of root
exudates (181). As a general rule, exudation phenomena studies to date have not clearly defined the plant root conditions (74, 181) under which exudation occurred. A large number of investigations on root exudates have been performed under non-sterile conditions (77). Many studies have been made with detached roots (56, 80, 244). Bowen and Rovira (182) have demonstrated that changes occur in root physiology when the roots are excised. Exudation studies, then should be restricted to axenic-undisturbed plant root systems. Chemically, however, they should also include the soluble, particulate and gaseous compounds.

Nature of Plant Root Exudates

The nature of plant root exudates has been extensively reviewed (23, 173, 178, 181). In these reviews the total carbon-nitrogen content of root exudates have been generally considered from the nutritional status only (75, 77, 94, 181, 199, 220). However, individual organic compounds have also been identified. Among those compounds are: sugars (2, 23, 132, 133, 172, 194, 197, 201, 223), amino acids (2, 23, 25, 80, 94, 109, 120, 132, 146, 173, 174, 197, 199, 201, 223), organic acids (2, 80, 197, 199, 201, 220, 223), enzymes (38, 100, 128, 210), vitamins (176, 234), alcohol (71), hydrocyanic acid (158), phosphates (185), nitrogen (237), kinetin like substances (96, 196), nematode hatching factors and nematode attractants (19, 36, 230), zoospore attractants (244), fungal spore germination stimulants (191, 192), stimulants of seed germination of the parasitic seed plants *Striga lutea* Lour (32) and *Orobanche minor* Sm. (33, 136), inhibitors of germination of fungal propagules and fungal growth (63, 219),
inhibitors of nitrogen fixing and nitrifying bacteria (phenolics) (125, 164, 165), seed germination and plant root growth inhibitors (21, 188). Rovira (181) compiled information from reports by 10 different workers on exudates from wheat roots, which showed 10 sugars, 20 amino acids, 10 organic acids, 3 nucleotides and flavones, and 3 enzymes, to be components of exudate.

Amounts of Exudates

The amounts of root exudates to date may have been underestimated (74). The non-diffusible materials have not been collected (134, 181). As a rule, qualitative analyses of exudates are determined more frequently than the quantitative. This is mainly due to the small amounts of compounds present (181). Collected exudates are usually concentrated 10 times the original concentration to yield appreciable quantities (26, 75, 87, 194). Calum, Raistrick and Todd (36) grew 150,000 tomato (Lycopersicon esculentum Mill.) plants in sand culture to obtain 12 g of the eelworm-cyst hatching factor, edipic acid.

Vancura (220) reported that root exudates of wheat (Triticum aestivum L.) and barley (Hordeum vulgare L.) plants were equivalent to 7-10% of the dry matter of the plant shoots. Miller and Schmidt (121) showed 1.0 to 2.4 mg amino acids per plant were released from Phaseolus vulgaris L. under sterile conditions. Richter, Wilms, and Scheffer (166) reported in their daily harvest (nutrient solution cultures) that 0.24 μMol. of amino nitrogen per 22 alfalfa plants (Medicago arabica (L.) All.) were collected.
Plants grown in sand release more exudates than plants grown in liquid cultures (7, 172). The periodic replacement of the ambient solution (194) versus periodical sampling (166) and one terminal collection compared with intermittent collections (7, 75) introduce possible variables in the amounts of exudates released by plants. Furthermore, plant roots are capable of re-absorbing released organic compounds in its root zone (204, 241, 242). This may be correlated to stage of plant growth in a "source-sink" manner (46). Young seedlings (172, 201) and young roots (199, 200) release more exudates than the older plant roots (22). Therefore, the expression of root exudates quantity should include a time unit (74), and the stage of plant growth.

Sites of Exudation

Plant root tips have formerly been considered as primary sites of exudation. In 1944 Brown and Edward (32) observed that germination of *Striga lutea* seeds occurred at the root tips of host plant, and they concluded that secretion of a stimulant occurs only at the root tips. Similar observations were reported in the case of chlamydospores germination of *Fusarium solani* (Murt.) Appel and Wall. f. sp. *phaseoli* (Burk.) Shyd. and Hans. in relation to bean root exudation (192).

Regions of plant roots, other than root tips have been shown to be sites of exudation. Zentmyer (244) reported the attractiveness of *Phytophthora cinnamoni* Rands. toward the region of elongation of avocado (*Persea americana* Mill.) roots. Nematodes were also observed attracted toward the rapidly elongating area of roots (19). Ninhydrin positive reacting compounds have been shown to be released behind the
root tips of germinating broad bean (*Vicia faba* L.) seeds (151). Frenzel (64) demonstrated, by using Neurospora mutants, that threonine and asparagine were released from the root tips, while leucine, valine, phenylalanine and glutamic acids were exuded from the root hair region. Radioactive tracers have been used successfully to detect sites of root exudation. Rovira and Bowen (185) showed that the losses of absorbed phosphate (\(^{32}\)P) from root into the ambient solution correspond with the patterns of the uptake of those compounds. McDougall (132, 133) and McDougall and Rovira (134, 135) utilizing \(^{14}\)CO\(_2\) concluded that exudation occurs mainly at root tips and basal portions of secondary root eruptions.

Root exudation may be a dual mechanism of passive release of absorbed materials, i.e. from the free space of root tissues and active excretion of cell compounds via the membranes and cell wall. Morre, Jones and Mollenhauer (126) observed the accumulation of droplets at the tips of corn roots. A similar phenomenon was also observed in the case of apple (*Malus sylvestris* Mill.) root hairs (78). They attributed this to the activity of Golgi bodies and positive turgor pressure in the root cap cells. Hiatt and Howe (80) postulated that within root cells, organic acids may electrostatically bind an equivalent quantity of K\(^+\) and that amino acids may bind equivalent quantities of both K\(^+\) and Cl\(^-\), otherwise excess of these ions will leak into the surrounding medium.
Factors Affecting Exudation

The factors affecting root exudation have been extensively reviewed by Hale, Foy and Shay (74). They emphasized three main areas, namely: internal and external environmental factors, plant-soil microbes interactions, and effects of foliarly applied compounds. Factors affecting root exudation will be briefly reviewed in relation to the present investigation.

Plant Species

Plant species produce different qualities and quantities of root exudates. Vancura and Hovadík (224) investigated six plant species from four different families and concluded that the less the plants are related phylogenetically the greater is the differences in the composition of their exudates. Similar results were obtained by Balasubramanian and Rangaswami (10) using another four different species. They reported that the number and nature of the 17 amino acids and 4 sugars were different according to plant species and age of the individual plant. Rovira (173) reported 22 and 14 different amino acids from root exudates of 21 day old peas and oat seedlings respectively. The proportions of various amino acids in these exudates, however, were different for each. Qualitative and quantitative differences in root exudates were demonstrated in root exudates of five different pine species (199).

An example of the specific differences in root exudates composition among plant species is the stimulation of Orobanche species seed germination. The seed germination of Orobanche species in vitro
requires a stimulant found in flax root exudates. Flax, however, is a non-host plant of Orobanche (33, 136). It has been speculated that there may be two compounds involved; one acts as germination stimulant, while the second is required in the process of sucker attachment (33). Tomato, which is a host, is postulated to produce the second compound or both of them.

**Plant Age and Stage of Plant Growth**

Plant root exudation patterns have been shown to be directly correlated to the plant's age and the stage of plant growth (89, 175, 223). Thus, the time and the methods of collection are very critical (74, 115) in root exudate studies.

In the accumulation of nutrient elements and organic compounds within a developing plant there are pronounced changes in the distribution patterns of those elements that occur in a "source-sink" fashion (46). Root exudations are apparently, directly or indirectly affected by these nutritional balances. Slankis, Runeckles and Krotkov (197) found similarities between exudations from young roots of mature white pine (*Pinus strobus* L.) trees and the roots of young seedlings. Working with spring barley, wheat, maize (*Zea mays* L.), cucumber (*Cucumis sativus* L.), turnip cabbage (*Brassica oleracea* L.), tomato and red pepper (*Capsicum annuum* L.) plants, Vancura and Hovadik (223, 224) have designated three phases of qualitative changes in root exudation during the life of these plant species. The first occurs during the transition from nutrition by the seed cotyledons to mainly photosynthetic nutrition, the second during flowering, and the
third during late fructification. Amino acid excretion increases parallel with growth until in late fruiting phases it is clearly diminished. They found β-pyrazolylalanine in the root exudates of cucumber only at the early seedling stage. With tomato and red pepper they found that tyrosine occurred in exudates only at fruiting and not at any other stage of growth. Sugar excretion was also still increasing until flowering phase but qualitative changes occurred and large amounts of rhamnose appeared. Only trace amounts of this sugar were found in the initial phases of growth.

With peas and oats, Rovira (172) found more amino acids and sugars exuded during the first 10 days of growth than during the second 10 days. Smith (201) compared root exudates from 55 year-old versus 3 week-old sugar maple. Carbohydrates from seedlings were more diverse and abundant than those from mature trees. Also, amino acids and organic acids were released in greater diversity and abundance from the unsuberized tips of mature tree roots. Miller and Schmidt (120) found a direct correlation between quantities of amino acids exuded and the size of Phaseolus vulgaris L. plants grown under sterile conditions.

Support Media and Root Damage

The use of sand or artificial soil resulted in greater amounts of the various amino acids exuded than nutrient solution culture (25). This was attributed to the possible damage of root hairs and tissues (7). Pea plants liberated greater quantities of amino acids in quartz sand than in solution culture. Homoserine, an amino acid from peas
was recorded from sand cultures only. Also, when the plants were removed from their rooting media and washed, the quantity of root exudation was increased significantly (7).

Root exudation of methane dithiol from *Albizzia lophantha* roots were not detected until plants were mechanically removed and roots were washed (44).

**Soil Microorganisms and Exudation**

Microorganisms may affect root exudation in several ways. These are: (a) an effect upon the permeability of root cells and the damage of root tissues or root injury, (b) an effect upon the metabolism of roots, and (c) absorption of certain compounds in root exudates and excretion of different forms of compounds by microorganisms (181).

A wide variety of microbial metabolites are reported capable of altering root cell permeability (236). Polymyxin (137), penicillin (116), victorin (235) and enzymes (102, 210) are examples of microbial metabolites that increased cell permeability and thus caused loss of organic constituents from the root cells.

Culture filtrates have been demonstrated to increase the root exudation of certain compounds, alter cell permeability, and increase leakage (137, 138). This work, however, has been criticized because the conditions under which the organisms were grown were different both physically and nutritionally from those under which a rhizosphere population grows. Also the concentrations applied were arbitrarily chosen and may not be comparable to natural conditions.
Mechanical damage of root tissues may result in increased exudation of organic compounds (7). Utilizing $^{14}$C-labeled carbon dioxide and $^{32}$P-labeled phosphate, it has been shown that the base of young, secondary roots (184, 185) and damaged cortical cells (135) are active sites of exudation. Nematodes, also, were found attracted toward damaged zones and cut ends of plant roots (19).

Rhizoplane microorganisms (142) may colonize the epidermal and cortical tissues of healthy appearing roots and other underground plant parts (142, 143). Roots of plants grown under reinfested conditions exhibited brownish appearance, less turgid condition and occasional dry rot (75, 107, 233).

Monoculture inoculations under gnotobiotic conditions present ideal conditions for the studies of the effects of the physical presence of the microorganism and its metabolites on root exudation. Hameed and Couch (75) compared root exudates of germ-free versus *Penicillium*-inoculated marigold plants. They found an increased concentration of total carbohydrates and amino acids in the exudates of the inoculated plants.

Nodulated leguminous plants have been reported to secrete nitrogen from their roots (239). Day, Sen and Sundara Rao (55) compared root exudates of uninoculated, but not germ free, versus *Rhizobium meliloti* inoculated Egyptian clover (*Trifolium alexandrinum* L.) and alfalfa (*Medicago sativa* L.). Aspartic acid only was found in the exudate at the 6 weeks and 9 weeks stages of growth of the uninoculated plants. The inoculated plants, however, contained three amino acids at 6 week stage and seven amino acids after 9 weeks. Exudates of inoculated
plants contained glucose and fructose at both stages, 6 weeks and 9 weeks, while the uninoculated plant root exudates contained glucose only.

**Soil Moisture**

It has been reported that temporary wilting of plants greatly increases the amounts of root exudates (85, 93, 98, 222). Dove (56), using detopped tomato root systems, found no changes in phosphate or the volume of exudates, under atmospheric desiccation. This, however, may have been due to the detopping (30).

Indirect evidence that soil moisture stress affect root exudation was obtained by Couch and Bloom (47). Exudates of orchardgrass, timothy and red clover collected after successive, temporary wilting and re-wetting, resulted in greater inhibition of egg hatch (*Meloidogyne incognita* (Kofoid and White) Chitwood) than the exudates of plants grown at continuous field capacity.

**Temperature**

Generally, an increase in temperature increased the amount of root exudates (175) and the exudation from germinating seeds (193) of several plant species. Vancura (221) found that the amount of exudates from maize and cucumber increased with increase in temperature, although exudation of individual sugars was lower at 28°C than 19°C. Plants growing at optimum temperatures and then exposed to a 3 days-cold shock exhibited qualitative and quantitative increases in exudates.
Light

The light intensity at which plants are growing affects the balance of amino acids released by the underground portion. With clover plants, serine, glutamic acid, and α-alanine were less at lower light intensities than under full daylight. Serine and asparagine, however, were increased in the exudates of tomato plants as light intensity decreased. Shading of wheat plants at early stages of development has been reported to cause decreased synthesizing activity of the root (4, 175).

Plant Nutrition

Mineral nutrition status in the root region may have a profound effect on the root physiology and cell permeability. Bowen (26) found that loss of amides and amino acids from pine roots was 2 1/2 times greater from roots grown in phosphate deficient nutrient solution than the ones grown under adequate phosphates. There was an increase in quantities of free amino acids and amides in the phosphate-deficient grown plants, which was held as a reason for the increased exudation.

Low levels of calcium in germ-free liquid cultures of peanut plants (Arachis hypogaea L.) increased cell permeability which resulted in an increase in sugar exudation (194). Rovira (175) found no significant differences in exudation of amino acids from roots of four plant species grown in 5 x 10⁻⁴M to 5 x 10⁻²M calcium solutions. Bowen (26) pointed out that even the lowest calcium level (5 x 10⁻⁴M) used was still adequate for plant growth.
Foliar application of nutrients have been reported to affect rhizosphere microflora (1, 9, 156, 229). This would seem to indicate profound effect on root exudation.

Other Environmental Factors

Anaerobic conditions appear to enhance excretion of mineral and organic substances from roots (70, 71, 80). Alcohol was formed under anaerobic conditions (6 hours) in the root region of corn and sunflower plants in amounts which was toxic to other plants (71). A change in pH of the eluting solution from 6.4 to 5.9 increased exudation from roots of wheat seedlings (131). Dubrov and Bulygina (58) observed an endogenous rhythm exists in the secretion of organic substances by roots with a characteristic position of diurnal maxima and minima. Similar phenomenon was observed with alfalfa (166). The rhythm was exhibited for amino acids and a yellow colored pigment, and it was sensitive to dinitrophenol treatments.

Foliarly Applied Chemicals

Generally the effects of foliarly applied compounds upon root exudation are identified by the changes in the population of the rhizosphere microorganisms of the treated plants (1, 9, 156, 157, 211, 227, 229). Balusubramarian and Rangaswami (10), however, found a direct effect of nutrient sprays on the root exudation patterns in four crop plants. Foliar application of 0.1% sodium nitrate solution increased the concentration of amino acids exuded by sorghum (Sorghum vulgare Pers.), sunhemp, ragi (Eleusine coracana (L.) Gaertin) and tomato. But, applications of phosphorous as sodium hydrogen phosphate
solution, reduced amino acids concentration. Exudation of sugars was reduced by sodium nitrate treatment and increased with the sodium hydrogen phosphate spray. In an earlier investigation, 0.28 M sodium hydrogen phosphate spray on tea plant (Thea sinensis L.) increased the rhizosphere fungal population, and 1.47 M sodium nitrate had no effect (227).

Foliar application of hormones (gibberellic acid, indol-3-acetic acid), urea, antibiotics (chlortetracycline, chloramphenicol) and substituted phenoxy acetic acids, altered the bacterial-fungal populations of the rhizosphere of many plant species (156, 157, 211, 229). These effects may be either direct, as the sprayed compound translocated and released in the rhizosphere (84, 150, 211), or indirect via altering the exudate pattern or the enzymatic activity of the rhizosphere (9).

**Effects of Plants on Soil Microorganisms**

The greater number of species and the higher population of soil microorganisms in the rhizosphere soil than the non-rhizosphere soil is generally attributed to the nutritional requirements of those organisms (42, 101, 109, 169, 174). The aspects of rhizosphere effects have been broadly reviewed in the preceding sections. The following are specific effects of plant roots upon soil microorganisms.

Fungistatic phenomena are common characteristics of natural soil (190). Propagules of such soil fungi as *Pythium irregulare* and *Verticillium albo-atrum* Rei. and Bev. germinate only when soil fungistasis effect is eliminated by seed exudates of *Pinus resinosa* Ait. (2), and root exudates of tomato plants (190), respectively.
Roots may directly stimulate the germination of soil inhabiting organisms. Schroth and Snyder (192) found that chlamydospores of *Fusarium solani* (Mart.) Appel and Woll. f. sp. *phaseoli* (Burk.) Snyd. and Hans germinated most consistently in the soil when in close proximity to germinating bean seeds and root tips of primary lateral and adventitious bean roots. Mature roots, however, had little effect on chlamydospore germination and growth when tested in soil. Nematode hatching factors were characterized in root exudates of several plant species (19, 36). Germination and establishment of parasitic relationship between parasitic seed plants (*Striga* lutea and *Orobanche* sp.) and their respective hosts are governed by root exudates (32, 33, 136).

Chemotaxis of zoospores, nematode and fungal growth to root exudates has been observed (19, 97, 230, 244). Zentmyer (244) found that the chemotaxis of the zoospores of *Phytophthora cinnamoni*, Ran. and chemotropy of their germ-tubes were directly related to infection and disease production. *Meloidogyne javanica* (Trenb) Chitwood and *M. hapla* Chitwood (19) and *Heterodera* sp. (230) are attracted by the roots of their specific hosts. Changes in the microbial physiology are likely induced by growing plants (63, 213). Buxton (35) showed that an avirulent isolate of *Fusarium oxysporum* Sch. f. sp. *pisi* became virulent after treatment with root exudates from resistant plants. The mechanism postulated was either the production of enzymes by the fungus to detoxify root exudates, or an increase in the ability of the fungus to use nutrient materials from the resistant plant.

Plant roots may inhibit germination, or growth or promote antagonistic effects upon certain soil microorganisms. Turner (219) found
44% of the sporangial germination and 100% of the zoospores activity of *Phytophthora palmivora* (Butler) Butler were inhibited by roots of *Zea mays* L. A variety of growth inhibitors were reported released by plant roots. Examples of those compounds are: hydrogen cyanide from sorghum seedlings (158), phenolic compounds and gallotannins from *Ambrosia elatior* L., *Euphorbia corollata* L. and *Helianthus annuus* L. (165), trans-cinnamic acid from guayule roots (21, 22), and ethanol which was produced by corn and sunflower roots under anaerobic conditions (71). Activities of soybean roots may change the pH of a nitrate rich soil by as much as one unit (168), which may have a profound effect on soil microflora.

Robinson (169) showed that in a mixed pasture of subterranean clover and lucerne, clover stimulated the growth of *Rhizobium trifolii* but not *Rhizobium meliloti*. However, lucerne stimulated the growth of both *Rhizobium* spp. The differential stimulation of growth of *Rhizobium* spp. by lucerne and clover was attributed to either a direct effect of the legume root exudates on the *Rhizobium* sp. or a stimulation of the growth of microorganisms antagonistic to *R. meliloti* by the exudates of clover. Rhizosphere bacteria and actinomycetes of wheat plants antagonized *Azotobacter* and prevented its growth (206).

Theron (213) found that nitrification was entirely repressed under the perennial grasses from the second season after its establishment onwards. Under the annual crop nitrification stopped when the crop was growing vigorously and picked up again when the crop was mature, then became normal after the crop was gone. During nitrification stoppage, ammonia was present. Therefore, it was stated that
plants affect the mineralization of nitrogen by paralyzing the autotrophic dehydrogenase system of the nitrifying organism. Similar observations were reported with corn and alfalfa, and ryegrass (*Lolium* sp.), wheat, salad rape, lettuce (*Lactuca sativa* L.) and onion (*Allium cepa* L.) by Molina and Rovira (124) and Moore and Waid (125), respectively.

**Effects of Soil Microorganisms on Plant Growth**

Appreciable attention has been given to the effects of rhizosphere microorganisms on plant growth. Accumulated information suggests that common rhizosphere microflora may be beneficial or detrimental to growth of higher plants. Mixed-inoculation with soil microorganism enhanced the growth of plant shoots and roots (107, 108, 119, 161, 167, 179, 205). Similar results were observed upon monoculture inoculation with bacterial and fungal rhizosphere isolates (8, 75, 108, 127, 141, 177, 180, 212). Increased tops, however, may be accompanied with a reduced root growth (28, 103, 140, 232, 233). Slight inhibition of root growth, however, under non-sterile conditions was coupled with greater shoot growth than the sterile plants (75, 232, 233). Plant growth was also reduced upon inoculation with rhizosphere microorganisms (8, 163). Bowen and Rovira (28) found that subterranean clover seedlings develop shorter and fewer root hairs under non-sterile conditions.

The apparent disagreement among the above studies can be explained by differences in plant species, age, supporting media, microbial population and other experimental variables (140). Monoculture inoculation with three soil fungi on peanut seedlings were detrimental to plant
growth. The same three species, however, combined together caused less damage than each one of them alone or combinations of two species at a time (41).

The non-sterile conditions have increased the levels of free amino acids in the sap of plants (119, 161), and enhanced physiological activities and enzymatic activities of plant roots, compared to sterile conditions (161). Earlier flowering was also observed after inoculation of axenic plants with rhizosphere isolates (205) and plants grown from inoculated seeds (179).

Effects of Soil Microorganisms on Plant Nutrition

The main role of soil microflora in plant nutrition is the transformation of minerals into more readily available forms.

Microorganisms participate in many different ways in the nitrogen cycles in nature. One phase of that participation is the release or mineralization of organically bound nitrogen, thus making it available to plants.

Microbial decomposition of organic matter releases not only its carbon and nitrogen, but the many other minerals contained in the organic matter as well (43, 69). Microbes also affect the availability of various minerals in their inorganic combinations. Iron (3), manganese (215), and sulfur are transformed from unavailable to available forms by microbial oxidations and reductions.

Products of microbial oxidation exert solubilizing effects upon soil parent material and insoluble forms of fertilizer. When rock phosphate is composted with sulfur and manure, the sulfuric acid
formed by biological oxidation makes the insoluble phosphate available (43). Non-sterile plants may obtain more phosphates from poorly soluble phosphate material than sterile plants (12, 13, 67, 119). Sulfur applied to soils as a corrective for excessive sodium salinity is ineffective until it has been oxidized to sulfate by the soil flora (43).

The soil microflora produce organic compounds that may act as chelating agents, which prevent mineral elements from fixation or flocculation in unavailable forms.

Under high carbon soil amendments, soil microorganisms may immobilize soil nitrogen and other mineral elements. Thus they may compete with higher plants in nutrient supply. Also, soil microorganisms may compete with plants for elements in low supply (147).

Microbes also affect transformations that make nutrients unavailable to higher plants. Manganous and ferrous compounds become highly insoluble when they are oxidized to manganic and ferric compounds (8, 215). Nitrate nitrogen may be reduced to gaseous nitrogen under anaerobic conditions as bacteria satisfy their oxygen demand (43).

Soil microorganisms may also influence the uptake and translocation of minerals from the root medium (143, 161, 183). Barber, Sanderson and Russell (13) found that under sterile conditions, cross sections of barley roots exhibited uniform accumulation of $^{32}$P at the central steel. The cross-sections of non-sterile roots, however, showed irregular, localized zones of $^{32}$P accumulation on the root surface, which was indicative of the bacterial colonies. MacDonald (114) suggested that contaminants on sugar beet discs produced compounds
that influenced the adsorption of sodium and potassium in a similar
manner to growth inhibitors.

Comparative analyses of plant foliage between sterile and non-
sterile plants revealed differences in the accumulation of various
elements. Non-sterile plants yielded less ash content than the sterile
ones (218, 232, 233). In spite of the increase in plant growth under
non-sterile conditions, there was a decrease in nitrogen, potassium
and phosphorus contents of those plants (127). It was speculated,
however, that soil microflora allowed the plants to use nutrients of
soil more economically.

Monoculture inoculations have been shown to result in decreased
uptake and translocation of $^{32}$P-phosphate, $^{35}$S-sulphate, and $^{14}$C-
bicarbonate (208).

Under non-sterile conditions, phosphate uptake was more efficient
(12, 184), and there was increased phosphate accumulation (27, 29, 67,
161). Rovira and Bowen (184) showed that the rhizosplane microflora
significantly increased phosphorus uptake and altered its distribution
in the various phosphorus containing fractions of the root. Higher
amounts of nitrogen (121, 161, 163) potassium and phosphorus (161) were
accumulated under non-sterile conditions. Recently, Miller and Chau
(119) reported that soybean plants (Glycine max Harosoy) grown in
re-infested soil (mixed inoculation), though they grew better and
showed similar nitrogen, phosphorus and potassium contents as germ-
free plants. Micronutrient analyses, however, revealed significantly
increased contents of calcium, magnesium, iron, aluminum, copper, zinc
and molybdenum under re-infested conditions.
Soil microorganisms synthesize a wide variety of extracellular compounds. These include growth promoting substances (18, 127, 233), antibiotics (137, 138), growth inhibitors (97, 207), toxins (129, 235), amino acids (204) and many other organic compounds. Microbial products are always suspected for certain effects observed on treated plants. The significance of these metabolites resides in the possibility of their activity on a microenvironmental scale (142, 147). Concentration scales, however, for such compounds in situ are still arbitrary and undefined (138).

Phytotoxic substances have been demonstrated in natural soil and amended soils (129, 148), and in soil sterilized by autoclaving (183). The toxicity due to autoclaving was nullified by soil reinfestation with mixed inoculations (183). Miller and Chau (119), however, showed that growth reduction in autoclaved soil was statistically insignificantly different from the unsterilized one. The presence of indole-3-acetic acid (IAA) in the root region was often observed causing the root hair deformation and branching (18). Relatively higher concentrations of IAA, when accumulated inside the root due to presence of soil microorganisms, may inhibit the growth of plant roots (233).

Several plant diseases are directly or indirectly related to microbial metabolites. For instance, the victoria blight on oat is caused by the metabolic product of Helminthosporium victoriae Mehan and Murphy, victorin (235), and the frencing of tobacco is caused by amino acids released by soil microorganisms (204).
Polymyxin, an antibiotic produced by soil bacteria *Bacillus polymyxa* and initially utilized in cation-uptake studies to suppress other bacterial contamination, inhibited root growth of barley seedlings (137). Norman (138) investigated several antibiotics. Presuming they are released in the rhizosphere, they may enter the plant or be adsorbed to root surfaces in the immediate vicinity of the loci of production.

Furthermore, soil microbial metabolites were investigated for possible root disease controls in an antagonistic relationship against primary root pathogens (97, 207).

Another important phase in the plant-soil microorganisms interrelationships is the influence of soil microorganisms on the exudation of organic compounds from plant root and the fate of these compounds in soil. The literature concerning this topic was reviewed under factors affecting exudation in an earlier section of this review.

**Plant Root Diseases and the Plant-Soil System**

Plant-soil interrelationship is a part of the environmental variable in the triple-phase interactions of host-pathogen-environment. Thus root diseases are ecological outcomes of plant-soil interactions (including the primary pathogens), rather than a mere interaction between a susceptible and pathogenic entity (66).

Considerable amounts of valid information have been accumulated about plant-primary pathogen interactions. However, root disease problems, in many cases, are incited by conglomeration of several soil microorganisms (saprophytes) rather than a single entity (104, 118, 240).
The close association between soil microflora and roots has raised controversial speculations among root disease scientists. The soil saprophytic microflora may predispose plant roots and thus open avenues for the more aggressive pathogens (149). Also, the rhizosphere and root-surface microflora may shield the plant root against the root pathogens, which may be the case with mycorrhiza and some free living fungi (66).

Garrett (66) reviewed the interaction of root-infecting fungi with root-surface and rhizosphere microflora. He theorized that the interaction may be controlled by the genetical constitution of the host plants, where resistant versus susceptible plant are compared in terms of differences in their rhizosphere and root surface microflora. Ophiobolus graminis (Sacc.) Sacc. on wheat, Thielaviopsis basicola (Berk. and Br.) Ferraris on tobacco and Fusarium sp. on flax were antagonized by the saprophytic microflora on the resistant plant species to a greater extent than the susceptible one (97). Similar conclusions were recently derived in case of Verticillium wilt of tomato (97, 207).

Soil type may encourage certain saprophytes which antagonize the root pathogens. In acid soils Trichoderma viride Pers. stops further colonization of pine roots by Fomes annosus (Fr.) Cooke (66).

Plant nutrition or accumulation of certain metabolites inside the root tissue may have direct effects on the pathogen or stimulate the growth of the competitive microflora. Maize plants inoculated with Phymatotricum omnivorum (Shear) Duggar in sterile sand-bentonite
were quickly invaded and killed; whereas, similar inoculated plants in unsterilized sand-bentonite remained perfectly healthy.

Toxic substances-producing microorganisms are commonly found among the rhizosphere microflora (15, 129, 225).

Soil-Borne Penicillium Species and Their Pathogenic Potentials

Species of Penicillium are abundant in the soil and decomposing organic matter (117, 159). They commonly become dominant upon re-infestation of sterilized soils (49). They are commonly isolated from rhizosphere (145), root region (144), and root surfaces (142). Under certain instances, foliar sprays of organic compounds stimulate the growth of Penicillium sp. in the rhizosphere of treated plants (156, 157).

Penicillium sp. are constantly associated with root disease complex of seed rot and seedling blight of sorghum (104), and root lesions of strawberry (118). Penicillium sp. have also been reported as primary pathogen in damage of peanuts (240) and corm rot of gladiolus (68, 130, 149, 152)

A wide variety of extracellular metabolites are produced by Penicillium spp. Phytotoxic substances were liberated from Penicillium sp. in soil (37, 123, 129, 139) and in culture filtrates (15, 20, 37, 105, 225).

Penicillium simplicissimum (Oud.) Thom is a soil inhabiting fungus, generally isolated from natural soil and old textile made equipment (159). This fungus exerts solubilizing effects on rock mineral due to its capability of producing organic acids (195).
Penicillium citrinum Thom is another soil inhabiting species (159), commonly known for its synthesis of citrinin, an antibiotic with the formula, 4,6-Dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3H-benzopyran-7-carboxylic acid (122, 123, 170, 216, 228). The potential for citrinin production, however, is nonspecific for this species and it differs from one isolate to another (155, 159, 243). Citrinin has been purified from culture filtrates of this species and investigated for a possible selective herbicidal activity on broad leaf plants (122, 123).

Penicillium sp. are potential producers of extracellular enzymes. Pectolytic and cellulytic enzymes were often demonstrated in culture filtrates (40, 61, 65, 72, 73, 83, 95, 105, 153, 231) and tissues colonized by Penicillium sp. (45, 99).
MATERIALS AND METHODS

Plant Materials

Dwarf marigold, Tagetes erecta L. cv 'First Lady'\(^1\) was used as the test plant species throughout the present investigation. The culture of Penicillium simplicissimum (Oud.) Thom used was isolated from soil obtained from a field used for intensive tobacco culture. The isolate of Penicillium citrinum Thom was obtained from Dr. O. R. Rodig of the Department of Chemistry, University of Virginia, Charlottesville, Virginia.

At the outset of the investigation a single spore culture of each Penicillium species was grown on potato dextrose agar slants. Conidia from each species respectively were placed on 3 mm sterile glass beads. The beads were then in turn placed in individual glass tubes sealed at one end. After the air from the tubes had been evacuated under 50 \(\mu\) Hg vacuum, the open end of each tube was sealed and the entire set stored at 5°C. These stored conidia served as the source of inoculum for the entire investigation. Sub-cultures for each experiment were prepared by placing a spore-laden glass bead on potato dextrose agar.

\(^1\) Obtained from Gero. G. Ball, West Chicago, Illinois.
General Experimental Procedures

The isolator chambers used in the investigation were constructed from 6 mil flexible plastic. Measuring approximately 60 cm x 60 cm x 120 cm, each chamber was equipped with an entry port at one end and air filter ports at each end. Manipulation of materials inside the chambers was accomplished by means of glove ports attached to the sides (Fig. 1). The individual chamber air filters were prepared from 3 to 5 layers of 1 1/4 cm thick FM 004 Glass Filter Media obtained from the same source as the chambers. The filter media was held in place by wrapping it around a stainless steel cylinder so that inlet air was forced through the filter media into the chamber by a fan and was exhausted through a second filter at the opposite end of the chamber.

Prior to sterilization of the chambers, the inlet and outlet openings of the air filters were sealed with mylar tape and the entire filter sterilized with dry heat at 150°C. The sterile filters were then attached to the filter ports and all inside surfaces of each chamber sprayed with 300 ml of freshly prepared 2% aqueous peracetic acid (PAA) (107, 112, 119, 194). After 5 hr the mylar seals on the filters were broken from inside the chamber and the PAA exhausted for 72 hr.

With the exception of living plants and fungus conidia, materials to be placed in the chambers were sterilized in the following manner. Items to be introduced into the chambers were placed in stainless steel

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Figure 1. Marigold plants growing in the individual plant containers inside an isolator chamber. Flexible plastic isolator chamber is mounted on movable cart and inflated with filtered air. Inlet air filter is attached to back end of the chamber and the exhaust filter to the front beside the entry port. Fluorescent and incandescent light sources are mounted on a rack hanging above the chamber.
cylinder measuring 30 cm diameter x 90 cm long. Both ends of the cylinder were then sealed with mylar film and the cylinder and the contents autoclaved for 4 hr at 15 psi and 121°C. After autoclaving, the cylinder was attached to the entry port of the gnotobiotic chamber by means of a plastic sleeve (Fig. 2). The inside of the sleeve and entry port were sterilized by spraying with PAA through the sleeve nipple. The nipple opening was then plugged with cotton to allow dissipation of the PAA. After at least 1/2 hr, the inner cover of the chamber entry port was removed and the mylar film on the cylinder broken. The sterile materials were then transferred into the main chamber.

The probability of chamber contamination was checked at weekly intervals. Sterile cotton swabs were rubbed across the surface of the inner chamber, plants and plant containers. Individual swabs were then placed in a tube of each of the following sterile media: potato dextrose agar (PDA), nutrient broth, Saboraud dextrose agar, thioglycolate broth and nutrient agar. The tubes were observed daily for the presence of microbial growth and if any was found the entire experiment was abandoned. Contamination was observed only once during the three years that various experiments were conducted.

Containers for supporting the growth of individual plants were constructed using an 80 mm x 100 mm pyrex glass soaking jar with a 400 ml polypropylene beaker seated in the opening of the jar with a __________

3/ All media were obtained from DIFCO Laboratories, Detroit, Michigan.
Figure 2. The stainless steel cylinder in position attached to the entry port of an isolator chamber by means of a plastic sleeve. Autoclaved materials inside the cylinder are carried inside the chamber via this connection. Aspirator can be seen in foreground and was used for the 2% PAA spray to sterilize the inside of the connection.
2 cm wide rubber gasket (Fig. 3 and 4). Glass wool was placed over the upper end of the drainage tube and held in place with a piece of aluminum wide screen. Three hundred and fifty g of coarse silica sand, previously ignited at 600°C for 6 hr to remove organic material, was placed in each beaker. Initial irrigation of the plants was accomplished by pouring 300 ml of 1/2 strength Hoagland and Arnon nutrient solution (81) supplemented with iron ethylenediamine tetra-acetic acid (Fe EDTA, 50 mg/l) into the beakers and allowing it to percolate through the sand. Recirculation of the nutrient solution in each container was accomplished by air pressure applied by means of a rubber bulb attached to the air pressure application tube (Fig. 4). This action forced the nutrient solution upward and onto the surface of the sand, after which it was allowed to percolate back into the jar.

In those experiments in which root exudates were measured weekly, 80 ml of the nutrient solution was collected each week from each of the plant containers. At the end of experiments in which exudates were not collected weekly, the sand with the plant root in place was eluted with ten successive 100 ml volumes of half strength nutrient solution. Each portion was poured into the beaker and pulled through the irrigation tube under vacuum while the pressure application tube was tightly clamped.

Well-formed seeds of marigold, free of defects, were selected with the aid of a steroscopic microscope. In order to decrease the surface area, the calyx of each seed was trimmed off with a pair of scissors. Seeds were then wrapped with a double layer of cheesecloth and soaked for 20 min in a 1:5 dilution of 5.25% commercial sodium
Figure 3. The container used for growing individual plants inside isolator chambers showing proper solution volume, irrigation, and circulation tube and the air pressure application tube on the far side.
Figure 4. A detailed line drawing of the individual plant container. Air pressure was obtained by using a rubber bulb (not shown). See the text for the operation of this container.
hypochlorite. The seeds were removed with sterile forceps from the bleach solution, rinsed three times with sterile distilled water and aseptically transferred to test tube slants containing potato dextrose medium diluted with an equal volume of Hoagland and Arnon supplemented nutrient solution and solidified with 0.7% agar (Fig. 5). After 5 days incubation in the dark at 25°C, the test tubes containing germinated seeds were placed on a window sill where they received natural daylight. Two weeks later, contamination-free seedlings were aseptically transferred into previously autoclaved screw capped jars. The jars containing the seedlings were placed inside the entry port of an isolator chamber, the entry port closed and the inside of entry port sprayed with PAA. Twelve hours later the seedlings were moved inside the chamber, removed from the jar and placed in glass tubes contained in a shallow aluminum pan half-filled with Hoagland and Arnon nutrient solution (Fig. 6 and 7). Ten days after planting, seedlings selected for uniformity were transplanted into the individual plant containers. The plants were then grown in continuous light from cool white fluorescent lamps supplemented with two 60 watt incandescent lamps. Light intensity 10 in above the plant container was 1100 ft. c. and the temperature inside the isolator chambers was 28 ± 1°C. Nutrient solution was pumped from the reservoirs to the surface of the sand of the individual plant containers twice daily. Loss in volume of nutrient solution was compensated for by adding fresh nutrient solution as needed.

*Penicillium simplicissimum* and *P. citrinum* were incubated at 24°C on 50 ml of PDA in 250 ml erlenmeyer flasks. After 5 days incubation,
Figure 5. Marigold seedling germinated from surface sterilized seeds on PDA slants containing 0.7% agar and 1/2 strength Hoagland and Arnon nutrient solution, under sterile conditions inside screw capped test tubes.
Figure 6. A line drawing of the transplanting tube used to support the marigold seedlings during acclimation prior to transfer into individual plant containers. Young seedlings were placed on the crossed aluminum wire with their roots in the nutrient solution.
GLASS TUBE

ALUMINIUM WIRE

SIDE WINDOWS FOR ROOT GROWTH
Figure 7. Axenic marigold seedling during acclimation inside the preparatory chamber. The test tubes which can be seen in the foreground contain the following 5 different microbial growth media: PDA, nutrient broth, nutrient agar, thioglycolate broth and Sabouraud dextrose agar. Prescription bottles contain 1/2 strength Hoagland and Arnon nutrient solution.
conidia were collected by adding 50 ml of sterile distilled water containing several solid glass beads and swirling the flasks. A millipore filter (pore size 0.45 μ; Millipore Filter Corp., Bedford, Massachusetts) was used to retain the conidia while they were washed three times with sterile distilled water.

One hundred ml of sterile distilled water was then added to the filter holder and the apparatus shaken to suspend the conidia. One ml aliquots of the suspension were counted in a haemocytometer to establish concentration of spores. The spore density was adjusted as needed by either adding sterile water or applying vacuum to remove water through the filter and then rechecking spore concentration.

One-hundred and fifty ml of the conidial suspension was transferred into each gnotobiotic chamber in the same manner as the transfer of seedlings. The individual plants were inoculated by placing 10 ml of conidial suspension on the surface of the sand in the plant containers.

Specific Experimental Procedures

Seven experiments were conducted. In experiments I and II axenic plants and plants inoculated with _P. simplicissimum_ were compared with respect to fresh weight, dry weight, plant height and chemical composition of plant tissues and chemical composition of root exudates at flower bud formation and at flowering, respectively.

In experiment III plants were inoculated with _P. simplicissimum_ and _P. citrinum_ and compared with axenic plants with respect to the same parameters as in experiments I and II.
In experiment IV axenic plants, treated with extract from axenic plant roots and *P. simplicissimum*-colonized roots, were compared for differences in growth, chemical composition of dried leaves and composition of root exudates.

In experiment V an exocellular metabolite of *P. citrinum*, *citrinin*, was added to the rooting medium of axenic plants.

In experiment VI, root systems of 30 day old axenic plant seedlings were inoculated with a *P. simplicissimum* conidial suspension and successive samples of roots examined histologically.

In experiment VII axenic plants were grown and inoculated in the same manner as experiment VI. Three weeks after inoculation, root tissues were harvested for assays for extracellular enzymes. Root tissues collected during this experiment were assayed in conjunction with frozen roots collected during experiments I and II.

In all experiments, the inoculated and axenic plant checks were housed in separate isolator chambers. Chambers containing axenic plants were subjected to the analysis of variance for statistical significance or variation within and among chambers.

**Experiment I**

A total of 20 axenic plants were grown inside two separate isolator chambers, 10 plants in each chamber. At the 36 day stage of growth, plants in one chamber were inoculated with *P. simplicissimum* conidial

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4/ Purified citrinin was obtained from Dr. O. R. Rodig, Department of Chemistry, University of Virginia, Charlottesville, Virginia and Dr. P. Craig, Smith Kline and French Laboratories, Philadelphia, Pennsylvania.
suspension at a rate of $7 \times 10^6$ conidiospores per plant. Twenty days after inoculation, when buds had formed, the plants were harvested.

Axenic plants and *P. simplicissimum*-colonized plants were compared with respect to the botanical characteristics and the morphology of the root system.

Root systems were shaken free of the sand and immediately stored at -20°C for use in exocellular enzyme assays in conjunction with materials obtained in experiment VII.

Gross measurements of growth was made on plants used during this experiment. These included (a) measurement of plant height above the surface of the rooting medium, (b) fresh weight of top growth and total fresh weight, and (c) dry weight of top growth. The treatment and control plants were subjected to analysis of variance for statistical variation within chambers and significance differences between treatment and check plants.

Dry weight determination was made by drying plant materials in an oven at 85°C for 48 hours. Dried plant materials were ground in a Wiley Mill and stored in jars. Leaves and stems were separately analyzed for total water soluble carbohydrates, reducing sugars, fructose alone and nutrient elements.

Chemical analyses of plant tissues and root exudates were made in triplicate and data were subjected to analysis of variance.

The 80°C water extractable carbohydrate content was determined by the anthrone method (57) and computed as mg/g of dry weight of leaves. Beakers containing 200 mg of dry, ground leaves and 50 ml of 80°C distilled water were placed in a water bath at 80°C and after one
hour the contents were filtered through Whatman No. 1 filter paper into 100 ml volumetric flasks. The volume was adjusted to 100 ml. It was necessary to further dilute the extract in a 1:1 ratio in order to obtain a precise anthrone color reaction. Glucose solutions, containing 50 to 200 µg/ml, were used as standards to estimate total carbohydrate equivalent. Absorbancies were read at 540 nm on a Spectronic 20 spectrophotometer.

Reducing sugars in undiluted extracts were determined by the Somogyi and Nelson test (202). Absorbancies of samples and standard glucose solutions containing 50 to 200 µg/ml were read at 505 nm in a Spectronic 20 spectrophotometer.

The resorcinol test (48) was utilized for measurement of fructose content. Fructose solutions containing 50 to 200 µg/ml were used as standards. Absorbancies were read at 540 nm in a Spectronic 20 spectrophotometer.

Nitrogen was measured by the micro-Kjeldahl method (24, 88, 113) at the Forage Analysis Laboratory, Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

For phosphorus, potassium and calcium analyses, 500 mg samples of dry, ground leaves were ashed in a 50 ml beaker for 2 hours at 475°C in a muffle furnace. Ashed materials were dissolved in 20 ml of 0.3 N HNO₃, transferred to 50 ml volumetric flasks and brought to volume with 0.3 N HNO₃. A 10 ml aliquot of this solution was pipetted into a 50 ml volumetric flask for subsequent determination of phosphorus by Linder's method (106).
Potassium and calcium contents were determined by flame photometry, using a Beckman model B spectrophotometer at 770 nm and 423 nm, respectively. For the calcium analyses, 2.5 ml of the 0.3 N HNO₃ ashed leaf sample solutions were mixed with equal volume of Na EDTA-KOH solution to increase the sensitivity of that test. The Na EDTA-KOH solution was prepared by dissolving 75 g of Na EDTA plus 60 g KOH in one liter distilled water at pH 12.0.

Diluted root exudates collected as described in the general procedures, were filtered through millipore filters of 0.45 μm pore size and temporarily stored at 5°C. Exudates were concentrated under vacuum to 1/10 their original volume in a Buchner flash evaporator at 39°C. Each concentrate was divided into 10 aliquots and stored at -20°C.

The total organic matter in the exudates was determined by the dry combustion method (221).

The anions of nitrate and nitrites present in the circumambient solution interfered with the initial phenol sulfuric acid test for total carbohydrates. This problem was eliminated by removal of the anions by passing exudate samples through anion exchange columns (1 cm² x 15 cm) of Amberlite IRA-400, Cl⁻ form (87).

The phenol sulfuric acid test (59, 82) was utilized to measure total carbohydrate present in exudates. Standard dilutions of glucose containing 10 to 100 μg/ml were prepared in nutrient solution of the same concentration as concentrated exudates and processed in the same manner as the exudate samples. Absorbancies were measured at 490 nm, in a Beckman DU II spectrophotometer.
Two subsamples from exudates collected per plant were handled according to the procedure outlined above and chemical tests were made in triplicate.

The Somogyi and Nelson test (202) was employed in determining the total reducing sugars in the concentrated exudate.

For protein determination, the Folin phenol test was used (5, 6). The dilutions of bovine serum protein containing 50 to 200 µg/ml in 0.1 N phosphate buffer pH 7.5 were treated similarly to the unknown samples and their absorbancy was read on a Beckman DU II spectrophotometer at 750 nm.

The total amino acid equivalent in root exudates was determined by the ninhydrin test (171). Dilutions of an amino acid, DL-Valine obtained from National Biochemical Corporation, Cleveland, Ohio, containing 0.02 to 0.4 µM were used as standard. Absorbancies were read on the Spectronic 20 spectrophotometer at 570 nm.

**Experiment II**

In the second experiment, a total of 24 axenic plants were grown inside two separate chambers, 12 plants in each chamber. At 36 day stage of plant growth, plants were inoculated with *P. simplicissimum* conidial suspension at a rate of $7.5 \times 10^6$ conidiospores per plant. This experiment was harvested 34 days after inoculation, when plants had flowered.

2/Obtained from Sigma Chemical Company, St. Louis, Missouri.
Axenic plants and *P. simplicissimum*-colonized plants were also compared with respect to the botanical characteristics and the morphology of the root system.

Root systems were shaken free of sand and immediately stored in freezer of -20°C to be used for the preparation of extracts of colonized roots used in experiment IV. Frozen root material was saved to be used for exocellular enzyme assays in conjunction with materials obtained in experiment VII.

Growth ratings were made with respect to plant height, fresh weight and dry weight. Chemical analyses of leaves for total water soluble carbohydrates, reducing sugars and fructose alone were conducted in the same manner of experiment I. Root exudates were collected at the end of the experiment and analyzed for total water soluble organic matter, total water soluble carbohydrates, reducing sugars, protein and total amino acid equivalent in the manner outlined in experiment I. Samples of dried and ground stems were analyzed for nitrogen, phosphorus, potassium and calcium.

**Experiment III**

Nine plants in the third experiment were inoculated with $8 \times 10^6$ conidiospores of *P. simplicissimum* per plant, in one chamber, and with $4 \times 10^6$ conidiospores of *P. citrinum* per plant in a second chamber. Six axenic plants were maintained inside two separate chambers, 3 plants per chamber, as controls.

Root exudates were obtained in six weekly collections of nutrient solution starting at the 35 day stage of plant growth. These exudates
were collected by evacuating 80 ml of the circumambient solution from each plant container. Three 80 ml samples represented one week sample per one replication per treatment. The weekly collections of exudates obtained during this experiment were filtered through Whatman No. 51 filter paper, concentrated to 1/10 the original volume and stored at 5°C. These exudates were analyzed within 24 to 48 hr from the time they were collected. Root exudate analyses for reducing sugars, protein and total amino acid equivalent were made in the same manner as described for experiments I and II. Data were subjected to analysis of variance.

The plants in this experiment were harvested 5 weeks after inoculation. Inoculated plants were compared with axenic plants with respect to plant height, fresh weight and dry weight. Leaves were analyzed for total water soluble carbohydrates, reducing sugars and fructose alone. Nitrogen content was computed as percent dry leaves. Chemical analyses were made according to the procedures described in experiments I and II.

Experiment IV

In the fourth experiment, sterilized extracts of axenic roots and *P. simplicissimum*-colonized roots were applied to the sand in the individual plant containers. Root extracts were obtained by mincing 80 g of plant roots with 80 ml cold nutrient solution in an Omnimixer, filtered through cheesecloth and centrifuged at 10,000 g for 20 min at 5°C. The supernatant was decanted and sterilized by filtration using a millipore filter, pore size 0.45 µ. The sterilized extracts were adjusted to 120 ml volume and transferred into the isolator chambers. Plants within each treatment received 40 ml of root extract.
During the course of this experiment root extracts were prepared and applied at the 3 different intervals. The first application of root extracts was made at the 35 day stage of plant growth. The second and third applications were made at the beginning of the first and fourth weeks from the time of the first application. There were three plants in each treatment, and all treatments were simultaneously replicated inside two chambers. The data were subjected to analysis of variance.

Six weekly collections of root exudates, starting at the 35 day stage of plant growth, were made in the same manner described in experiment III. Chemical analyses of these exudates for reducing sugars, protein and total amino acid equivalents were made the same as the manner described in experiments I and II.

Five weeks from the time of the application of the first dosage of root extract, the experiment was terminated and comparisons were made between the extract-treated plants and axenic non-treated plants grown in the same chambers. Plants were compared with respect to heights, fresh weights and dry weights. Dried leaves were analyzed for total carbohydrates, reducing sugars and percent nitrogen in the same manner as described in experiment I.

Experiment V

A total of 24 plants were treated with 0.0, 0.1, 1.0 and 10.0 mg/l of citrinin. Three plants per treatment per isolator chamber were replicated simultaneously inside 2 separate isolator chambers. Data obtained were subjected to analysis of variance and compared by means of Duncan's multiple range test.
Concentrations of citrinin were computed on the basis of 275 ml, the volume of the circumambient nutrient solution in the individual plant containers. A stock solution of citrinin was prepared by dissolving 41.25 mg in 300 ml of 0.1 M sodium citrate buffer, pH 7.5 to 8.0. Twenty ml of this stock solution were poured onto the sand in the plant containers to produce 10.0 mg/l concentration. Dilutions of this stock solution were used in the same manner to obtain 1.0 mg/l and 0.1 ml/l citrinin concentrations per plant container. The control plants received 20 ml of sodium citrate buffer only. All stock solutions above were sterilized by passage through a 0.45 µ pore size millipore filters and then introduced into the isolator chambers.

After application to the sand in the plant containers, the remaining aliquots of citrinin solutions were bioassayed for bacterial growth inhibition. A standard bioassay procedure was used with Bacillus mycoides and Bacillus mesentericus (122, 123). The presence of bacterial growth inhibition zone in the bioassay plates indicated that active citrinin was present in the rooting medium.

Citrinin concentrations were prepared and applied in 4 weekly dosages in the manner outlined above.

At the 53 day stage of plant growth, plants were harvested. Comparison of plant growth in the different citrinin concentration treatments was made with respect to plant height, fresh weight and dry weight. Nitrogen and protein contents were computed as percent leaf dry weight. Chemical analysis of leaves was made in the same manner as experiment I.
Experiment VI

The root systems of 30 day old axenic plants were inoculated by dipping them in conidial suspensions of \textit{P. simplicissimum} prior to transplanting into aluminum pans containing perlite wetted with nutrient solution. \textit{Penicillium simplicissimum} cultures grown on potato dextrose agar slants were introduced into the isolator chambers in the same manner as seedlings. Spore suspensions were obtained by washing the slant culture surfaces with 100 ml nutrient solution and decanting into an open container.

Inoculated plants were removed for histological study at 0, 4, 8, 16, 18, 20, 28 and 36 hours and 9 days after inoculation. The excised roots were fixed with a freshly prepared 3.2% glutaraldehyde in .025 M phosphate buffer of pH 6.8 at 0°C to 1°C. Fixed root tissues were then dehydrated in an ethyl alcohol series from 10% to 50% and then stored in 70% ethyl alcohol at 0°C to 1°C. Root tissues were cleared and stained by holding them just below boiling under lactophenol containing 0.01% acid fuchsin (modified from the cotton blue clearing and staining procedure) (63). Photomicrographs of the whole mounts of roots were made with the aid of a light microscope equipped with a green filter.

Experiment VII

Three weeks after inoculation in experiment VI, root systems were harvested and used in conjunction with frozen root tissues from experiments I and II for macerating, cellulase and polygalacturonase (PG) enzyme assays.

Crude enzyme extracts were obtained by grinding 1 part of fresh or frozen roots in an Omni mixer with 1 volume of 0.5 M NaCl. The
extract was filtered through cheesecloth, centrifuged at 10,000 g for 20 min, and then dialized against several hundred volumes of deionized water for 30 hours at 5°C. After dialysis, the enzyme extraces were immediately tested for activities.

The loss of coherence of potato (Solanum tuberosum L.) tuber discs was used to determine macerating enzymes activity (16, 17). Potato tubers were surface sterilized for 10 min in a 1:10 solution of commercial bleach containing 5.25% sodium hypochlorite. They were then peeled and cut into 50 µ slices with a hand microtome. A cork borer 10 mm in diameter, was used to cut the slices into discs, which were then rinsed and placed in distilled water until used.

Macerating enzyme activity was compared at different pH values ranging from 3.0 to 9.5 using a 0.1 M sodium citrate buffer (pH 3.0, 4.0, 5.0, 6.0) and a 0.2 M Tris buffer adjusted with 0.2 M HCl to the appropriate pH (pH 7.0, 8.0, 9.0, 9.5). Two ml of buffer with 3.0 ml of the enzyme preparation and six potato discs mounted on a toothpick were placed in each test tube. After incubation at 30°C for 10, 18 and 24 hours intervals, loss of coherence was determined using a scale of 0 to 4 (0 = no maceration, 4 = complete loss). Boiled enzyme preparations and enzyme preparations from axenic roots and buffer solution alone served as checks. Each treatment was replicated three times.

Viscometric and reducing sugar methods were used to assay for cellulase, polygalacturonase (PG) and polymethylgalacturonase (PMG) (11, 16, 17, 60, 128). Viscometer reaction mixtures contained the enzyme preparation and the respective buffered substrate which were pipetted into Oswald and Franske viscometer (size 300) and incubated
in a water bath of the respective temperature. Percent loss in viscosity was measured in relative values to the viscosity of the crude enzyme preparation which represented 100% loss in viscosity (11).

Reducing end-group analysis was performed on the reaction products formed in test tubes run simultaneously with the viscometer assays for cellulase by using the Somogyi and Nelson test (202). Reducing end-group analysis with respect to PG and PMG was performed on the reaction products formed in the viscometers after 24 hours by using the Somogyi and Nelson test (202). The standard solutions of glucose or galacturonic acid containing 50 mg/l of 200 mg/l were used to determine the equivalent residues released respectively. Each pH value and crude enzyme preparation were replicated twice and chemical tests were conducted in triplicate.

For the cellulase assays, two different species of carboxymethylcellulose were used as substrates. Carboxymethylcellulose-7HP served as a substrate for the loss in viscosity assays of cellulase activity. The substrate was prepared by mixing 0.5% (W/V) CMC-7HP in 0.05 M acetate buffer of pH 5.0 in an Omni mixer for 30 seconds at a high speed. The tank of the mixer was surrounded with boiling water bath during this step. The substrate solution was then filtered through glasswool before use. Five ml of substrate, plus 1 ml of the enzyme preparation, were incubated in Oswald and Fenske viscometer in a water

6/ Obtained from Hercules Powder Company, Wilmington 99, Delaware, U.S.A.
bath at 40°C. Loss in viscosity was recorded at 2 hours intervals from 0 to 24 hours of incubation.

The CMC-7LP was used as a substrate in the reducing end-group assays of cellulase activity. The substrate was prepared by mixing 3.33% (W/V) of CMC-7LP in 0.05 M acetate buffer of pH 5.0 in an Omni mixer, without heating. One ml of this solution was incubated with 1 ml of the crude enzyme preparation in a water bath of 30°C. Triplet samples were analyzed at 2 hour intervals for the reducing groups content utilizing Somogyi and Nelson test.

Pectin NF\(^7\)/ and sodium polypectate\(^7\)/ solutions prepared as 1.5% concentration in 0.1 M sodium citrate buffer of pH 3.0, 4.0, 5.0 and 6.0 were used as substrate for the PG and PMG activities. Two ml of the substrate at the respective pH, plus 2 ml of the crude enzyme preparation plus 2 ml of deionized distilled water were pipetted into Oswald and Fenske viscometer. After 24 hours incubation at 30°C in a water bath, viscosity was measured and reducing power equivalent to polygalacturonic acid standard dilutions in the reaction mixture was determined by Somogyi and Nelson test (202). The enzyme preparation alone served as the 100% loss in viscosity and maximum reducing power.

\(^7\)/ Obtained from Sunkist Grower, Inc. Corona, California, U.S.A.
RESULTS

Axenic marigold plants were consistently obtained and maintained under sterile conditions for 5 to 6 weeks inside the isolator chambers. During these periods of plant growth, the sterility checks from axenic plants on all 5 of microbial growth media showed no bacterial or fungal contamination. Checks from the inoculated plants revealed the presence of only the *Penicillium* species which had been introduced. Statistical analyses of all the data showed that there were no significant variations within and among isolator chambers used during the investigation.

The axenic plants, and plants with *P. simplicissimum* or *P. citrinum* colonized roots, exhibited no discernable visual symptoms of environmental stresses which might have existed in the isolator chambers. There was also no visible difference in the morphology of the haulms of axenic plants and root colonized plants. The root colonized plants, however, showed enhanced overall growth, earlier flowering and considerable degradation of their root systems (Fig. 8).

Experiment I

Comparisons were made between axenic plants and plants with *P. simplicissimum*-colonized roots with respect to plant height, fresh weight, dry weight, and color and turgidity of roots. Root colonized plants showed greater dry weight and plant height and there was a trend toward greater fresh weight (Table I). Axenic roots were white and turgid. Colonized roots, however, were brown, less turgid and showed considerable degradation (Fig. 8). The level of degradation of colonized roots was estimated to be 30 to 40%.
Figure 8. Comparative plant size, number of flowers, and size and color of root systems between germ-free (axenic) and *P. simplicissimum*-colonized marigold plants 34 days after inoculation.
Table I. Height, fresh weight and dry weight of 58 day old axenic plants and *P. simplicissimum*-colonized plants 20 days after inoculation.  

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<th>Plant height</th>
<th>Fresh weight</th>
<th>Dry weight</th>
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<tr>
<td></td>
<td>cm</td>
<td>Shoot g</td>
<td>Total g</td>
</tr>
<tr>
<td>Axenic plants</td>
<td>37.7</td>
<td>73.5</td>
<td>109.3</td>
</tr>
<tr>
<td><em>P. simplicissimum</em>- colonized plants</td>
<td>40.9</td>
<td>79.6</td>
<td>116.7</td>
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1/ Data compiled by taking average of 10 plants.
Chemical analyses of dried leaves for total water soluble carbohydrates, total reducing sugars and fructose alone revealed that there were more total reducing sugars and fructose in the leaves from plants with *P. simplicissimum*-colonized roots than from axenic plants (Table II). Also, there was a trend toward more water soluble carbohydrate content in the leaves from root colonized plants. The ratio of the μg/mg of total water soluble carbohydrates to reducing sugars in the dried leaves of the axenic plants, 3.5 to 1, was higher than the ratio of the same compounds in *P. simplicissimum*-colonized plants, 3.1 to 1.

Chemical analyses for nutrient elements in dried leaves showed lower percentages of phosphorus and potassium in leaves from plants with *P. simplicissimum*-colonized roots than in leaves from axenic plants (Table III). The percentages of nitrogen and calcium were similar in leaves from axenic and from root-colonized plants.

Chemical analyses showed less total water soluble organic matter and protein content in the exudate of plants with *P. simplicissimum*-colonized roots than in the exudate from axenic plant roots (Table IV). Apparently the amounts of total water soluble carbohydrates, reducing sugars and total amino acid equivalents were not affected at this stage of colonization.

**Experiment II**

Results of this experiment showed increased plant height and dry weight of plants with *P. simplicissimum*-colonized roots compared to the axenic plants, but the fresh weights showed only a trend in this
Table II. Total water soluble carbohydrates, reducing sugars, and fructose content of leaves from 58 day old axenic plants and *P. simplicissimum*-colonized plants 20 days after inoculation.\(^1\)

<table>
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<tr>
<th></th>
<th>Total water soluble carbohydrates</th>
<th>Reducing sugars</th>
<th>Fructose</th>
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<tr>
<td></td>
<td>µg/mg</td>
<td>µg/mg</td>
<td>µg/mg</td>
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<tr>
<td>Axenic plants</td>
<td>65.4</td>
<td>16.9</td>
<td>9.8</td>
</tr>
<tr>
<td><em>P. simplicissimum</em>-</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>colonized plants</td>
<td>73.9</td>
<td>24.2(^{**})</td>
<td>13.2(^*)</td>
</tr>
</tbody>
</table>

\(^1\)/Data compiled by taking average of 10 plants.
Table III. Nitrogen, phosphorus, potassium, and calcium content as percent dry weight of leaves of 58 day old axenic plants and \textit{P. simplicissimum}-colonized plants 20 days after inoculation.\(^1\)

<table>
<thead>
<tr>
<th>Nitrogen</th>
<th>Phosphorus</th>
<th>Potassium</th>
<th>Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Axenic plants</td>
<td>4.70</td>
<td>0.52</td>
<td>3.23</td>
</tr>
<tr>
<td>\textit{P. simplicissimum}- colonized plants</td>
<td>4.59</td>
<td>0.48*</td>
<td>2.89**</td>
</tr>
</tbody>
</table>

\(^1\) Data compiled by taking average of 10 plants. All chemical tests were made in triplicate.
Table IV. Chemical composition of root exudates of 58 day old axenic plants and *P. simplicissimum-* colonized plants 20 days after inoculation.  

<table>
<thead>
<tr>
<th></th>
<th>Total water soluble</th>
<th>Total water soluble</th>
<th>Reducing sugars</th>
<th>Protein equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mg</td>
<td>μg</td>
<td>mg</td>
</tr>
<tr>
<td>Axenic plants</td>
<td>409.71</td>
<td>2.49</td>
<td>269.87</td>
<td>4.02</td>
</tr>
<tr>
<td><em>P. simplicissimum-</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>colonized plants</td>
<td>361.55**</td>
<td>2.07</td>
<td>265.36</td>
<td>3.39*</td>
</tr>
</tbody>
</table>

1/Amount of chemical compounds per plant as average of 10 plants. Chemical tests were made in triplicate.
direction. The fresh weight of shoots only, however, was significantly higher for root colonized plants than for axenic plants (Table V).

The roots of plants grown under gnotobiotic combination with *P. simplicissimum* were brown, flaccid and showed considerable degradation (Fig. 8). The amount of degradation at this stage of plant growth was estimated to be 40 to 60% of the total root system.

Results of foliar analyses showed more total water soluble carbohydrates in the leaves of plants with *P. simplicissimum*-colonized roots than in leaves of axenic plants. Also, there was a trend toward more reducing sugars in the leaves from root colonized plants. The fructose concentration of the leaves from the two plant groups, however, were similar (Table VI). The ratio of the µg/mg of the total water soluble carbohydrates to reducing sugars in the dried leaves of the axenic plants, 3.4 to 1, was higher than the ratio of the same compounds in leaves of the *P. simplicissimum*-colonized plants, 2.5 to 1.

Dried leaves from plants with *P. simplicissimum*-colonized roots showed higher calcium percentages than leaves from axenic plants (Table VII). There were no significant differences in percentages of nitrogen, phosphorus and potassium in leaves from axenic and from root-colonized plants (Table VII). The percentages of nitrogen, potassium and calcium were not different in dried stems of axenic and root-colonized plants. Phosphorus percentage, however, was lower in stems of the root-colonized plants than in the axenic plants (Table VIII).

Observations on the date of flowering revealed that inoculated plants developed floral buds 5 to 6 days earlier than axenic plants.
Table V. Height, fresh weight and dry weight of 72 day old axenic plants and *P. simplicissimum*-colonized plants 34 days after inoculation.  

<table>
<thead>
<tr>
<th></th>
<th>Plant height cm</th>
<th>Fresh weight</th>
<th>Dry weight g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoot</td>
<td>Total</td>
<td>Shoot</td>
</tr>
<tr>
<td>Axenic plants</td>
<td>40.5</td>
<td>109.5</td>
<td>186.8</td>
</tr>
<tr>
<td><em>P. simplicissimum</em>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>colonized plants</td>
<td>50.3**</td>
<td>143.4**</td>
<td>227.2</td>
</tr>
</tbody>
</table>

1/ Data compiled by taking average of 12 plants.
Table VI. Total water soluble carbohydrate, total reducing sugars and fructose content in µg/mg of leaves from 72 day old axenic plants and *P. simplicissimum* colonized plants 34 days after inoculation.¹/

<table>
<thead>
<tr>
<th></th>
<th>Total water soluble carbohydrates µg/mg</th>
<th>Reducing sugars µg/mg</th>
<th>Fructose µg/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axenic plants</td>
<td>77.2</td>
<td>31.9</td>
<td>20.8</td>
</tr>
<tr>
<td><em>P. simplicissimum</em>-colonized plants</td>
<td>88.1**</td>
<td>33.2</td>
<td>19.0</td>
</tr>
</tbody>
</table>

¹/ Data compiled by taking average of 12 plants. Chemical tests were made in triplicate.
Table VII. Nitrogen, phosphorus, potassium and calcium content computed as percent dry weight of leaves from 62 day old axenic plants and *P. simplicissimum*-colonized plants 34 days after inoculation.\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>Nitrogen</th>
<th>Phosphorus</th>
<th>Potassium</th>
<th>Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Axenic plants</strong></td>
<td>4.31</td>
<td>0.48</td>
<td>2.87</td>
<td>2.45</td>
</tr>
<tr>
<td><strong>P. simplicissimum-</strong></td>
<td>4.01</td>
<td>0.47</td>
<td>2.71</td>
<td>2.73**</td>
</tr>
<tr>
<td><strong>colonized plants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Data compiled by taking average of 12 plants. All chemical tests were made in triplicate.
Table VIII. Nitrogen, phosphorus, potassium and calcium content computed as percent dry weight of stems from 72 day old axenic plants and *P. simplicissimum*-colonized plants 34 days after inoculation.¹/

<table>
<thead>
<tr>
<th></th>
<th>Nitrogen</th>
<th>Phosphorus</th>
<th>Potassium</th>
<th>Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axenic plants</td>
<td>1.02</td>
<td>0.28</td>
<td>3.27</td>
<td>1.40</td>
</tr>
<tr>
<td><em>P. simplicissimum</em>- inoculated</td>
<td>1.10</td>
<td>0.23**</td>
<td>3.33</td>
<td>1.43</td>
</tr>
</tbody>
</table>

¹/ Data compiled by taking average of 10 plants.
Also, those floral buds opened 10 days ahead of the ones on axenic plants (Fig. 8).

Dry weight of seeds from plants grown under axenic and infested conditions was 7.5 g, and 1.1 g, respectively. Neither seed lot germinated in a viability test.

Results of the root exudate analyses showed higher amounts of total water soluble carbohydrates, reducing sugars, protein content and total amino acid equivalents in the exudate of *P. simplicissimum*-colonized roots. The amounts of total water soluble organic matter were not different in the exudate of axenic and root-colonized plants (Table IX).

**Experiment III**

Plant height and fresh and dry weights of plants with *P. simplicissimum*-colonized roots and plants with *P. citrinum*-colonized roots were compared with those of axenic plants (Table X). There was a trend toward increased plant heights and dry weights of *P. citrinum*-inoculated plants. The fresh weights of the same plants, however, showed an apparent decrease. The growth measurements of the *P. simplicissimum*-inoculated plants were not different from those of axenic plants.

There was a trend toward increased total water soluble carbohydrates and reducing sugars in the dried leaves of *P. simplicissimum* and *P. citrinum*-inoculated plants compared to the leaves from axenic plants (Table XI). Percent nitrogen, however, was the same for all three plant groups (Table XI).
Table IX. Chemical composition of root exudates of 72 day old axenic plants and *P. simplicissimum-* colonized plants at 34 days after inoculation.  

<table>
<thead>
<tr>
<th></th>
<th>Total water soluble organic matter</th>
<th>Total water soluble carbohydrates</th>
<th>Reducing sugars (µg)</th>
<th>Protein (mg)</th>
<th>Total valine-equivalent amino acid (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axenic plants</td>
<td>462 mg</td>
<td>1.36 mg</td>
<td>224.99 µg</td>
<td>2.77 mg</td>
<td>2.42 µM *</td>
</tr>
<tr>
<td><em>P. simplicissimum-</em> inoculated</td>
<td>419 mg *</td>
<td>2.80 ** µg</td>
<td>664.67 ** µg</td>
<td>4.19 * µg</td>
<td>3.02 * µg</td>
</tr>
</tbody>
</table>

1/ Amounts of chemical compounds per plant as average of ten plants. Chemical tests were made in triplicate.
Table X. Height, shoot fresh weight and shoot dry weight of 75 day old axenic plants and *P. simplicissimum* - and *P. citrinum*-colonized plants 35 days after inoculation.\(^1\)

<table>
<thead>
<tr>
<th>Plant height</th>
<th>Fresh weight</th>
<th>Dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>cm</strong></td>
<td><strong>g</strong></td>
<td><strong>g</strong></td>
</tr>
<tr>
<td>Axenic plants</td>
<td>41.6</td>
<td>121.6</td>
</tr>
<tr>
<td><em>P. simplicissimum</em>- colonized plants</td>
<td>41.6</td>
<td>125.8</td>
</tr>
<tr>
<td><em>P. citrinum</em>- colonized plants</td>
<td>52.5</td>
<td>110.5</td>
</tr>
</tbody>
</table>

\(^1\) Data compiled by taking averages of 6 axenic plants and 9 colonized plants.
Table XI. Total water soluble carbohydrates, reducing sugars and percent nitrogen of dried leaves from 75 day old axenic plants, *P. simplicissimum*- and *P. citrinum*-colonized plants 35 days after inoculation.  

<table>
<thead>
<tr>
<th></th>
<th>Total water soluble carbohydrates</th>
<th>Reducing sugars</th>
<th>Nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/mg</td>
<td>µg/mg</td>
<td>%</td>
</tr>
<tr>
<td>Axenic plants</td>
<td>58.7</td>
<td>21.6</td>
<td>4.36</td>
</tr>
<tr>
<td><em>P. simplicissimum</em>-</td>
<td>67.3</td>
<td>35.0</td>
<td>4.18</td>
</tr>
<tr>
<td>colonized plants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. citrinum</em>-</td>
<td>59.7</td>
<td>34.7</td>
<td>4.44</td>
</tr>
<tr>
<td>colonized plants</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1/ Data compiled by taking averages of 6 axenic plants and 9 colonized plants.
Root exudates collected prior to inoculation showed no significant variation in amounts of reducing sugars, proteins and total amino acid equivalents among plants used in this experiment.

The amounts of reducing sugars increased at comparable rates in the exudates of the inoculated and axenic plants during the first and the second weeks after inoculation (Fig. 9). Three weeks after inoculation, the amounts of reducing sugars in the exudates of axenic and *P. citrinum*-inoculated plants remained constant while the amounts of the same compounds in the exudate of *P. simplicissimum*-inoculated plants continued to increase through the fifth week.

The protein content was not different in the exudates of axenic and *P. simplicissimum*-inoculated plants during the first and second weeks after inoculation. By the third week after inoculation, the protein content in the exudate of *P. simplicissimum*-inoculated plants increased and continued to increase through the fifth week after inoculation. The protein exudation of the roots of the axenic plants, however, did not increase until the fourth week after inoculation and thereafter was similar to that of the inoculated plants. Amounts of protein in the exudate of *P. citrinum*-inoculated plants showed no specific trends (Fig. 10).

Concerning the exudation of amino acids, there were similar total amino acids equivalents in the exudate of axenic and *P. simplicissimum*-inoculated plants during the first and second weeks after inoculation. From the third through the fifth week after inoculation, however, the total amino acid equivalents were higher in the exudates of *P. simplicissimum*-inoculated than the axenic plants. The exudate of
Figure 9. Amounts of reducing sugars in 240 ml of nutrient solution, as root exudates, collected in 80 ml portions from three plants each treatment each week.
Axenic plants

P. simplicissimum – inoculated

P. citrinum – inoculated

µg REDUCING SUGARS

1,300

1,000

700

400

100

50

0

1

2

3

4

5

WEEKS
Figure 10. Amounts of protein in 240 ml of nutrient solution, as root exudates, collected in 80 ml portions from three plants each treatment each week.
Axenic plants

P. simplicissimum – inoculated

P. citrinum – inoculated

μg PROTEIN vs. WEEKS
P. citrinum-inoculated plants showed a pattern similar to the exudate of axenic plants (Fig. 11).

The exudation of reducing sugars, protein and total amino acid equivalents from axenic plants decreased 3 weeks after inoculation, when plants were flowering. Such a decrease did not occur in the exudates of P. simplicissimum-inoculated plants (Figs. 9, 10, 11).

**Experiment IV**

Plant height, fresh weight and dry weight measurements showed a trend toward decreased growth rates of axenic plants treated with sterilized extract of roots of axenic plants or with sterilized extract from P. simplicissimum-colonized roots. The greatest reduction in growth, however, appeared to be in the plants treated with sterilized extract from P. simplicissimum-colonized roots (Table XII).

Concentration of total water soluble carbohydrates and reducing sugars in leaves showed increased amounts of these compounds in leaves of plants treated with sterilized extracts from axenic roots and plants treated with sterilized extracts of P. simplicissimum-colonized roots. The highest concentrations of total water soluble carbohydrates and reducing sugars, however, were found in leaves from plants treated with sterilized extracts of P. simplicissimum-colonized roots (Table XIII).

Compared with the exudate from roots of axenic plants, there were higher amounts of reducing sugars in the exudates of plants treated with sterilized extract of roots of axenic plants or with sterilized extracts of P. simplicissimum-colonized roots (Fig. 12). The highest amounts of reducing sugars and protein were observed in the exudates.
Figure 11. Amounts of total amino acid equivalent in 240 ml of nutrient solution, as root exudates, collected in 80 ml portions from three plants each treatment each week.
Axenic plants

P. simplicissimum–inoculated

P. citrinum–inoculated

µ Mole Amino Acid Equivalent

WEEKS

5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Table XII. Comparative heights and fresh weights and dry weights of shoots of plants treated with extracts from roots of marigold plants grown axenically and colonized with *P. simplicissimum* respectively.1/

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plant height</th>
<th>Fresh weight</th>
<th>Dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cm</td>
<td>g</td>
<td>g</td>
</tr>
<tr>
<td>Control</td>
<td>41.6</td>
<td>121.6</td>
<td>16.3</td>
</tr>
<tr>
<td>Plants treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with extract of axenic root</td>
<td>39.7</td>
<td>113.9</td>
<td>15.5</td>
</tr>
<tr>
<td>Plants treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with extract of <em>P. simplicissimum</em>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>colonized root</td>
<td>34.9</td>
<td>101.1</td>
<td>12.1</td>
</tr>
</tbody>
</table>

1/ Data compiled by taking averages of 6 control plants and 9 treated plants 35 days after first treatment. Plants were 74 days old at time of harvest.
Table XIII. Comparative total water soluble carbohydrates, reducing sugars and percent nitrogen of dried leaves from plants treated with extracts of roots of marigold plants grown axenically and colonized with *P. simplicissimum* respectively.¹

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total water soluble carbohydrates µg/mg</th>
<th>Reducing sugars µg/mg</th>
<th>Nitrogen %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>58.7</td>
<td>21.6</td>
<td>4.36</td>
</tr>
<tr>
<td>Plants treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with extract of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>axenic root</td>
<td>56.3</td>
<td>22.1</td>
<td>4.39</td>
</tr>
<tr>
<td>Plants treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with extract of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. simplicissimum-</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>colonized root</td>
<td>70.5</td>
<td>27.8</td>
<td>4.22</td>
</tr>
</tbody>
</table>

¹Data compiled by taking averages of 6 control plants and 9 colonized plants 35 days after inoculation. Plants were 74 days old at time of harvest. All chemical tests were made in triplicate.
Figure 12. Amounts of reducing sugars in 240 ml of nutrient solution, as root exudates, collected in 80 ml portions from three plants each treatment each week. (Arrows indicate the time at which application of root extracts was made).
of plants treated with sterilized extract from *P. simplicissimum*-colonized roots.

Concerning protein in the exudate, there were higher amounts of protein in the exudate of plants treated with sterilized extract from *P. simplicissimum*-colonized root than the exudates of plants treated with sterilized extract from axenic roots and the control. The exudates of the latter two plant groups, however, showed similar amounts of protein (Fig. 13).

Total amino acid equivalents in the exudates from plants treated with sterilized extract of axenic roots, *P. simplicissimum*-colonized roots, and the control, revealed no difference in total amino acid equivalents (Fig. 14).

There was a common trend of a sharp decrease in the amounts of reducing sugars, protein and amino acids in the exudates of both treatments and of the control plants 3 weeks from the first application of root extracts (Figs. 12, 13, 14).

**Experiment V**

Axenic plants treated with low concentrations of citrinin revealed a trend of enhanced growth. Plant height, fresh weight and dry weight showed a trend toward higher values at 0.1 mg/l and 1.0 mg/l citrinin than at 10 mg/l citrinin and the control (Table XIV).

Percentages of nitrogen and protein in plants treated with 1.0 mg/l citrinin were significantly higher than those of the control and the 10 mg/l citrinin treatment, but they were similar to those of the
Figure 13. Amounts of protein in 240 ml of nutrient solution, as root exudates, collected in 80 ml portions from three plants each treatment each week. (Arrows indicate the time at which application of root extracts was made).
Control
Extract of axenic root
Extract of *P. simplicissimum* - colonized root

μg PROTEIN

WEEKS
Figure 14. Amounts of amino acid equivalents in 240 ml of nutrient solution, as root exudates, collected in 80 ml portions from three plants each treatment each week. (Arrows indicate the time at which application of root extracts was made).
Extract of axenic root

Extract of *P. simplicissimum*-colonized root

WEEKS

μ MOLE AMINO ACID EQUIVALENT

Control

- Extract of axenic root

- Extract of *P. simplicissimum*-colonized root
Table XIV. Height, shoot fresh weight and dry weight of 53 day old axenic plants treated with various concentrations of citrinin at 4 weekly intervals.\(^1\)

<table>
<thead>
<tr>
<th>Treatment (\text{mg/l})</th>
<th>Plant height (\text{cm})</th>
<th>Fresh weight Shoot (\text{g})</th>
<th>Dry weight Shoot (\text{g})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>28.5</td>
<td>44.3</td>
<td>6.9</td>
</tr>
<tr>
<td>0.1</td>
<td>43.0</td>
<td>82.0</td>
<td>13.1</td>
</tr>
<tr>
<td>1.0</td>
<td>40.2</td>
<td>61.9</td>
<td>9.8</td>
</tr>
<tr>
<td>10.0</td>
<td>36.5</td>
<td>59.9</td>
<td>9.4</td>
</tr>
</tbody>
</table>

\(^1\) Data compiled by taking averages of 6 plants, three plants per treatment per chamber, replicated inside two chambers.
0.1 mg/l citrinin treatment. Ten mg/l citrinin concentration showed no effect on percentages of nitrogen and protein (Table XV).

Experiment VI

Examination of whole mounts of cleared and then stained roots of axenic plants and *P. simplicissimum*-inoculated plants obtained during this experiment and experiments I and II and roots of *P. citrinum*-inoculated plants from experiment III revealed that conidial germination and fungal mycelial growth occurred on the root surfaces. Conidial germination of *P. simplicissimum* under gnotobiotic conditions was first observed 16 to 18 hours after inoculation. All conidial germination was observed in the root hair zone only. There was no direct penetration of intact plant tissues. However, new hyphae grew in close association with root hair surfaces and along the epidermal surfaces (Fig. 15B and 16A). Colonization of old root hairs and weakened epidermal and cortical tissues was evident by the fifth and the sixth day after inoculation (Fig. 15A and 15D).

Extensive disorganization of epidermal and cortical tissue was occasionally observed on axenic roots. In case of the inoculated roots, disorganized tissues and sloughed off tissues were observed with a number of conidia adhering to them (Fig. 15F). These tissues later on became centers for fungal activities. A dense mycelial growth formed on these root tissues and then the hyphae ramified and colonized adjacent cortical tissue. Extensive mycelial growth was observed covering considerable length of individual roots. Roots from experiments II and III were covered with mycelial growth and the conidiospores (Fig. 15C and 15E).
Table XV. Nitrogen and protein content as percent of dry weight leaves from 53 day old axenic plants treated with various concentrations of citrinin at 4 weekly intervals. 1/

<table>
<thead>
<tr>
<th>Treatment mg/l</th>
<th>% Nitrogen</th>
<th>% Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>3.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.61&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.1</td>
<td>3.17&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>21.00&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.0</td>
<td>3.67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.38&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10.0</td>
<td>3.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1/ Data compiled by taking averages of 6 plants, three plants per treatment per chamber replicated inside two chambers. Values with different lower case letters are significantly different at 0.05 level of confidence using Duncan's multiple range method.
Figure 15. Stages of marigold root colonization by *P. simplicissimum* and *P. citrinum*, under gnotobiotic conditions: (A) initial colonization of epidermal cells, x 2400; (B) fungal hypha growing on surface of the epidermis along the junction between two epidermal cells, x 2600; (C) conidiophore of *P. simplicissimum* on root surface, x 1600; (D) same as A, x 1600; (E) *P. citrinum* mycelial growth on root surfaces, x 703; (F) conidia adhering to the slime on the root surface, x 1600.
Figure 16. Morphological changes of root hairs of marigold plant colonized with *P. simplicissimum* and *P. citrinum* under gnotobiotic conditions: (A) germinated conidia and deformed tips of root hairs, x 2100; (B) invagination of root tips in a pocket formation, x 2240; (C) x 2120; and, (D) dichotomous growth at early and late stages of development, x 2240.
Root hairs of the inoculated plants exhibited morphological deformations 20 and 34 days after inoculation. These included invagination of root hair cell wall, initiation of multiple growing tips and dichotomous branching of root hairs (Fig. 16A and 16D).

**Experiment VII**

The potato disc assays for macerating activity in the extracts of *P. simplicissimum*-colonized root showed a positive increase in maceration after 10, 18 and 24 hours of incubation at 25°C (Fig. 17). The crude enzyme preparation exhibited higher macerating activities at pH 5 for all incubation periods. By 18 hours, at pH 5, the activity reached a maximum value of 4. Maceration at pH 4 and 6 was still increasing at the 24 hour incubation period. This may indicate a suppression of those enzymes at pH 4 and 6. There was no maceration of potato discs incubated in boiled crude enzyme preparation, preparations from axenic roots and buffer solutions alone.

Release of reducing groups and changes in viscosity were followed in carboxymethylcellulose (CMC) plus enzyme preparation mixtures (Fig. 18). Results showed a gradual increase in amounts of reducing groups liberated throughout 24 hours. Similar increase in percent loss in viscosity of substrate observed in the CMC plus enzyme preparation mixtures of two separate samples. Boiled preparation showed no release of reducing group throughout the same period. These results are evidence for the presence of cellulolytic enzyme in the extract of *P. simplicissimum*-colonized roots. During the activity of this enzyme, there was a hydrolysis of the end groups rather than a random hydrolysis of the substrate molecules.
Figure 17. Maceration activity of the extract of *P. simplicissimum*-colonized root on potato discs incubated at 25°C.
Figure 18. Percent loss in viscosity and reducing sugars released as mg glucose equivalent from carboxymethylcellulose (CMC-7HP and CMC-7LP, respectively) by the action of the extracts of *P. simplicissimum*-colonized root. Reaction mixtures incubated at pH 5 and 40°C. Samples 1 and 2 obtained from two adjacent chambers.
REDUCING SUGARS RELEASED

% LOSS IN VISCOSITY

HOURS

mg GLUCOSE

P. simplicissimum - colonized active
P. simplicissimum - colonized active -2
P. simplicissimum - only
CMC only
The release of reducing groups and changes in viscosity of pectin NF and sodiumpolypectate (NA-PP) were assayed at pH 3, 4, 5 and 6 (Fig. 19 and 20).

In comparing the release of reducing groups and loss in viscosity of pectin substrate, results showed high amounts of reducing group were liberated at pH 3 and 4, whereas the viscosity was not affected at these pH values (Fig. 19).

When Na-PP was utilized as a substrate, the liberation of reducing groups was greater at pH 4 and 5 than the pH 3 and 6 (Fig. 20). Results showed an increased percent loss in viscosity of Na-PP in the reaction mixture incubated with each increase in pH. The release of reducing groups and percent loss in viscosity showed similar trends from pH 3 to 5.
Figure 19. Percent loss in viscosity and reducing sugars released as mg polygalacturonic acid equivalent from pectin NF by the action of extracts of _P. simplicissimum_ -colonized root at pH 3, 4, 5 and 6. Reaction mixtures incubated 24 hours at 30°C.
REDUCING SUGARS RELEASED

- P. simplicissimum - colonized active
- P. simplicissimum - colonized boiled
- Pectin only

% LOSS IN VISCOSITY

- P. simplicissimum - colonized active
- P. simplicissimum - colonized boiled

pH

% LOSS IN VISCOSITY

μg/ml REDUCING SUGARS
Figure 20. Percent loss in viscosity and reducing sugars released as mg polygalacturonic acid equivalent from sodiumpolypectate by the action of extracts of *P. simplicissimum*-colonized roots at pH 3, 4, 5 and 6. Reaction mixtures incubated 24 hours at 30°C.
REDUCING SUGARS RELEASED

- P. simplicissimum - colonized active
- " " - " " boiled
- " " - " " : NaPP only

% LOSS IN VISCOSITY

P. simplicissimum - colonized active

% LOSS IN VISCOSITY

pH

g/ml REDUCING SUGARS
DISCUSSION

Since it has been recognized that rhizosphere soil supports a higher microfloral population than non-rhizosphere soil, there has been an increasing interest in the role of microflora in the life of higher plants. Plant pathologists, especially those who work with root diseases, have been giving more attention to the interactions among plant roots and saprophytic microflora, to determine what their role is under natural growing conditions. The present investigation established that there was an increase in top growth of axenic marigold plants after inoculation with one or the other of two *Penicillium* sp., in spite of the considerable degradation of the roots of the inoculated plants. Lindsey (108) reported similar results from monoculture inoculation with saprophytic soil fungi of several plant species. Though Lindsey's conclusions and conclusions of the present investigation were derived from model systems, they showed that saprophytic fungi in the rhizosphere can act as primary pathogens on plant roots. Such fungi may also predispose plant roots to other pathogens or they may follow an already established pathogen and accelerate pathogenesis.

In the present investigations, the presence of cellulolytic and pectolytic enzymes in the extracts of colonized roots as well as the root degradation of the inoculated plants were indicative of the pathogenic potentials of the fungi used. Such activities with *Penicillium* sp. had been previously reported only from culture filtrates and from extracts of post harvest diseased tissues (45, 61, 72, 73).
Since there was increased water soluble carbohydrates in the foliage of the inoculated plants, it may be inferred that root coloniza-
tion may have an effect on foliage reactions to pathogens. The coloniza-
tion of plant roots by saprophytic fungi was not detrimental to the
growth of plant shoots under these monocultural experimental conditions. 
While the improved growth of plants with Penicillium sp. colonized roots 
might not hold true for natural conditions, it seems reasonable to 
postulate that if the comparison were made with plants under natural 
conditions that axenic conditions may not be the optimal growth conditions 
for marigold plants.

The enhanced growth of inoculated plants coinciding with a lowered 
or similar foliar phosphorus and potassium content as axenic plants 
supports several hypotheses. Of those hypotheses are higher plants 
may accumulate an excess of nutrient elements, and inoculated plants 
may make more efficient use of the available elements. Similar conclu-
sions were made by Rempe (162) after examining the influence of root 
 microflora on physiological processes in oat and maize.

Decreased percentages of phosphorus and potassium at the early 
stages of colonization might have been caused by an adverse effect 
exerted by the fungus upon active absorption. At later stages of 
colonization passive absorption might have increased as the free space 
of the root absorption sites increased through changes in membrane 
permeability and disruption of cellular structure. Therefore, inter-
pretation of results on nutrient uptake under sterile versus nonsterile 
growth conditions could be erroneous if the studies were limited to 
seedlings and/or early stages of colonization (107). Also, the effects
of colonization were not universal for all stages of plant growth. Enhanced growth was indicated by increased dry weight 20 days after inoculation. Thirty-four days after inoculation, however, all growth of shoots increased significantly. From this it can be concluded that results of experiments limited to seedlings cannot be compared to results obtained from prolonged colonization throughout the life cycle of the plant (107).

In their experiments, soil microbiologists and root pathologists have usually attempted to duplicate natural conditions. Therefore, inocula of a mixed population, such as natural soil, have commonly been used to reinfest the rooting media (107, 119). However, once soil or a plant growth medium is sterile, it is difficult, if not impossible, to return the system to the same condition found in nature. Often, the development of the mixed species inoculum has been made without knowledge of the population levels in so called natural soil. The reinfestation of a sterilized medium results in a succession of soil organisms and, at certain times, a single or few organisms are dominant. Frequently, differences between axenic plants and plants grown in rooting media reinfested with natural populations may well be caused by a single or a few unknown organisms rather than a climax population.

The system consisting of an axenic plant and a single isolate of a saprophytic soil fungus, though admittedly artificial, can be used to illustrate the potential of a single fungus in influencing the physiology of a plant.

The release of organic compounds from plant roots is a major factor in the interrelationships between plant roots and soil microorganisms.
The condition of the root in relation to this phenomenon was not clearly defined. Histological observations in the present investigation, as well as others (75, 108), showed root tissues sloughed from intact, undisturbed, axenic roots. Measurements of total organic matter in the rooting medium showed considerable amounts of water soluble organic matter were present. Root colonization of axenic plants by _P. simplicissimum_ increased the amounts of organic compounds in the rooting medium. However, no attempt was made to differentiate between compounds coming from roots injured as a result of colonization and those arising from fungal metabolism. One can conclude from the data that "diffusible" and "nondiffusible" components (132, 133, 178, 182) of exudates may be different for axenic roots than for colonized roots and furthermore, the degree of colonization and kinds of colonizing microorganisms may also modify the exudation pattern.

It has been shown that the stage of plant growth is a major factor in the quality and quantity of exudation (175, 224). The present investigation showed decreasing amounts of organic compounds in the exudates at the flowering stage of plant development. This phenomenon could not be observed for root colonized plants. Therefore, the identification of the root exudation with the colonizing microorganism and with the stage of plant development can explain many of the conflicting observations about qualities and quantities of exudation. A survey of root exudates from axenic plants of several species, at different times throughout their life cycles should reveal common patterns of exudation which may reflect changes in the soluble pool of organic compounds in the plant. For example, in the present
investigation decreased amounts of organic compounds in the exudates at flowering were coincident with the possible creation of a metabolic sink in the tops, as a result of bud formation and flowering. At the same stage of plant development, exudates of axenic plants treated with extracts of axenic roots or with extracts of colonized roots showed a sharp decrease in content of organic compounds. This may indicate absorption of such compounds from the confined medium. An association between increased exudation of amino acids and increased levels of free amino acids in root tissues has been reported (26). The increase in exudation from colonized roots, in the present investigation, was associated with an increased carbohydrate content in the foliage. Analysis of root tissues for soluble carbohydrates and amino acids might have furnished more direct evidence than foliar analysis. The decrease in total organic matter of the exudates of root colonized plants 20 days after inoculation might be attributed to the immobilization of organic compounds by incorporation into fungal mycelium, or the re-absorption of those compounds by the plant.

Since qualitative changes in components of the exudates were not measured, the role of changing patterns of exudation on root colonization by penicillia and root disease etiology remain unresolved. In many instances, enhanced or depressed growth of higher plants has been attributed to metabolic products of saprophytic soil fungi (161, 162, 232). The presence of citrinin was not determined in the exudate of *P. citrinum*-colonized roots, but it was demonstrated that purified citrinin applied at 1.0 mg/l stimulated the growth of axenic marigolds. This citrinin concentration may be produced by the fungus on the
colonized root and hence stimulate plant growth. Since fungal isolates which produce citrinin on a glucose media were reported producing from 0.5 to 4.7 g/l citrinin (123, 243), concentrations higher than 10 mg/l citrinin were not tested. However, this compound has been reported to have herbicidal effects on broad leaf plants when applied at 40 to 50 mg/l. Because there was no effect of citrinin treatments at 10 mg/l, the concentration was probably above the stimulatory range but below the inhibitory range of concentration. The extracts of P. simplicissimum-colonized roots employed in the present investigation may have been highly concentrated because they depressed the growth of axenic plants. No attempt was made to purify any biologically active compounds or to test various dilutions of the extract.

The present investigation has demonstrated the potential of saprophytic soil fungi in affecting plant growth, increasing root exudation, and affecting the concentration of different organic compounds and nutrient elements in marigold tissues.
SUMMARY

The role of the saprophytic microfloral component of the rhizosphere in plant nutrition, root exudation and root disease etiology is not clearly understood. Under field conditions, many of the observed differences in plant economy in different soils are unexplained because of the undefined biotic conditions. Comparative studies with plants grown axenically versus plants inoculated with soil microorganisms, though artificial, provide information which can eventually be compiled and interpreted.

The influences of *Penicillium simplicissimum* (Oud.) Thom and *Penicillium citrinum* Thom on growth, chemical composition of dried leaves and root exudates of marigold (*Tagetes erecta* L.) were investigated under gnotobiotic conditions.

Axenic marigold seedlings were obtained from surface sterilized seeds and then transferred into a previously sterilized gnotobiotic isolator chamber. Plants were grown in open containers, specially designed to facilitate drainage, circulation of nutrient solution and sampling of root exudate under controlled conditions of 28° ± 1°C and 1100 foot candles. Quartz sand free of organic matter and one-half strength Hoagland and Arnon nutrient solution (plus iron chelate, 50 mg/l) served as the rooting medium.

Gnotobiotic combinations were established by introducing washed conidiospores at a rate of 7 x 10^6 to 8 x 10^6 conidiospores per plant of *P. simplicissimum* and 4 x 10^6 conidiospores per plant of *P. citrinum* into the rooting medium. Seven main experiments were made. In
experiments I and II axenic plants were compared with plants with \textit{P. simplicissimum}-colonized roots. In experiment III axenic plants were compared with plants with \textit{P. simplicissimum}-colonized roots and plants with \textit{P. citrinum}-colonized roots. In experiment IV axenic plants were treated with extracts from axenic roots and extracts from \textit{P. simplicissimum}-colonized roots. In experiment V axenic plants were treated with various concentrations of citrinin. In experiment VI, the roots of axenic plants were inoculated with \textit{P. simplicissimum} and sampled for histological study. In experiment VII axenic roots and \textit{P. simplicissimum}-colonized roots were used for cellulase, PG and PMG enzyme assays. Plants were harvested and rated 20 days after inoculation in experiment I, and 34 days after inoculation in experiments II and III. Root exudates were collected at the end of the experiments I and II by leaching the rooting medium with ten 100 ml volumes of one-half strength nutrient solution, and also were directly sampled during experiments III and IV at weekly intervals for 5 weeks after inoculation.

Axenic marigolds in experiment V were treated with 0.1, 1.0 and 10 ppm citrinin (antibiotic produced by \textit{P. citrinum}), 4 weekly applications, and in experiment IV axenic plants were treated with extracts of 80 g axenic and \textit{P. simplicissimum}-colonized roots, at the second and fourth weeks from the first application.

Inoculated roots in experiment VI were sequentially sampled (0 hr to 42 hr to 5 weeks from inoculation time), fixed, cleared, stained and then examined for root colonization. Extracts of axenic and colonized roots in experiment VII were assayed for macerating, cellulolytic and pectolytic enzyme activities.
Results indicated that the presence of each species of Penicillium influenced the growth and development of marigold. There was an increased dry weight, fresh weight and height of plants with \textit{P. simplicissimum}-colonized roots. In case of \textit{P. citrinum} inoculations, similar effects were observed with the exception of a decreased fresh weight. The enhanced plant growth and earlier flowering was accompanied with increased total water soluble carbohydrates and reducing sugars contents 20 and 34 days after inoculation. The percentage of P and K in the dried leaves of root-colonized plants were decreased 20 days after inoculation. However, N, P and K concentrations were not affected 34 days after inoculation. Calcium concentration was significantly increased at the latter stage. While treatment with low concentration of fungal metabolite (citrinin) stimulated plant growth, there was no effects at 10 mg/l citrinin concentration. Extract of \textit{P. simplicissimum}-colonized root repressed the growth of treated plants.

Root exudate analyses for total water soluble organic matter, total water soluble carbohydrates, reducing sugars, proteins and total amino acid equivalents revealed a dynamic change in exudation patterns which were correlated with plant development and fungal growth. The decrease in total organic matter and protein in the root exudates 20 days after inoculation was attributed to reabsorption of organic compounds by the plant and the immobilization of those compounds by mycelial growth. Results showed increased amounts of organic compounds in the exudates 34 days after inoculation. This was correlated with an increase in sugar content of the foliage of the inoculated plant, colonization of root tissues and fungal metabolism. Analyses of
weekly collected exudates from axenic and root colonized plants supported the results outlined above. There was a decrease in the amounts of organic compounds in the exudates of axenic roots at flowering. This phenomenon was not detected in the presence of \textit{P. simplicissimum} which indicated an increase in exudation.

Conidial germination of both fungi was mainly observed at the root hair region. There was no direct penetration of germ tubes nor fungal hyphae into intact root cells. However, both fungi showed extensive colonization of sloughed tissues, weakened root hairs and areas of damaged epidermal cells. These areas became infection centers for further colonization of epidermal and cortical tissues.

Enzyme assays revealed active maceration of potato discs within 18 hours of 25°C incubation at pH 5 and 4. No maceration was detected in the extracts of axenic roots or the boiled check. Polygalacturonase activity was detected at pH 4 using pectin NF and sodium polypectate. Cellulase activities were detected by using CMC 7HP and CMC 7LP at pH 5 in percent loss in viscosity and release of reducing end groups tests, respectively.

These investigations have demonstrated the potential of \textit{P. simplicissimum}, \textit{P. citrinum} and their metabolic products in altering the physiology of marigold under gnotobiotic conditions. It further illustrates the significance of saprophytic microflora in plant nutrition and in root disease etiology. Also, it has demonstrated the importance of stage of plant growth and presence of microorganisms in the measurement of root exudates.


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ABSTRACT

Axenic marigold (Tagetes erecta L.) were grown in quartz sand free of organic compounds and one-half strength Hoagland and Arnon nutrient solution plus chelated iron, in individual plant containers, designed for collecting root exudates, inside sterile gnotobiotic flexible plastic isolators. Plant roots were treated with washed conidiospores of Penicillium simplicissimum (Oud.) Thom (7 to 8 x 10^6 per plant) or Penicillium citrinum Thom (4 x 10^6 conidiaspores per plant), citrinin (0.1, 1.0 and 10 mg/l), and extracts of 80 g axenic or P. simplicissimum-colonized roots. Experiments were harvested 20 days (prior to flowering) and 34 days (flowering) after inoculation. Root exudates were collected at the end of experiments or also weekly obtained for 5 weeks. Inoculated plants were larger in size, contained more dry matter and flowered earlier than the axenic plants. Concentration of 1.0 mg/l citrinin stimulated plant growth and 10 mg/l citrinin had no effect. Extract of P. simplicissimum-colonized roots suppressed plant growth. Roots exhibited 40-60% degradation 34 days after inoculation. Fresh weight of P. citrinum-inoculated plants was lower than the non-inoculated. Total water soluble carbohydrates and reducing sugars were significantly higher in the foliage of root colonized plants 34 days after inoculation. Total organic matter and protein were decreased in root exudates 20 days after inoculation. These variations were attributed to reabsorption of organic compounds by the plants and immobilization of those compounds by the mycelial growth. Thirty-four days after inoculation there was increased amounts of organic compounds
in the exudates as root colonization progressed. Analyses of periodically obtained exudates indicated a decrease in exudates at flowering from axenic plants only. This phenomenon was not detected in the presence of P. simplicissimum. Percentages of P and K in dried leaves were decreased 20 days after inoculation. However, N, P, and K concentrations were not affected 34 days after inoculation. Calcium concentration was significantly increased at the latter stage. Germination of conidiospores occurred in the root hair region, but no direct penetration of intact living cells was observed. There was extensive mycelial growth from infection centers at weakened root hairs, damaged epidermal cells and sloughed tissues. Macerating enzyme, polygalacturonase, and cellulase were detected in extracts of P. simplicissimum-colonized roots.