AN ABSTRACT OF THE THESIS OF

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Title: A COMPOUND FROM ONION ROOTS INHIBITORY TO PYRENOCHAETA TERRESTRIS

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Abstract approved: _____ E. K. Vaughan

In vitro tests showed mycelial growth of Pyrenochaeta terrestris was inhibited by root and bulb extracts from the pink root resistant onion cultivar Nebuka, but not by extracts from the susceptible Southport White Globe. The active principle was shown to be fungistatic rather than fungicidal. It is present in both infected and non-infected roots indicating invasion by the pathogen is not required for its production.

Root and bulb extracts from Nebuka were passed through a Bio-Gel P10 column and 25 fractions, 10 ml each, were collected. Bioassay tests showed inhibitory activity was confined to two fractions. Active extract applied to Silica Gel-G plates and developed in a mixture of methyl ethyl ketone, pyridine, water and glacial acetic acid (70:15:15:2) indicated the inhibitory compound had an Rf value of 0.44, and reacted positively to ninhydrin, ammoniacal silver nitrate,

tetrazotized benzidine and diazotized sulphanilic acid suggesting an amino-phenolic complex. Quantitative estimation of total phenols in extracts from onion cultivars ranging from highly resistant to completely susceptible showed resistant cultivars contained more total phenols than susceptible ones.

The inhibitory effect of the compound was destroyed by heating or by exposure to extreme acid or base conditions.

Volatile oils liberated by extracts from Nebuka and Southport White Globe onions had little affect on mycelial growth of P. terrestris.

Attempts to demonstrate that a fungistatic compound is extruded from roots of Nebuka were unsuccessful.

A Compound from Onion Roots Inhibitory to *Pyrenochaeta terrestris*

by

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INTRODUCTION

Onions are worldwide in distribution and are an important food crop. They are grown commercially to some extent in almost every state in the United States and in nearly every home garden. Although Egypt, Turkey and Japan have greater acreage devoted to onion production, the United States is the world's largest producer because of higher yields per acre (32). The largest portion of the crop is grown in the northern states from New York to the west coast (74). In the United States, more than a million tons of onions are harvested annually from approximately 100,000 acres (46, 74).

In Oregon, approximately 145,300 tons of onions are produced annually with a farm value of about 5-1/2 million dollars, ranking the state sixth in the nation. Production is concentrated mainly in Malheur County with about 3,000 acres devoted primarily to Yellow Sweet Spanish onions grown on mineral soils, and in the Willamette Valley with approximately 2,000 acres of Yellow Danver onions grown on peat soils.

Almost all of the onions in both eastern and western Oregon are grown on land infested with the onion pink root organism Pyrenochaeta terrestris (Hansen) Gorenz, Walker and Larson. The disease is a

problem in both areas, but more severe losses occur in eastern Oregon. Fields in Malheur County planted to onions two or three years in succession have become so infested with the fungus that onion production is no longer profitable.

Long crop rotations with corn, potatoes and sugar beets have been used to minimize disease losses. Also in some areas, early plantings are made so that much of the growth takes place before the soil temperature becomes favorable for disease development (7).

P. terrestris is worldwide in distribution. Chupp and Sherf (7) noted that the disease has been found in Australia, Bermuda, Canada, Netherlands, New Zealand, South Africa and in all onion growing areas in the United States. Pink root is a problem wherever onions are grown and is minimized only in those areas where soil temperatures remain low throughout the growing season.

The most satisfactory means of control of this disease would be the use of immune or highly resistant commercially acceptable varieties. Thus far, none has been produced that is adapted to Oregon conditions. Breeding programs conducted on a nationwide scale are hampered by environmental conditions peculiar to each area and by market requirements. Varieties that are moderately resistant in one area often are susceptible when grown in another. Varieties grown in the same area for several years may be resistant in some seasons and susceptible in others.

One of the objectives of this study was to determine whether differences exist in chemical composition of living uninfected roots of various onion cultivars that would offer a possible explanation for resistance. A second objective was to isolate the fractions responsible for resistance, study their physical properties and attempt to characterize the compound or compounds involved.

REVIEW OF LITERATURE

Geographical Distribution

Taubenhaus and Johnson (62) in 1917 prepared a preliminary report on a disease of onions in Texas, which they named pink root, and in 1919 Taubenhaus (61) extended the description. In 1921, Taubenhaus and Mally (63) published a complete description of the disease and reported on methods of control. In 1921, Edgerton (14) reported that in Louisiana there were records of the pink root disease dating back to 1909.

After the disease had been described, reports concerning its occurrence were received from many other areas. Chaves and Erickson (6) reported the disease in Brazil in 1960. In 1959 and 1960, Klingner and Pontis-Videla (34) noted that the disease occurs extensively in Mendoza province of Argentina. According to Chupp and Sherf (7), pink root has been found in the Netherlands, Canada, New Zealand, Australia, Bermuda, South Africa and all onion growing areas in the United States.

Causal Organism

The causal organism was first described as Fusarium malli Taub. but Taubenhaus and Mally (63) obtained evidence in 1921 that a new species of Fusarium was the causal agent. In California, four new

species of Fusarium were isolated and named by Sideris (55) in 1924. He concluded that several species of Fusarium were involved in pink root infection and that Fusarium malli alone was not the causal organism.

Hansen (20) working with pink root in Texas and California was unable to incite the disease with Fusarium malli or with any of ten other isolates of Fusarium tested. He observed that when turgid and firm pink roots were cultured, the number of fungi was greatly reduced in contrast to the large number of fungi obtained from partially decayed roots. He also found that firm pink onion roots immersed in a 1:500 mercuric chloride solution for three minutes and then cultured yielded only one fungus which belonged to the genus Phoma and not Fusarium. Additional research by Hansen (21) showed that a Phoma sp. used as inoculum incited the disease and could be re-isolated from the onion roots. He then concluded that Fusarium sp. acted only as secondary invaders and in 1921, named the causal organism Phoma terrestris.

In 1948, careful morphological studies of the pink root organism by Gorenz, Larson and Walker (18), showed that pycnidia of fertile isolates always bore setae which are characteristic of Pyrenochaeta and not Phoma. On the basis of these findings, they changed the name to Pyrenochaeta terrestris (Hansen) Gorenz, Larson and Walker. According to Tims (65), Fusarium sp. may be involved in the root

rotting process associated with pink root disease even though Fusarium has been eliminated as the primary causal organism. Sideris (56) found that fusaria are commonly associated with onion roots infected with P. terrestris. Research conducted by Davis and Henderson (12) showed that Fusarium vasinfectium var. zonatum could attack onion roots only after invasion by the pink root fungus or another pathogen. Kehr, O'Brien and Davis (33) obtained evidence that F. oxysporium f. cepae could act as a primary pathogen of onion roots. Ten onion varieties tested by Lorbeer and Stone (45) showed differences in susceptibility to F. oxysporium f. cepae which incites basal rot, but all were equally susceptible to P. terrestris.

Hess (25) showed that onion stands were significantly reduced by Fusarium spp. and by P. terrestris. He found that both organisms were capable of causing onion root disease and that P. terrestris alone and mixtures of P. terrestris and Fusarium spp. produced pink root symptoms, while Fusarium spp. alone failed to produce typical symptoms.

Formation of Pycnidium

Caravajal (4) showed that P. terrestris formed pycnidia when grown on potato dextrose agar (PDA) under reduced light intensity at room temperature. Several workers (26, 27) have used near ultraviolet light to induce sporulation of the fungus.

Hansen (21) observed pycnidia formation of the pink root fungus on corn meal agar but few were formed on Czapek's, Pfeffer's and other synthetic media. Kulik (40) and Kulik and Tims (41) obtained some pycnidia on bean pod agar, but failed to observe pycnidia formation on corn meal agar. Of the 91 isolates tested, eight formed pycnidia after 57 days in culture, ten others developed pycnidia without spores or the presence of setae and all the remaining isolates were void of fruiting structures. An apparent correlation was noted between pathogenicity and formation of pycnidia or pycnidia-like bodies. Hansen (22) observed that mycelial transfers produced fewer pycnidia than did spore mass transfers. Gasiorkiewicz (16) found pycnidia on onion roots in the field and he and Wilhelm (76) were able to induce pycnidial formation on the surface of sterilized onion roots grown in sand for approximately one to three months.

Variability of Isolates

In 1929, Hansen (21) noted variation in isolates of P. terrestris. Further investigation by Hansen (23) revealed binucleate spores in P. terrestris were not necessarily genetically identical. In studies by Struckmeyer et al. (59), on the histology of the susceptible and resistant reactions of onions, no mention was made as to the number of nuclei per spore.

Gasiorkiewicz (16) used nitrogen mustard to induce isolate

variation. Some of the mutants originating from slightly pathogenic types produced symptoms equally as severe as those caused by naturally virulent isolates but most were less pathogenic. He found no evidence that naturally occurring variation among isolates was a result of binucleate spores. Microscopic examinations showed that spores as well as the vegetative mycelium and pycnidia were uninucleate.

Symptoms

P. terrestris is found commonly in soils where onions have been grown and may attack the roots of onion plants during any stage of growth. The disease is less evident in young seedlings when the temperatures are relatively cool, but as temperatures increase and the growing season advances, infection becomes more severe. The most obvious symptom of infection is pink color followed by necrosis of the roots. Infected plants usually continue to initiate new roots which are subsequently attacked and killed by the fungus.

Disease development and symptom expression are influenced by various factors. Nichols (47) found that concentration of inoculum, pathogenicity of the fungus isolate, moisture conditions and temperature all affected disease symptoms. Gorenz, Larson and Walker (19) found that resistance in some varieties depended on the quantity and virulence of the pathogen.

Kreutzer (36) showed that the color of infected onion roots was influenced by the hydrogen ion concentration of HCl and NaOH. He observed a color change from shades of red and purple at pH 8.5 to yellow brown at pH 4.5. Pigments extracted from the fungus were used in more sensitive tests and the color change took place between pH 7.0 and 7.86. Gorenz, Larson and Walker (19) found that growth rates were different, but all isolates grew at H-ion levels from pH 4 to pH 8.

Watson (75) developed a quick method for the identification of P. terrestris. Cultures of the fungus were transferred to petri dishes containing a low nutrient medium and wheat straw. The fungus produces a dark red pigment on wheat straw. Certain strains of Fusarium will also produce a red pigment in the agar medium but will not color wheat straw.

Hosts

In his original description of the pink root disease in 1918, Taubenhaus (61) noted that onions and their relatives such as chive, shallot, garlic and leek were susceptible. Kreutzer (37) found barley, cane sorghum, cantaloupe, carrot, cauliflower, corn, cucumber, egg plant, millet, pea, soybean, spinach, squash and wheat harbored the fungus; but some lines of the various plants tested were resistant to the pink root organism. Hansen (21) isolated the fungus from cowpea,

lima beans and potatoes. Thornberry and Anderson (64) observed tomatoes apparently infected with P. terrestris in soils which had been recently planted to onions. Wilhelm (76) found that the fungus was capable of attacking hairy nightshade and strawberry. Tims (66) added crabgrass, jungle rice and pigweed to the list. Hess (26) isolated the fungus from alfalfa, asparagus, beet, cabbage, chrysanthemum, cucumber, lettuce, oats, radish, sunflower, timothy, turnip and other hosts. According to Sprague (58), P. terrestris should be recognized as a minor but widespread parasite on roots of various grasses, cereals and other plants.

Mechanisms of Resistance

Struckmeyer et al. (59) observed differences in root penetration when resistant and susceptible onion varieties were grown in sand cultures. Root penetration in resistant varieties was limited to the epidermis and outer cortex; while hyphal penetration of susceptible varieties eventually decomposed the roots. Further studies of resistant varieties revealed the formation of pegs where the fungus failed to penetrate the cell wall. The pegs consisted of a hyphal tip enclosed by the cell wall of the host. Gorenz, Larson and Walker (19) found that an onion variety resistant to the pink root fungus might become susceptible by increasing pathogen concentration or by using a more virulent isolate.

Kreutzer (37) observed small irregular colonies of P. terrestris on the roots of Yellow Globe Danver onions grown in artificially

infested soil. Hyphae developing from fungal colonies proceeded in all directions once entrance was gained, growing throughout the cortex both inter- and intra-cellularly. He noted that the red pigment produced by the fungus was usually confined to the hyphae, but some diffusion from the hyphae into the invaded cell occurred. Cells not invaded by the fungus showed no color change. Further studies by Kreutzer (36) showed that healthy cells of the root-cap invaded by the fungus soon collapsed while plasmolysis and distortion of nuclei were found in cells penetrated by fewer hyphae. Cells adjacent to those attacked by the fungus showed slight plasmolysis even though no direct penetration occurred.

Siemer (57) showed that various onion varieties ranging from highly resistant to very susceptible expressed at least three levels of resistance when artificially inoculated with P. terrestris. He found that the fungus was incapable of root colonization or penetration of the highly resistant variety Nebuka. However, the fungus colonized and penetrated all root tissue except the xylem vessels of Yellow Sweet Spanish, a moderately susceptible variety; yet the plants continued to grow vigorously indicating tolerance to the pathogen. The roots of Southport White Globe (SWG), a highly susceptible cultivar, were completely ramified with mycelium usually resulting in death of the plant.

Phenolic compounds have been implicated in plant resistance to

many fungi. Several workers have presented reviews on the nature of the defense reaction and on the possible function of phenolic constituents in disease resistance (11, 15, 17, 24, 35, 38, 50, 53, 67, 73). Link, Angell and Walker (43), Link and Walker (44) and Walker (71) have shown resistance in pigmented onions to Colletotrichum circinans was due to protocatechuic acid and catechol. In this case, preformed phenolics protect the dead onion scales from the pathogen. No information was available on the possible role of phenolic compounds in onion roots until Siemer (57) showed a functional polyphenol oxidase enzyme system in a number of cultivars. However, differences in enzyme concentration between resistant and susceptible cultivars were insufficient to account for resistance.

GENERAL METHODS AND MATERIALS

Growth of Onion Plants

Approximately 50 seeds of each onion cultivar to be tested were planted in clean Del Monte EI-20 mesh quartz sand in 10 x 10 cm square plastic pots. At least four pots were planted to each cultivar to assure an abundance of plant material. A select few of the cultivars were planted in soil infested with P. terrestris and used to determine total phenolic content in infected onion roots. The seeds were planted 0.25 inches deep in each pot and watered with Hoagland's solution A and B (28), then covered with Kraft paper to retard evaporation and to promote even germination. After six days, the paper was removed and the plants watered every other day and Hoagland's solution was added once a week. Grow-Lux Fluorescent tubes were used to increase the day length to 14 hrs. Greenhouse temperatures varied between 24 and 32 C.

Extraction of Onion Juice

All root extracts were prepared in the following manner. Plants were removed from the pots and the roots washed thoroughly with tap water to remove excess soil or sand. A razor blade was used to sever the roots just below the basal plate taking care to not include any portion of the bulb. A mortar and pestle were used to grind the

root tissue along with a small amount of sand to aid in maceration. The pulp was then squeezed through cheesecloth and the juice collected in a 50 ml beaker. This process was continued until 35 ml of extract had been collected from 42 g of fresh onion roots. The extract was then centrifuged at 2000 rpm for 2 min. The supernatant was poured off and vacuum filtered through a Büchner funnel using 4.25 cm Whatman Glass paper. The extract was kept at 4 C and used within two days after extraction. Extraction of juice from onion bulbs was accomplished in a similar manner.

Column and Thin Layer Chromatography

Bio-Gel P-10, 50-150 mesh obtained from Bio-Rad Company was used to pack a 2 x 68 cm chromatographic column. The dried Bio-Gel P-10 was hydrated by slowly adding 10 g to 400 ml distilled water then stirred continuously with a magnetic stirrer for 12 hrs. The fully hydrated gel, together with the excess water was transferred to a vacuum flask, evacuated until bubbling ceased and then poured slowly into the column. After the gel had settled a few centimeters, the closed outlet at the bottom of the column was gradually opened to allow tighter packing of the column. The gel was poured continuously into the column resulting in a final bed level of 2 x 52 cm.

Further purification and separation of root and bulb extracts were accomplished by column chromatography. The extract (10 ml)

was collected from 10 g of fresh onion roots and carefully added to the top of the bed using a 10 ml pipette and gradually releasing the extract to slide down the side of the column. The sample was then drained down to bed level and washed carefully into the top of the gel bed with a small amount of distilled water. Approximately 5 ml of distilled water was added to the top of the bed then the column was attached to a water reservoir. The flow rate (0.5 ml/1 min) of materials being eluted out of the column was governed by a Beckman Model 746 Solution Metering Pump connected to the column. An Isco Fraction Collector was used to collect 25 or 11 fractions, 10 ml each, in 18 x 150 mm test tubes. Each fraction was stored immediately at 4 C to discourage microbial activity. Extract from most cultivars was passed through the column before being used in any further tests. After each use, at least 500 ml of distilled water was passed through the column to rid the gel of any residual extract, or the gel was removed, washed thoroughly, evacuated and then used to repack the column.

Thin layer chromatography on microscope slides was used for preliminary work in searching for a solvent system that would provide good movement and separation of onion extract. Silica Gel-G (35 g) was mixed with 100 ml of Chloroform-Methanol (2:1 v/v) and shaken vigorously for about 2 min. Two microscope slides 25 x 75 mm and 1 mm thick held back to back were dipped into the slurry then withdrawn slowly to

allow excess gel to drain off. The slides were then separated, dried for about 5 min and stored in a desiccator until used. This method of microscope slide preparation was similar to that described by Bobbitt et al. (3).

Thin layer chromatography (TLC) was used to aid in characterization of onion extract constituents. Silica Gel-G was prepared by mixing in a 500 ml flask for about 1-1/2 min 30 g of silica gel in 65 ml distilled water. Plates of two thicknesses were used (250 μ and 2 mm). The slurry was spread with a Desaga applicator on 20 x 20 cm uniformly thick glass plates, dried at 100 C and stored in a desiccator until used. In order to obtain an even advance of the solvent, 5 mm of silica gel was removed on both sides of the TLC plates parallel to migration direction and 5 mm removed from the end opposite to the one to be spotted. A plastic template was used to straddle the plate without coming in contact with the silica gel layer. The developing solvent used in these studies was allowed to ascend 134 mm above the spot before the plates were removed and air dried.

Bioassay Tests

Preliminary tests involved extract from the roots of Nebuka, a highly resistant cultivar of Allium fistulosum and SWG, a very susceptible cultivar of Allium cepa. Extract from each of the onion cultivars was centrifuged, then vacuum filtered through a Büchner funnel using

Whatman Glass paper and assayed immediately without passing the extracts through a chromatographic column.

A total of 35 ml was extracted from 42 g of fresh onion roots; the extract from each cultivar was filter sterilized by passing it through a 0.45 μ millipore filter. The pH of the extracts was not measured. Sterile pipettes were used to transfer the sterilized extract into 125 ml Erlenmeyer flasks containing 40 ml of potato dextrose broth (PDB). Five flasks were used and 1 ml of the onion root extract was pipetted into the first flask, 2 ml into the second, etc., until the last flask contained 5 ml extract. The control flask contained PDB plus 5 ml of sterile distilled water. Each concentration level from each cultivar was replicated twice.

Fungal plugs of P. terrestris were taken from the edge of seven day old cultures in areas free of pycnidia and used to inoculate all liquid cultures. The cultures were then incubated on a gyrotory shaker at 23 C.

Cultures were removed from the shaker after six days and vacuum filtered through a Büchner funnel using Whatman #1 filter paper that had been previously dried in an oven for 4 hrs at 75 C. The filter papers with the fungal mats were placed in uncovered petri dishes, dried at 80 C for 48 hrs and dry weights were determined.

Where column chromatography was involved in separation of root extract from Nebuka and SWG, a more refined technique was used

in bioassay tests. The extracts (10 mg/10 g fresh root tissue) were passed through the column and 25 fractions collected. The pH of each fraction was recorded. An aliquot from each fraction was filter sterilized through a 0.45 μ millipore filter into a 60 x 150 mm plastic petri dish. An equal volume of potato dextrose agar (PDA) was pipetted into each plastic petri dish and the agar mixed with the extract. The control consisted of 3 ml PDA plus 3 ml of distilled water. After the medium solidified, fungal plugs 4 mm in diameter were transferred to the center of each plate. The cultures were incubated at 20 C and growth measurements were made after six days. A similar procedure was used in preparation of liquid cultures by filter sterilizing 3 ml of extract into 3 ml PDB contained in 18 x 150 mm test tubes inoculated with a fungal plug. The control included 3 ml PDB plus 3 ml of distilled water and a fungal plug. The liquid cultures for this experiment and all subsequent experiments were incubated at 20 C in a slant rotary drum apparatus which made one revolution every 5 min. After six days' growth, the pH of each fraction was checked and dry weights taken by the method previously described. Bioassay tests were replicated at least two times.

A sample from each fraction collected was scanned on a Beckman Spectrophotometer in the ultraviolet (240-350 nm) and in the visible (350-720 nm) regions of the spectrum.

Preparation of Folin-Denis Reagent for Quantitative Tests

This reagent, used for quantitative estimation of total phenolics extracted from a wide range of onion cultivars, is comprised of 50 g of sodium tungstate, 10 g of phosphomolybidic acid and 25 ml of phosphoric acid mixed with 370 ml of distilled water contained in a 1000 ml flask (48). A hole was bored through a cork stopper and a 1.5 x 130 cm piece of glass tubing was driven through the cork and to about 7 cm below its base. This apparatus was then placed snugly into the mouth of the flask. The mixture was placed in a Thermoline Stir-Plate, brought to a boil then the temperature immediately reduced allowing the solution to boil slightly. Refluxing was continued for 2 hrs. The flask was then removed from the hot plate and allowed to cool for about 10-15 min before 130 ml of distilled water was added to the refluxed material.

Statistical Analysis

Results of all assay tests were analyzed by computer for analysis of variance. A one or two factorial analysis was computed for most experiments. The mean square error was used to determine the least significant difference (LSD) between any pair of points. One LSD (.05) for each test is presented in each table or figure.

RESULTS

Purification and Chromatographic Analysis of Onion Extract

Preliminary results using onion root extract from Nebuka and SWG without passing the material through a chromatographic column were encouraging. Cultures containing 1 and 2 ml SWG susceptible extract appeared to have a stimulatory effect on growth of P. terrestris. As concentrations increased, some inhibition was noted although fungal dry weight of cultures containing 5 ml of extract was somewhat greater than that of controls after six days' growth (Figure 1).

Some inhibition of P. terrestris was noted in cultures containing 1, 2 and 3 ml of Nebuka resistant extract when compared to controls, and inhibition was pronounced in flasks containing 4 ml of extract. Fungal growth was completely inhibited at the 5 ml concentration and dry weight consisted only of the fungal plug used initially to charge the culture (Figure 2).

Extract (10 ml/10 g of fresh onion roots) from Nebuka plants 75 days old was passed through a chromatographic column and the 25 fractions collected were assayed for the presence of an inhibitor to P. terrestris. Linear growth and growth in liquid medium was completely inhibited in tests involving fractions six and seven. Some

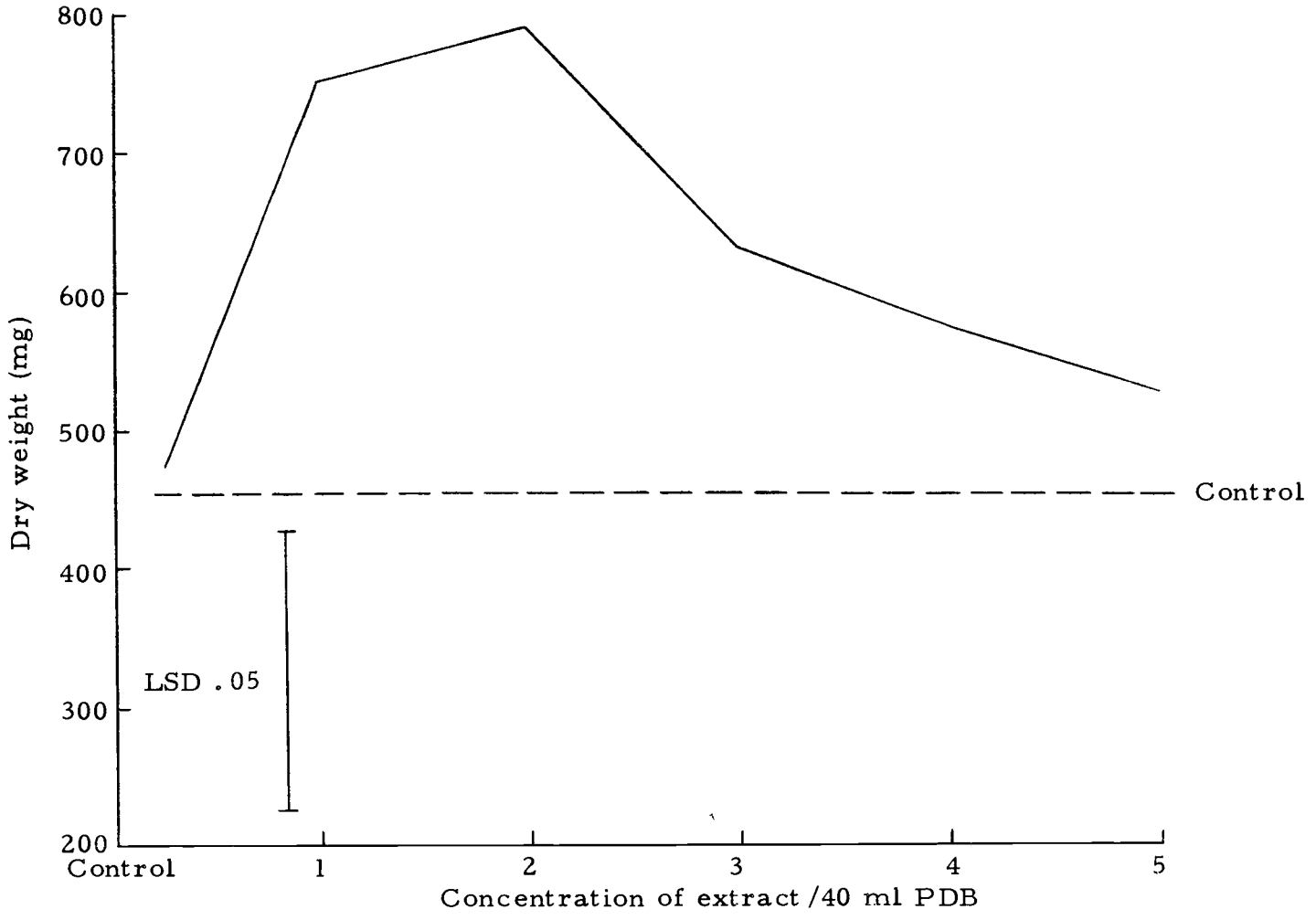

Figure 1. Effect of extract from roots of SWG onions on growth of P. terrestris in liquid medium after six days.

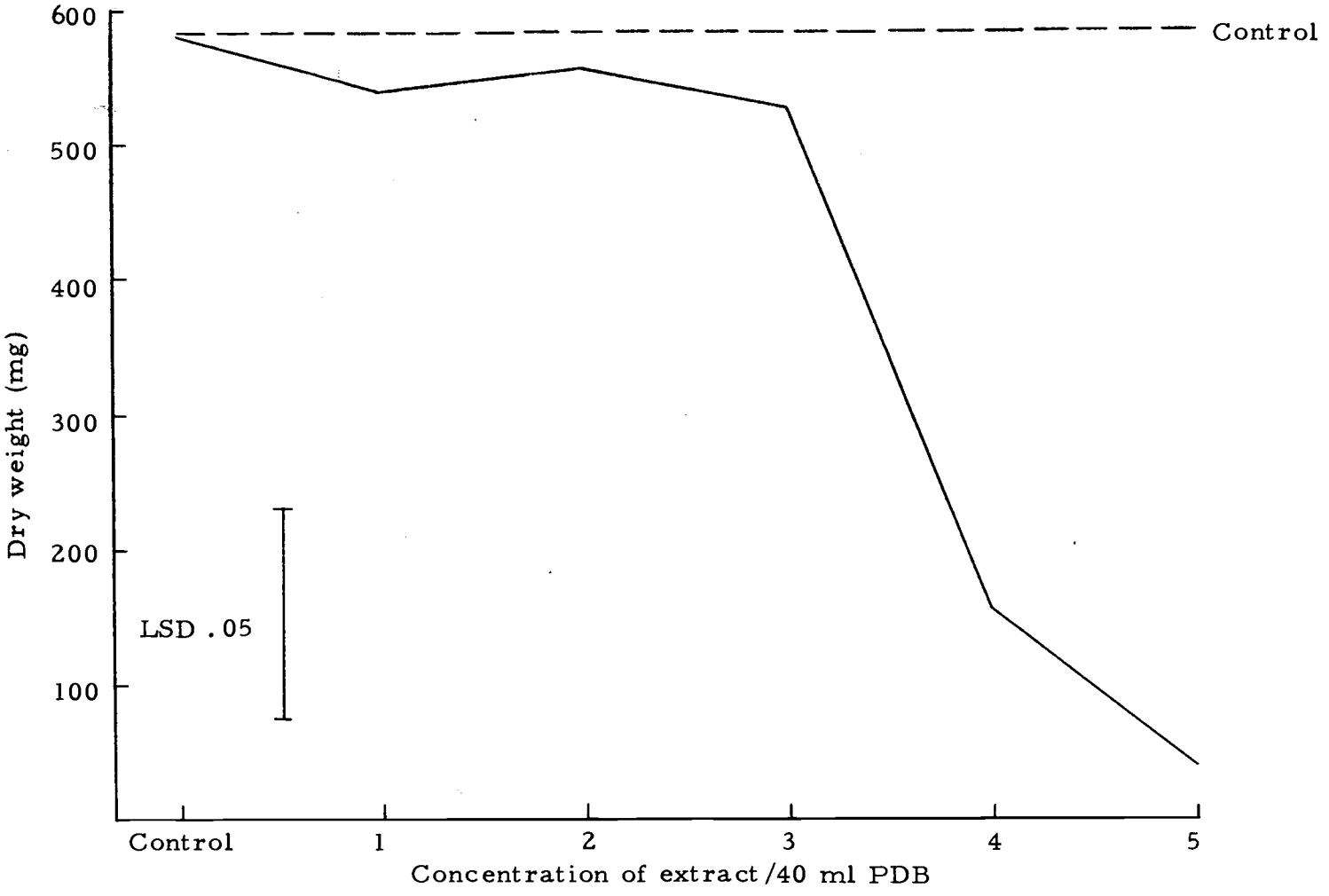

Figure 2. Effect of extract from roots of Nebuka onions on growth of P. terrestris in liquid medium after six days.

inhibition of linear growth was also associated with fraction five, while fungal growth in liquid cultures was slightly inhibited with fractions eight and nine. The other fractions did not inhibit fungal growth (Figures 3 and 4). Severe fungal inhibition of P. terrestris was associated also with fractions seven and eight collected from bulbs of Nebuka. Some inhibition was also noted in fractions six and nine (Figure 5). Fractions collected from SWG root extract (10 ml/ 10 g of fresh onion roots) and assayed showed some inhibition of linear growth in fractions five, six and seven. Some inhibition of fungal growth in liquid cultures was noted in fractions five, six, seven and eight as shown in Figures 6 and 7. Inhibition of P. terrestris by any fraction of the extract from SWG was less pronounced than that caused by extracts from Nebuka.

Samples from all fractions of extracts from SWG and Nebuka were monitored on a spectrophotometer but no specific absorption peaks were recorded.

As a means of further purification and separation of Nebuka extract, chromatographic analysis was undertaken. Before this technique could be used, a proper developing system had to be found which would provide good movement and separation of the extract. To aid in selection of a developing solvent, silica gel-coated microscope slides were used. The extract (10 ml) was collected from 10 g of fresh onion roots and passed through a chromatographic column.

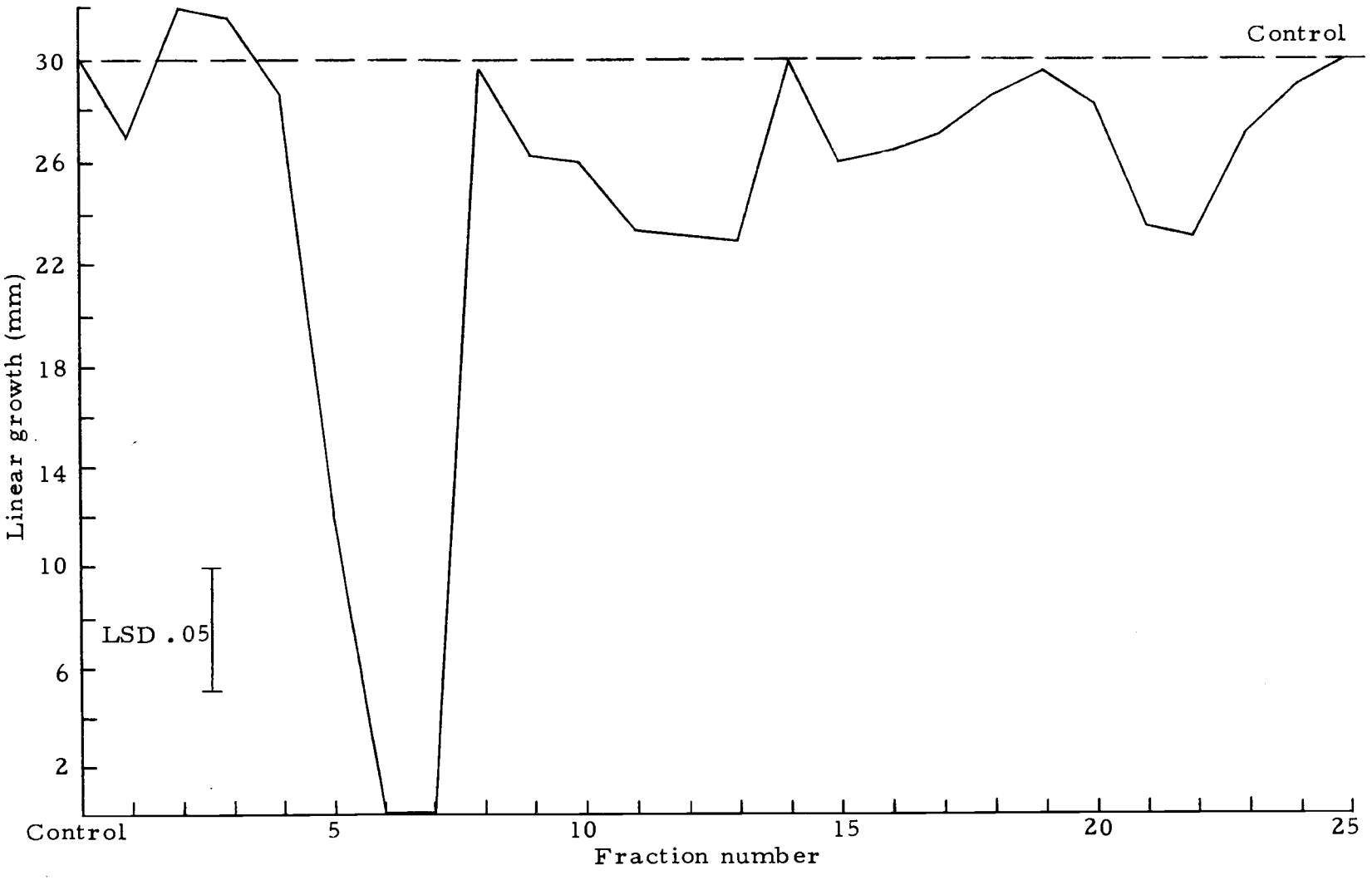

Figure 3. Effect of various fractions of an extract from roots of Nebuka onions on linear growth of P. terrestris after six days.

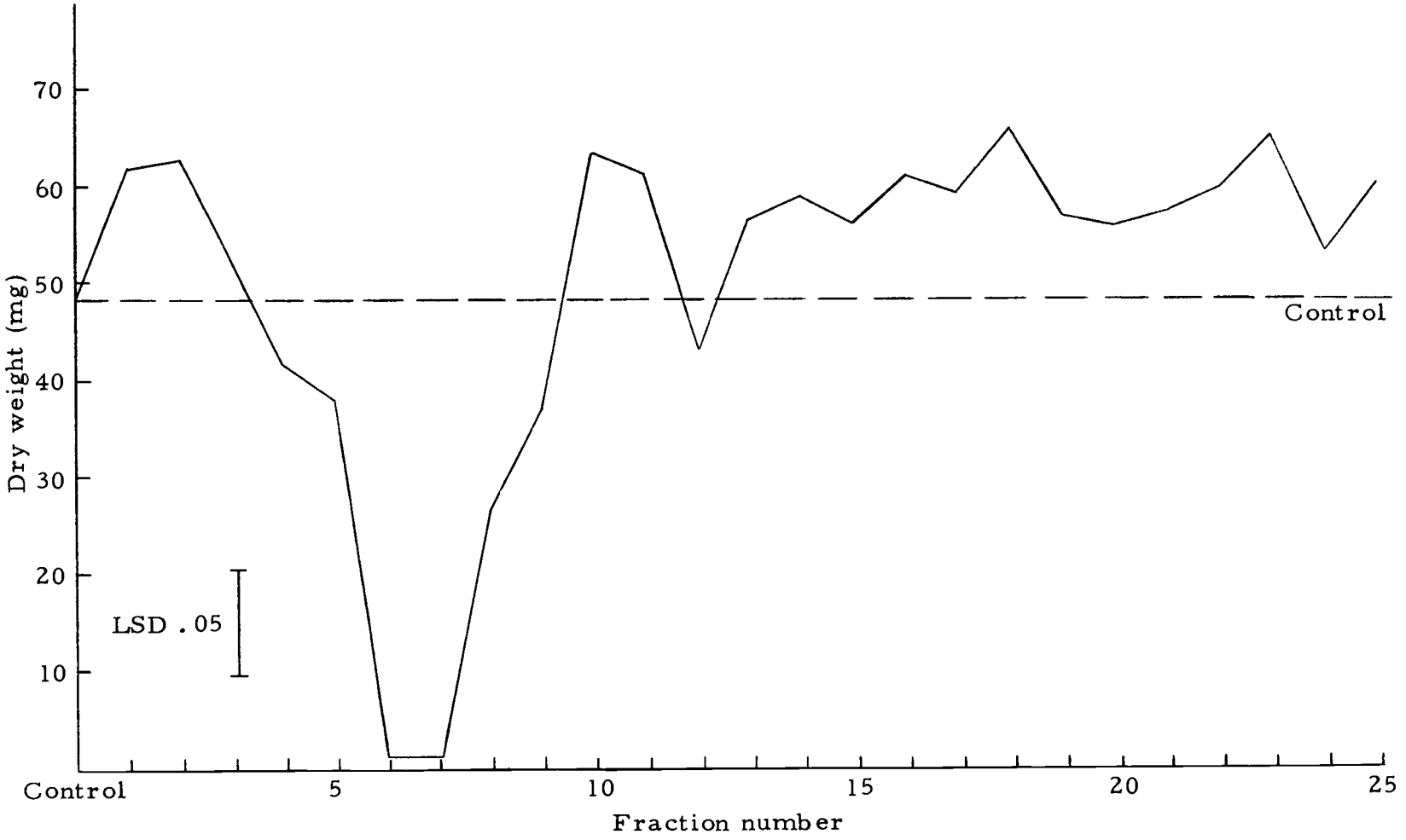

Figure 4. Effect of various fractions of an extract from roots of Nebuka onions on growth of P. terrestris in 3 ml liquid medium after six days.

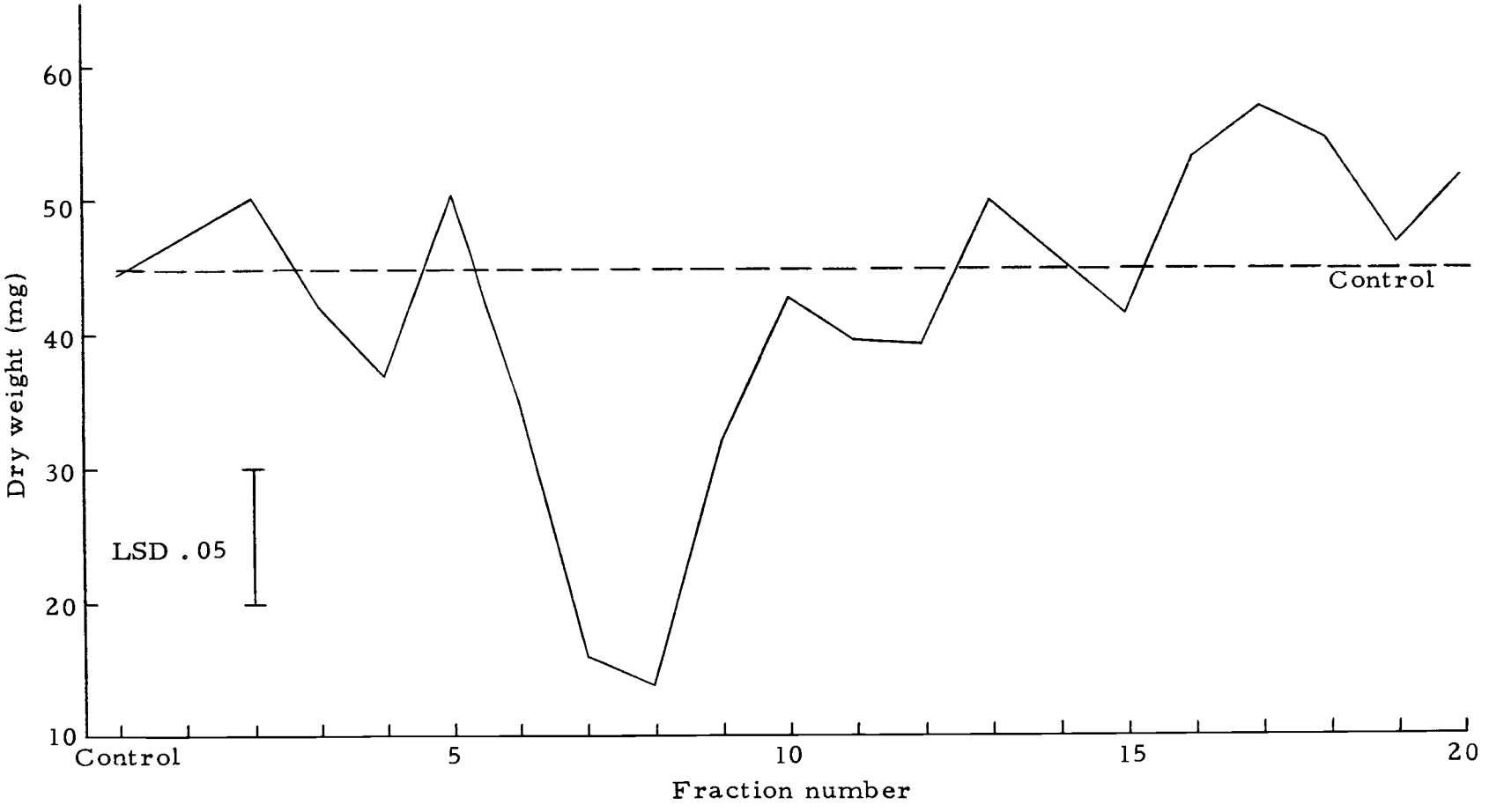

Figure 5. Effect of various fractions of an extract from bulbs of Nebuka onions on growth of P. terrestris in 3 ml liquid medium after six days.

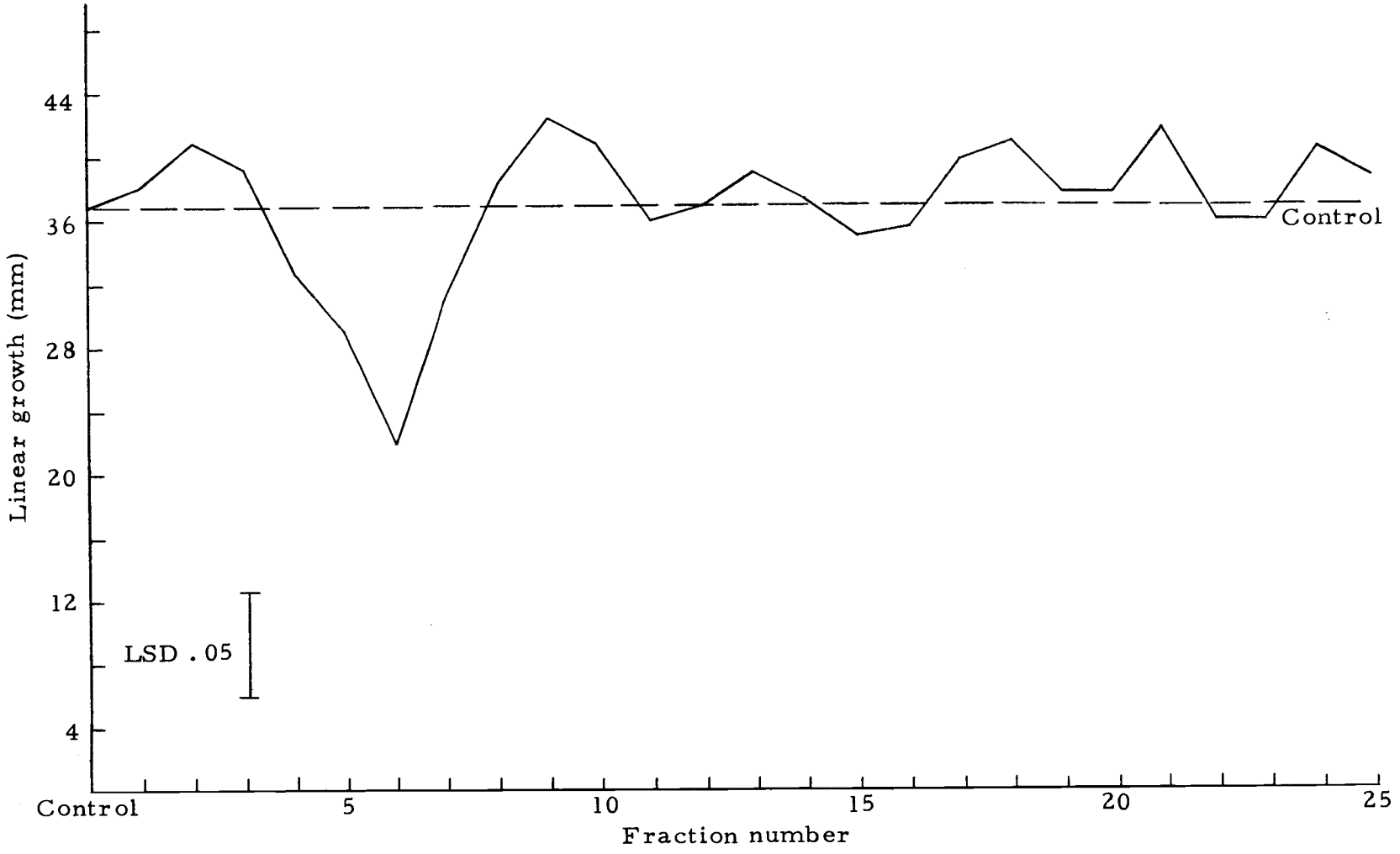

Figure 6. Effect of various fractions of an extract from roots of SWG onions on linear growth of P. terrestris after six days.

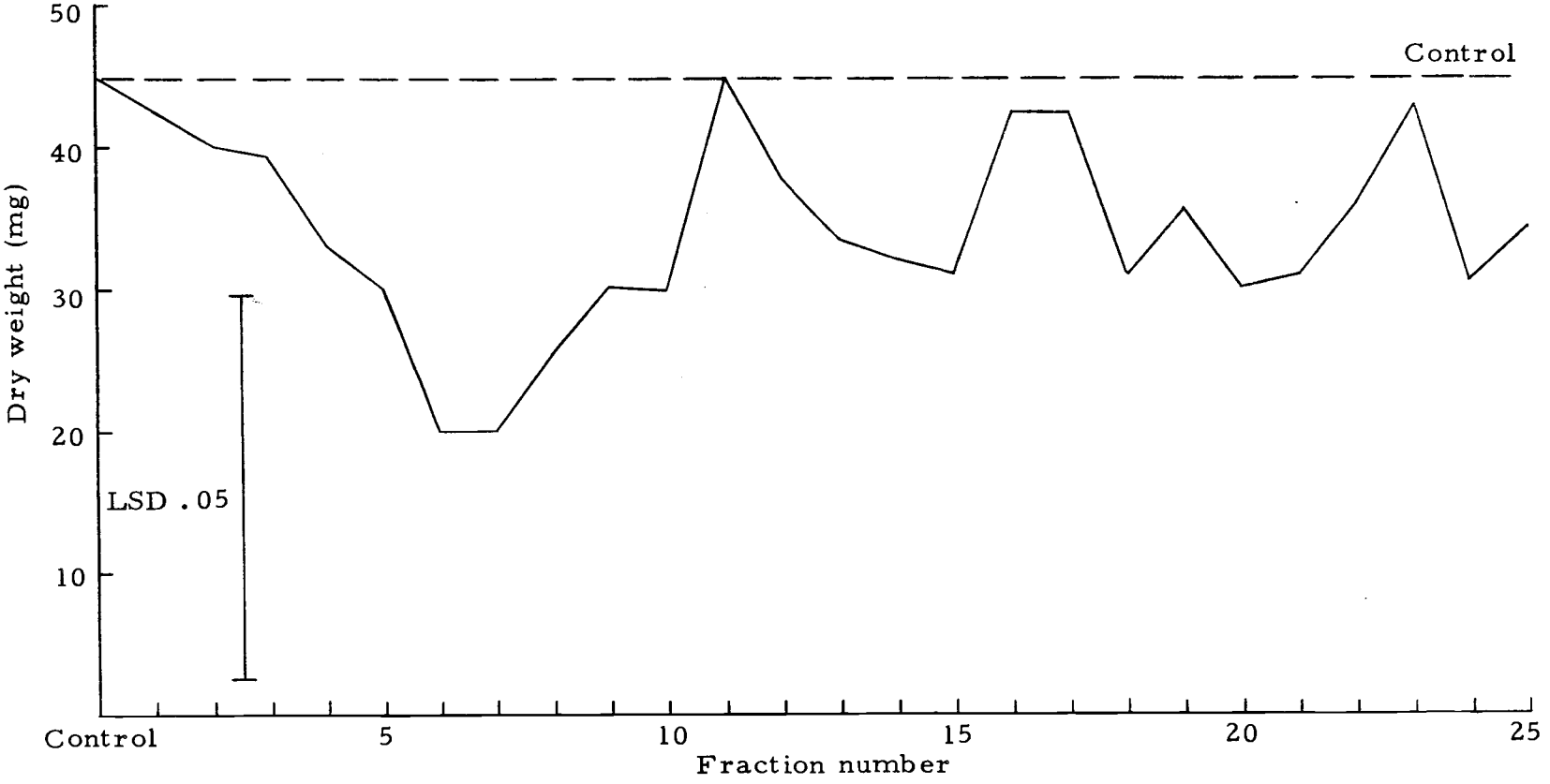

Figure 7. Effect of various fractions of an extract from roots of SWG onions on P. terrestris in 3 ml liquid medium after six days.

The slides were spotted with 50 µl of extract taken from an active fraction known to inhibit fungal growth. A hot air gun was used to facilitate drying and confine the spot to the smallest area possible. A series of different solvent systems were tested. The solvent was allowed to ascend 6 cm above the spot before the slides were removed and air dried. Detection of spot movement and separation was accomplished by exposing the chromatogram to iodine vapors and long wave ultraviolet light. Results from these tests showed that a developing solvent consisting of methyl ethyl ketone, pyridine, water and glacial acetic acid (70:15:15:2 v/v) was most effective in separation and movement of the extract.

Thin layer chromatography was used to further study the inhibitory substance. For these studies, extract (10 ml/10 g of fresh onion roots) taken from Nebuka plants three months old was used. An active fraction from Nebuka (3 ml) was streaked about 1 cm above the edge of a preparative TLC plate (Silica Gel-G 2 mm thick). The solution was applied with a 100 µl micro-pipette by slowly moving it along the edge of the plate maintaining as narrow a band as possible. The streaked plate was air dried and then placed in a glass developing tank containing the standard development solvent. The tank was lined with solvent saturated Whatman #1 paper to provide a saturated atmosphere. The solvent was allowed to ascend 134 mm above the streak before the plate was removed and air dried. Fluorescent

substances on the preparative TLC plate were observed by viewing under long and short wave ultraviolet lamps. A white fluorescent band was observed under the long wave ultraviolet lamp (352 nm) about 8 cm above the original streak.

Silica gel on the preparative TLC plate was divided into ten bands, each about 1.3 cm wide. Each was carefully scraped off the plate and placed in a 125 ml Erlenmeyer flask. A glass rod was used to pulverize the large pieces of gel. Forty ml of 95% ethanol was added to the flask and placed on a gyrotary shaker for 5 min, then removed and vacuum filtered through a Büchner funnel using Whatman #1 paper. The gels were washed two more times by the same procedure. The three filtrates of each TLC zone were combined and transferred to a 200 ml evaporation flask. The flask was then connected to a Buchler Flash-Evaporator Model PF-9GN. The unit was adjusted to allow a portion of the evaporating flask to be submerged in a water bath maintained at 40-45 C. The solutions were evaporated to dryness, then 10 ml of distilled water was added to the flask and shaken vigorously for a few minutes. An aliquot (3 ml) from each TLC zone was filter sterilized through a 0.45 μ millipore filter into 3 ml PDB and inoculated with a fungal plug. The control consisted of 3 ml PDB plus 3 ml of distilled water plus a fungal plug. Additional portions were used in checking pH and monitoring on the spectrophotometer. The same procedure was used on extracts taken from the

bulb portion of Nebuka plants, care being taken to exclude any root fragments.

Results of assay tests showed that growth of P. terrestris was inhibited with extracts from all bands associated with roots or bulbs of Nebuka but inhibition was more pronounced in extracts from bands five and six corresponding to the area of the plate which fluoresced when viewed with ultraviolet light (Figures 8 and 9). These tests were replicated twice with similar results.

Results of preparative TLC separations and subsequent bio-assays indicated the presence of a fungal inhibitory substance and initiated further investigation of the constituents involved. Six TLC plates (Silica Gel-G 250 μ thick) were spotted with an inhibitory fraction obtained from the column separation of 10 ml/10 g fresh onion roots of Nebuka and six were spotted with an inhibitory fraction from bulbs of the same cultivar. Five spots, 100 μl each, were applied to each plate. One plate was developed in the standard solvent system, air dried and then placed in a chromatographic tank containing iodine vapors. Several brown spots became visible almost immediately upon exposure to the iodine vapors. The locations of the spots on the plate were marked and used to designate spot location on all the other plates, thereby eliminating exposure to iodine vapors. A similar process was used for plates spotted with extracts from bulbs. Five separate spots with Rf values of 0.24, 0.44, 0.74, 0.79 and 0.89 for

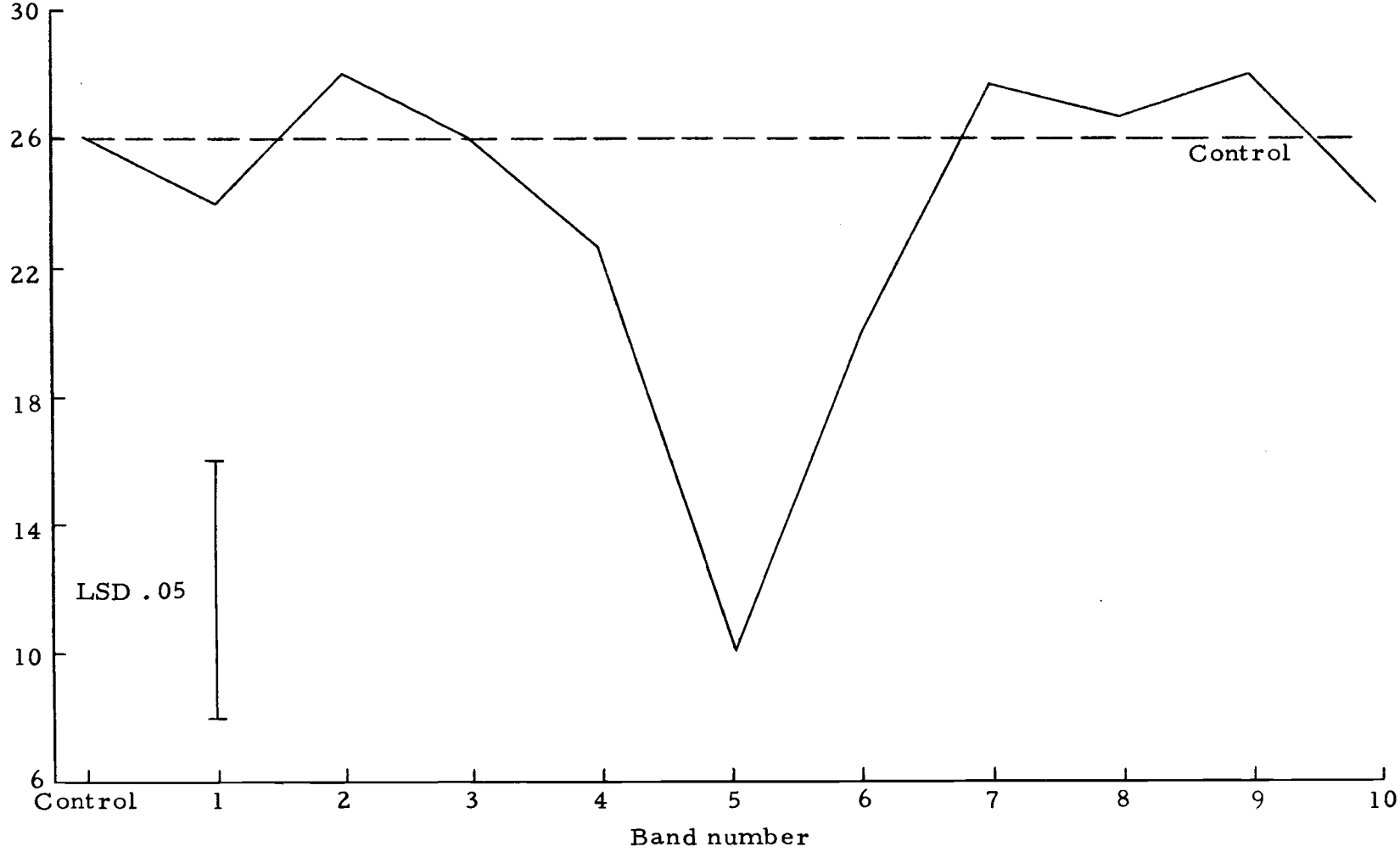

Figure 8. Effect of elutants from bands of a preparative TLC plate streaked with Nebuka root extract on growth of P. terrestris in 3 ml liquid medium after six days.

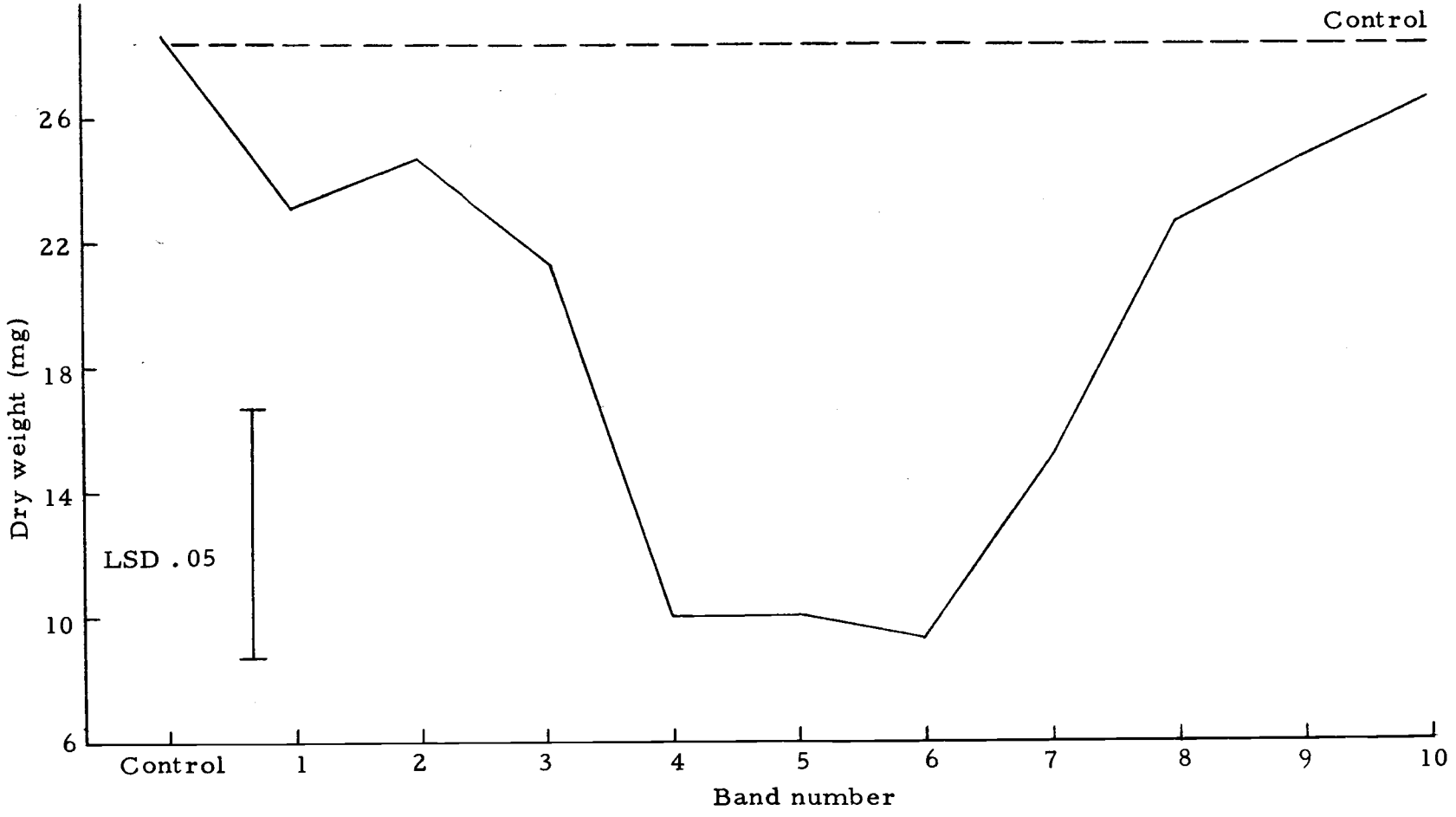

Figure 9. Effect of elutants from bands of a preparative TLC plate streaked with Nebuka bulb extract on growth of P. terrestris in 3 ml liquid medium after six days.

both root- and bulb-spotted plates were clearly defined by the iodine vapors (Figure 10). Silica gel on the plates was divided into four bands. Those spots with Rf 0.74 and 0.79 were combined and included in a single band. Corresponding spots from all six plates spotted with root extract, and six plates spotted with bulb extract with similar Rf values were scraped off and combined into a single flask. Material contained in the spots was eluted from silica gel bands in the same manner as that from preparative plates. A portion of the eluted material was used in bioassay tests to determine the effect on fungal growth in liquid medium. P. terrestris was severely inhibited by the elutant from a spot with an Rf value of 0.44. This was true for both root and bulb extract of Nebuka (Figure 11).

The possibility that inhibition is a pH phenomenon was investigated. The pH of silica gel bands from thin layer plates was checked after the bands were eluted with ethanol, flash evaporated and taken up in 10 ml of water, and was checked again at the end of the bioassay test. Table 1 shows pH values of bands from root and bulb extracts of Nebuka prior to use in assay tests and after the completion of the experiment. These results indicate that inhibition of P. terrestris cannot be attributed to pH. Portions of the eluted extract from roots and bulbs of Nebuka were monitored on the spectrophotometer but no unusual absorbance peaks were noted.

Several different chromatographic indicator sprays were used in

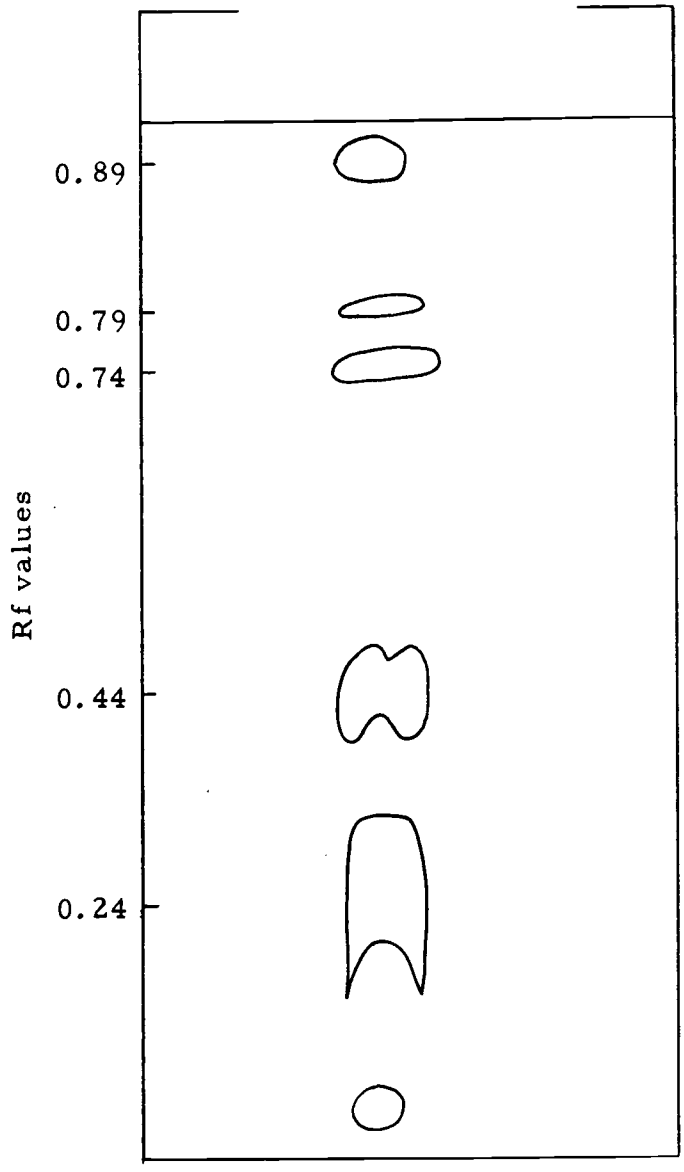

Figure 10. TLC distribution pattern obtained with extracts from roots and bulbs of Nebuka onions (methyl ethyl ketone:pyridine: glacial acetic acid:water (70:15:15:2)). Spots detected by iodine vapor.

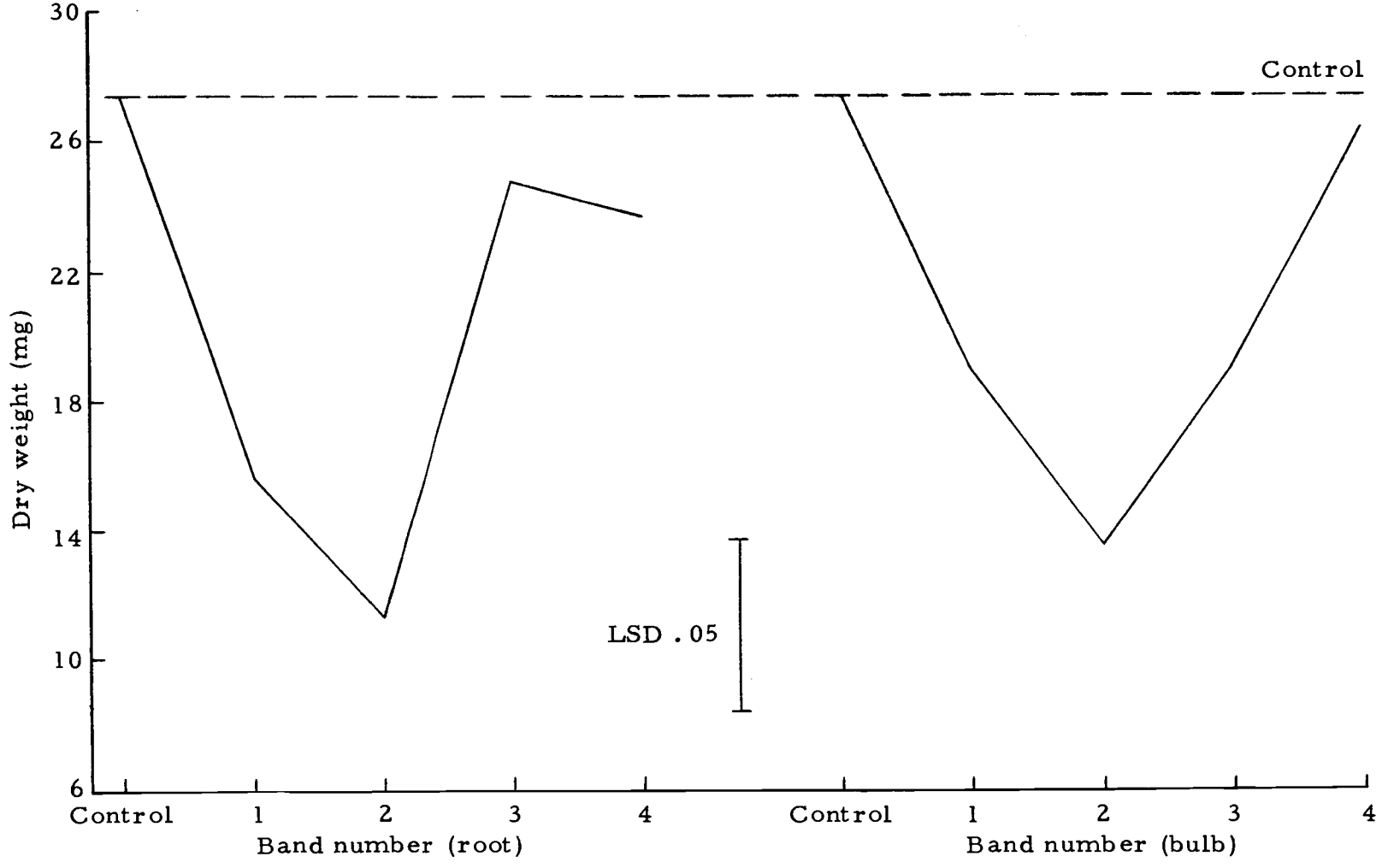

Figure 11. Effect of elutants from various spots contained in bands of TLC plates spotted with Nebuka root or bulb extract on growth of P. terrestris in liquid medium after six days.

Table 1. pH of eluted materials from various spots contained in bands from TLC plates spotted with an inhibitory fraction from root and bulb extract of Nebuka onions.

No. of bands removed from TLC plates	Rf of spots contained in band	pH of root extract prior to use in assay test	pH of liquid cultures[1] after 6 days' growth	pH of bulb extract prior to use in assay test	pH of liquid cultures[1] after 6 days' growth
1	0.24	4.7	5.1	4.7	5.6
2	0.44	5.3	5.3	4.8	5.9
3	0.74 0.79	6.0	5.4	5.0	5.7
4	0.89	5.7	5.3	5.2	6.1

[1]Original pH of liquid medium = 5.5

an attempt to identify the inhibitory substance. Thin layer chromatography plates were spotted with 200 µl of the active extract from the roots and bulbs of Nebuka-highly resistant, from roots of Yellow Sweet Spanish (YSS) Utah strain K x L selection - moderately susceptible, and from roots of SWG - susceptible. The TLC plates were developed in the standard solvent system and then air dried. The TLC plates were sprayed with ninhydrin to detect amino acids (70), bromocresol green for carboxylic acids (49), antimony trichloride for steroids (70), tetrazotized benzidine for phenolic compounds (49), dihydroxynaphthalene for sugars (70), and nitroferricynide for sulfur-containing compounds (70). The inhibitory spot Rf of 0.44 reacted positively only with ninhydrin and tetrazotized benzidine suggesting an amino phenolic complex. A compound having these TLC and chromogenic reactions was found in extracts from Nebuka, YSS and SWG cultivars. Since these preliminary tests suggested that the inhibitory substance may be phenolic in nature, sprays specific for several different phenols were used to treat another set of TLC plates spotted with 200 µl of extract from the same cultivars used previously. These sprays included: vanillin-toluene-p-sulphonic acid, ammoniacal silver nitrate, tetrazotized benzidine (52), diazotized sulphanilic acid (70), and ferric chloride-potassium ferricyanide (70). The spot with Rf of 0.44 reacted positively to ammoniacal silver nitrate as did the spot with Rf 0.89; a strong positive reaction was noted with

tetrazotized benzidine and a slight reaction with diazotized sulphanilic acid. Ferric chloride-potassium ferricyanide reacted with all the spots on the chromatogram.

Inhibition of P. terrestris was associated with extracts from the resistant cultivar Nebuka and with a spot with Rf of 0.44. Little inhibition was noted using similar extracts of a susceptible cultivar but the spot with the same Rf reacted positively to the same chromatographic sprays. These results indicated that the differences found in resistant and susceptible cultivars may be attributed to concentration of phenolic substances. Studies were undertaken to determine quantitatively total phenols in a number of healthy onion cultivars.

Seeds of various commercial onion lines were obtained from the University of Idaho Branch Experiment Station at Parma, Idaho and from Harris Seed Company. The seed was stored at 5 C until used. Table 2 shows the pedigree of all onion lines used in this study.

When plants of all onion lines to be tested were 100 days old, the roots were severed below the basal plate, thoroughly washed and patted dry. Tissue from each of the cultivars (10 g of fresh weight) was transferred to separate 20 x 200 mm test tubes, covered with 95% ethanol and kept at -15 C until used.

The methods involved in root extraction and purification were the same as those used in previous tests. Bioassay tests included mixing the extract from the various cultivars with PDA in 60 x 150

Table 2. Genetic identification of onion cultivars studied showing pedigree if known, and pink root susceptibility.

Pedigree	Pink root susceptibility[1]
Nebuka	VR
PUSS 371 x P54-306 prrT	VR
P52-371 x P54-306 prrTo	R
El Capitan Reciprocal	SS
Yellow Sweet Spanish (YSS) Utah strain K x L Selection Langer Seed Co.	MS
6334 x P53-350 prrO(2)	VS
Southport White Globe (SWG)	VS

[1] VR = very resistant; R = resistant; SS = slightly susceptible; MS = moderately susceptible; VS = very susceptible.

mm petri dishes and placing a fungal plug 4 mm in diameter of P. terrestris in the center of the solidified agar. Linear growth measurements were taken at the same time each day for six days to determine possible differences between cultivars and fungal inhibition.

The extract (10 ml/10 g of fresh onion roots) was passed through a chromatographic column and 12 fractions collected using only fractions 5 through 11 in bioassay tests. A portion of each fraction from all of the cultivars was used to determine quantitative estimation of total phenols.

Quantitative Estimation of Total Phenols

Gallic acid was used in preparation of a standard curve because of its molecular stability and purity. Standard solutions were prepared containing 10, 20, 30, 40, 50 and 60 μg gallic acid per 1 ml. The reaction mixture contained 1 ml standard gallic acid solution, 7.5 ml water and 0.5 ml Folin-Denis reagent. This mixture was thoroughly shaken then allowed to stand for 3 min after which time 1 ml of 20% sodium carbonate was added. The sample was again thoroughly mixed and left at room temperature for 45 min. Absorbance was determined at 725 nm (60). The mean of two replicates for a standard curve of gallic acid is shown in Figure 12.

The same procedure was used in determining total phenols in the various onion lines by replacing gallic acid with portions of

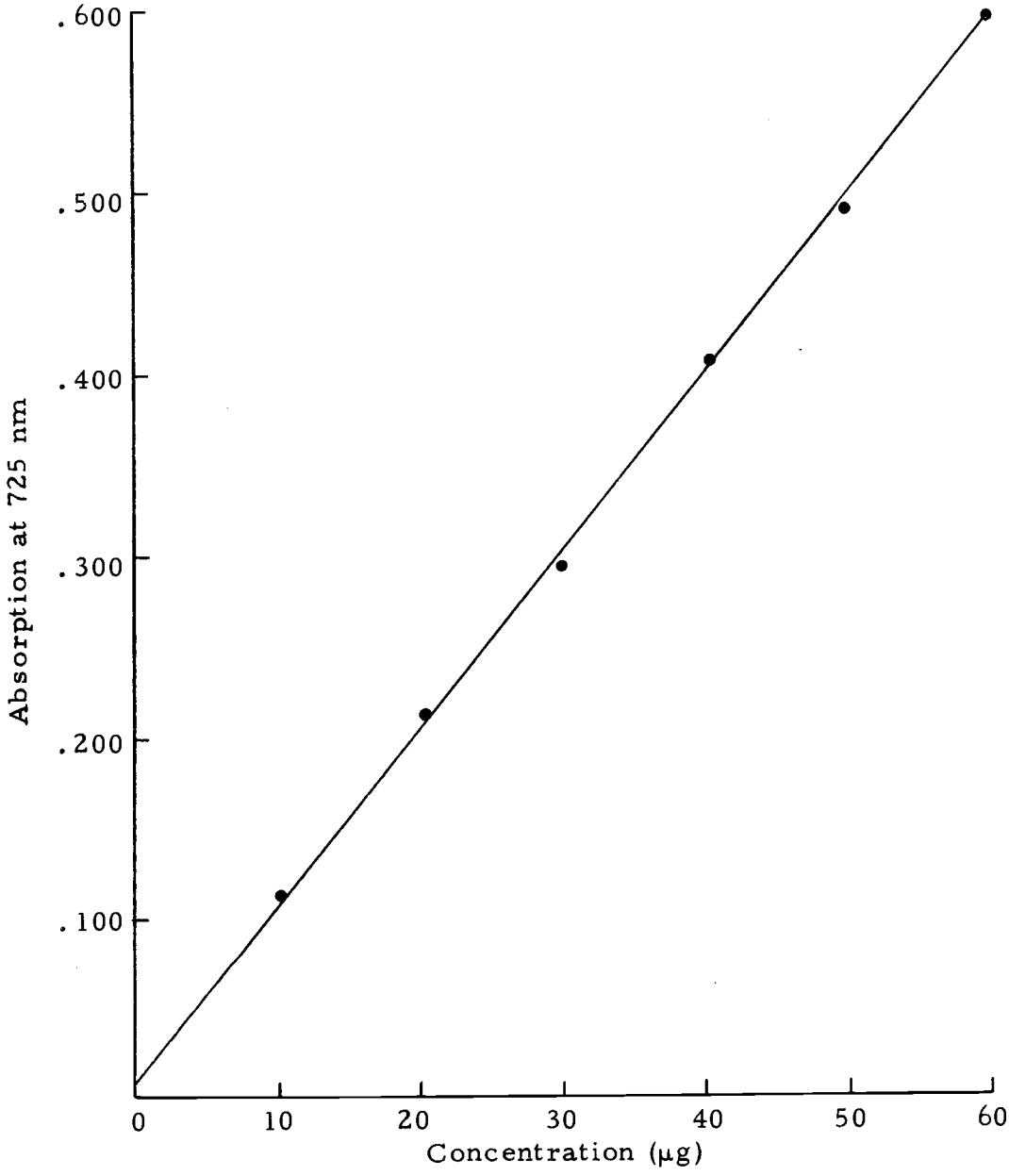

Figure 12. Standard curve for the phenolic acid, gallic acid.

extract from the various fractions collected and reading absorption at 725 nm. Quantitative determinations were made on column fractions 6 through 11 for each of the cultivars studied.

Total phenols extracted from Nebuka equalled 252 μg/g, fresh tissue compared to 187 μg from the susceptible cultivar 6334 x P53-350 prr O (2). The greater portion of phenolic compounds was contained in fractions eight and nine with minor amounts in fractions 6, 7, 10 and 11. Linear growth of P. terrestris was inhibited by fractions eight and nine from all seven of the cultivars tested. Little difference in total phenols was detected between four cultivars that varied in degrees of susceptibility (Table 3). Repeated determinations showed only minimal differences in total phenols in healthy and severely diseased roots of the moderately susceptible cultivar YSS.

Fractions eight and nine of the extract from all cultivars tested consistently resulted in more inhibition of P. terrestris than fractions 6, 7, 10 or 11. Fraction eight of the extract from Nebuka inhibited fungal growth to 1.3 mm/24 hrs while fraction eight of the extract SWG allowed the fungus to grow 5.5 mm/24 hrs compared to a control growth of 7.9 mm/24 hrs. Only slight differences in linear growth of the fungus were detected in extracts from cultivars that varied only slightly in degrees of susceptibility. Table 4 shows the effect of extracts from the onion cultivars tested on linear growth of P. terrestris.

Table 3. Quantitative estimation of total phenols in onion cultivars as determined by Folin-Denis method.

Pedigree	Susceptibility to *P. terrestris*[2]	µg Phenolic compounds[1] in fractions 8 & 9 (1 g fresh wt. tissue)	µg Total phenols[1] (1 g fresh wt. tissue)
Nebuka	VR	141.4	253.0 e
PUSS x P54-306 prrT	R	135.0 e	247.2 e
P52-371 x P54-306 prrTo	R	125.4 d	235.0 d
El Capitan Reciprocal	SS	107.6 bc	195.5 bc
Yellow Sweet Spanish (YSS) Utah strain K x L selection Langer Seed Co.	MS	107.0 b	196.5 bc
6334 x P53-350 prr o (2)	VS	109.0 bc	186.4 a
Southport White Globe (SWG)	VS	102.2 a	195.4 bc
LSD[3].05		3.2	6.2

[1] As gallic acid.

[2] VR = very resistant; R = resistant; SS = slightly susceptible; MS = moderately susceptible; VS = very susceptible.

[3] Figures followed by the same letter are not significantly different at the 5% level.

Table 4. Effect of onion root extract from various cultivars on linear growth of P. terrestris.

Variety	Susceptibility to P. terrestris[1]	Growth (mm/24 hr) Fraction number							Mean values
		6	7	8	9	10	11	control	
Nebuka	VR	7.2	3.4	1.3	3.9	7.3	7.0	7.4	5.35
PUSS x P54-306 prrT	R	8.5	4.5	2.0	3.0	8.6	9.0	9.5	6.30
P52-371 x P54-306 prrTo	R	7.3	7.5	3.5	3.0	8.3	8.4	9.0	6.70
El Capitan Reciprocal	SS	6.9	6.1	4.4	6.9	7.2	7.4	6.7	6.51
Yellow Sweet Spanish	MS	6.3	6.0	5.0	6.0	7.4	7.3	7.0	6.28
6334 x P53-350 prr o (2)	VS	7.6	6.2	5.2	7.0	7.1	7.2	7.0	6.75
Southport White Globe	VS	7.0	6.3	5.5	7.2	7.6	7.4	8.4	6.98
Mean values		7.25	5.71	3.98	5.28	7.64	7.67	7.78	

[1] VR = very resistant; R = resistant; SS = slightly susceptible; MS = moderately susceptible; VS = very susceptible.

Effect of Autoclaving on the Active Compound

The extract (2 ml) from fraction eight from Nebuka was filter sterilized and added to a test tube containing 2 ml PDB. A similar prep was sterilized by autoclaving for 15 min at 120 C and 15 psi. The control contained 2 ml PDB and 2 ml distilled water. A fungal plug 4 mm in diameter of P. terrestris was transferred to each tube and the liquid cultures were incubated at 20 C in a slant rotary drum apparatus which made one revolution every 5 min. Dry weights taken after six days' growth indicated extreme inhibition of fungal growth by the filter sterilized sample. In contrast, fungal growth on the autoclaved extract medium was nearly as great as on the control medium, indicating that most of the inhibitory substance was destroyed or modified by heating (Figure 13).

Effect of pH on the Active Compound

Inhibitory fractions (2 ml) of a root extract from Nebuka were placed in 10 ml beakers. The pH of the extract was 5.9. The pH of the extract in one beaker was raised to 12.6 with 1N KOH solution; 1N HCl was used to lower the pH of the extract in the other beaker to 1.6. Each aliquot was kept at room temperature for 5 hrs, then readjusted to pH 5.9, filter sterilized into 2 ml PDB, inoculated with a fungal plug 4 mm in diameter and then incubated at 20 C for six days.

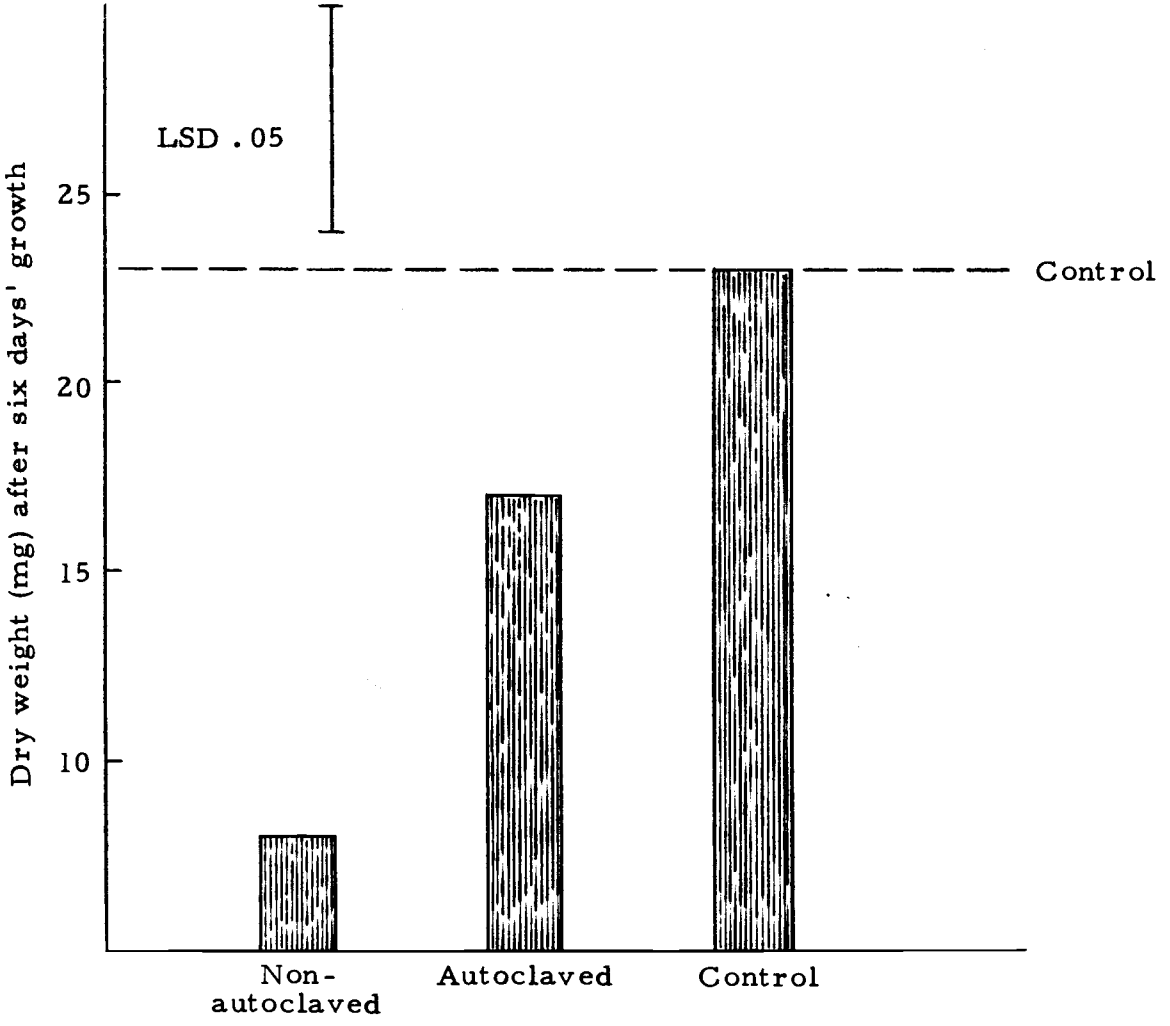

Figure 13. Effect of autoclaving on the ability of the active fraction of a root extract from Nebuka to inhibit growth of P. terrestris in liquid medium.

The control consisted of 2 ml PDB and 2 ml distilled water inoculated with the fungus. The cultures were kept in a rotary slant drum apparatus which made one revolution every 5 min. The inhibitory substance was altered by both the acid and base treatments to such an extent that only slight fungal inhibition was noted (Figure 14).

Effect of Dilution on the Active Compound

Root extract from Nebuka was diluted 1:3, 1:10, 1:30 and 1:100 by filter sterilizing into 3 ml PDB contained in test tubes and inoculated with a fungal plug 4 mm in diameter. The control consisted of 3 ml of water plus 3 ml PDB. The cultures were incubated at 20 C in a slant rotary drum apparatus which made one revolution every 5 min. Dry weights were taken after six days' growth. Each dilution was replicated three times. Complete fungal inhibition occurred at 1:3 dilution and slight inhibition at 1:10. At dilutions of 1:30 and 1:100, dry weights of the fungus were slightly greater than in the PDB controls (Figure 15).

Attempts to Demonstrate Presence of Fungistatic Substance in Root Exudates

Siemer (57) noted that on the surface of the roots of a susceptible plant, P. terrestris colonizes, forms a mycelial mat, then

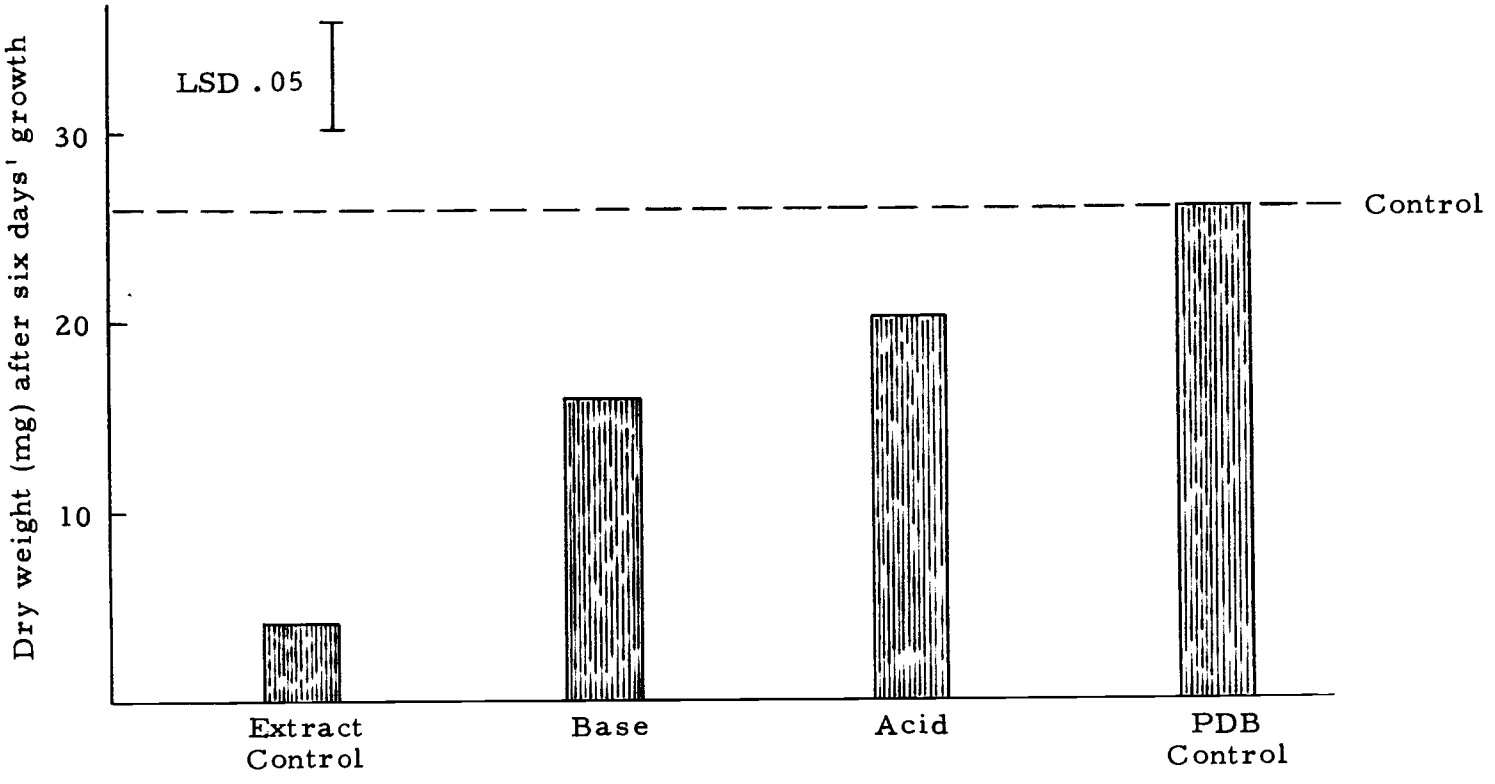

Figure 14. Effect of pH on ability of the compound from roots of Nebuka to influence growth of P. terrestris in liquid medium.

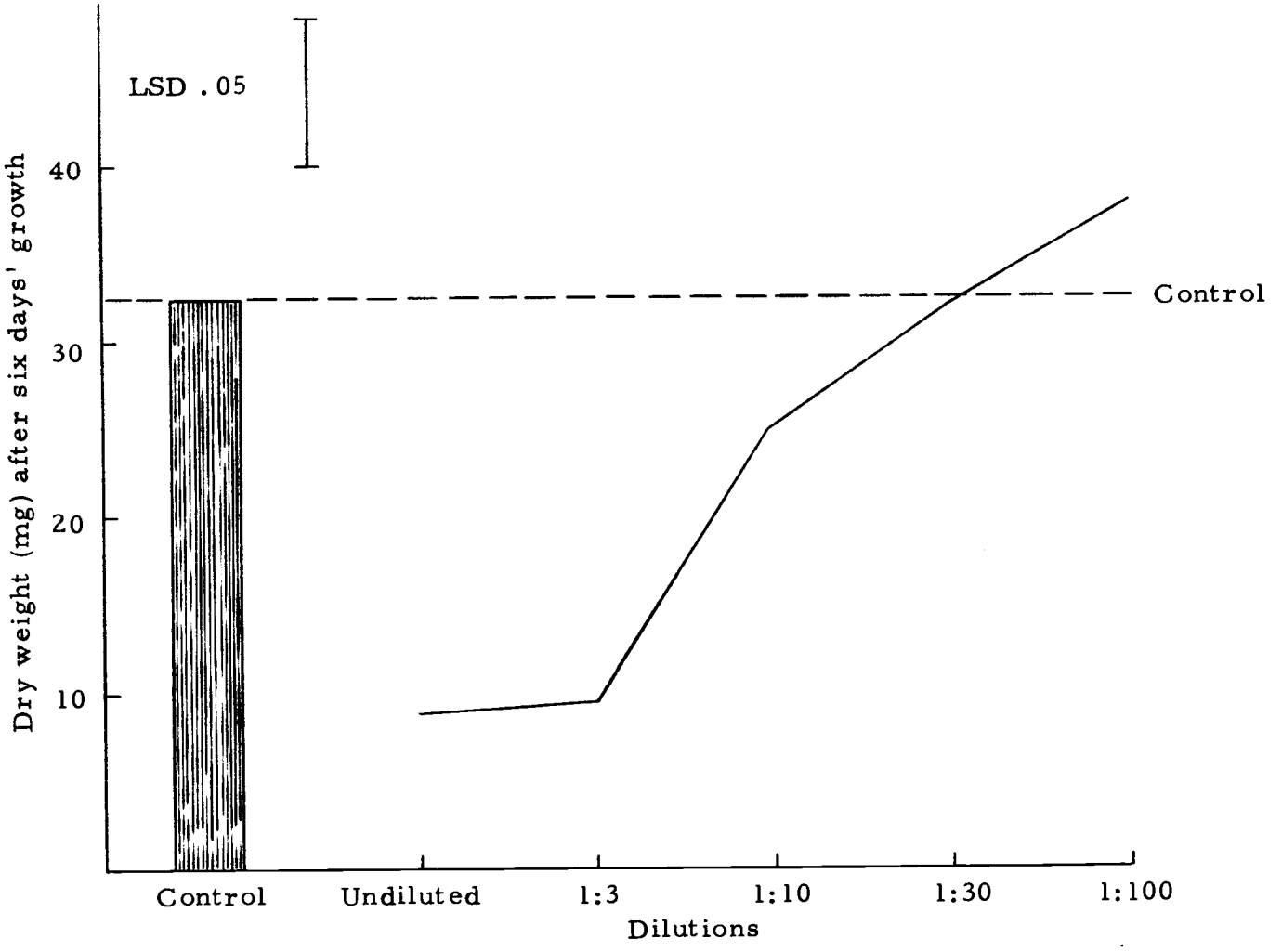

Figure 15. Effect of dilution on ability of a root extract from Nebuka to inhibit growth of P. terrestris in liquid medium.

penetrates. However, on Nebuka roots, it does not colonize, form a mycelial mat or penetrate. Struckmeyer et al. (59) also observed the same type of phenomena. This work was undertaken to try and determine whether failure to colonize was caused by fungistatic substances exuded from the roots. Two Nebuka plants 60 days old were carefully removed from soil infested with P. terrestris. The excess soil around the plants was removed with a small spatula leaving only that soil immediately surrounding the roots. Care was taken to reduce or eliminate root injury. The plants were then transferred to a glass bottle and the roots suspended in Hoagland's solution (28). Cotton was stuffed around the plant in the neck of the bottle to hold the plants in position. The system was kept at room temperature for three days. The plants were removed and the roots washed with 20 ml distilled water. This solution was vacuum filtered through Whatman #1 paper and 3 ml of the filtrate was passed through a 0.45 μ millipore filter and collected in a test tube containing 3 ml of PDB plus a fungal plug 4 mm in diameter. The cultures were incubated at 20 C in a slant rotary drum apparatus which made one revolution every 5 min. The control consisted of 3 ml of PDB and 3 ml of water plus a fungal plug. Dry weights were taken at the end of six days' growth. The test was replicated four times. No fungal inhibition was found. The tests were continued using a different procedure. Excess soil from Nebuka plants was removed and only that soil adhering to the root

system was thoroughly washed with 150 ml distilled water. The liquid was flash evaporated to dryness and taken up in 10 ml water and assayed for activity. Fungal inhibition was not demonstrated.

Another attempt was made to obtain evidence of a fungistatic substance from germinating Nebuka seed. Moistened filter paper was placed on the bottom of three standard size petri dishes. Approximately 100-150 seeds per plate were placed on the paper and covered with another piece of moistened paper, then enclosed with the top half of the dish. The papers were wetted periodically and kept at room temperature.

After five days, the germinating seeds from each incubation dish were combined and placed in a 250 ml flask containing 100 ml water. The flask was transferred to a gyrotary shaker for 5 min, removed and the water vacuum filtered. The filter papers used to cover the seed were washed with 60 ml of 95% ethanol and mixed with the water obtained from the washed germinating seed. The filtrate was flash evaporated to dryness and taken up in 10 ml water. Results from bioassay tests failed to show any evidence of inhibition of P. terrestris in liquid medium.

Effect of Volatile Oils from Onion Roots on Growth of P. terrestris

Walker (72) noted that volatile oils liberated from macerated

fresh onion tissue were fungicidal to Collectotrichium circinans spores, but fungistatic to mycelial growth. The procedure used to study the effects of volatile oils from onion roots on P. terrestris mycelial growth was similar to that used by Walker.

Mycelial plugs of P. terrestris 4 mm in diameter were placed in the center of 12 petri dishes containing PDA. After three days, five of the petri dishes were inverted and 0.5, 1.5, 2.5, and 5 ml (5 ml equivalent to 5 g of fresh onion root tissue) of the inhibitory fraction from the roots of Nebuka was placed in the inverted lid of each plate. Similar concentrations of raw extract from roots of Nebuka were placed in inverted lids of the remaining five petri dishes. The control plates consisted of a fungal plug on PDA. The same procedure was used to test raw extract and column fractions from roots of SWG. Linear growth measurements were taken each day for three days.

Liberation of volatile oils from the active fraction collected from the column and from raw extract of both Nebuka and SWG resulted in slight fungal inhibition only when 5 ml extract per plate was used (Table 5). These results indicate it is unlikely that volatile oils liberated from roots of onions have any effect on growth of P. terrestris in the soil.

Table 5. Effect of volatile oils from root extracts of Nebuka and SWG on linear growth of *P. terrestris*.

Extract (ml)	Growth (mm/24 hrs)			
	SWG		Nebuka	
	raw extract	column fraction	raw extract	column fraction
0.5	7.8 c	7.5 c	6.5 b	6.2 a
1.0	6.6 bc	7.3 bc	7.0 b	6.6 a
1.5	6.0 ab	6.8 abc	6.3 ab	7.1
2.5	6.3 ab	6.1 ab	6.1 ab	5.8 a
5.0	5.2 a	6.0 a	5.2 a	6.3 a
Control	6.5 bc	6.8 abc	6.3 b	7.0 a
LSD .05 [1]	1.2			

[1] Figures followed by the same letter are not significantly different at the 5% level.

DISCUSSION

One of the objectives of this study was to search for possible defense mechanisms in roots of various onion cultivars that might play a role in resistance to infection by P. terrestris. The cultivars studied ranged from highly resistant to completely susceptible to infection by P. terrestris. Differences in levels of certain chemical groups would offer a possible explanation for resistance. Assuming such differences were found, a second objective was to isolate the fractions responsible for fungal inhibition, study their physical properties and attempt to characteriaze the compound or compounds involved.

Preliminary tests indicated that some type of biochemical mechanism is functional in extracts from the roots of highly resistant cultivars and to a lesser degree in extracts from susceptible ones but only when higher concentrations of extract were used. In fact at lower concentrations, extract from SWG, a susceptible cultivar, appeared to stimulate growth of the fungus. No attempts were made to study the latter phenomenon but it may be worth purusing in the future.

Siemer (57) showed that all onion lines tested had a functional polyphenoloxidase system in the roots but activity was low and the differences between resistant and susceptible cultivars were not sufficient to explain resistance. However, he also noted that the phenolic pattern in the highly resistant cultivar Nebuka was so

different from the others tested that this could offer a basis for resistance.

Bioassay tests of root extract from Nebuka passed through a chromatographic column showed that only two fractions were involved in fungal inhibition. Linear growth of P. terrestris and growth of the fungus in liquid medium was completely inhibited. However, if the fungal inoculum plugs from either liquid medium or agar plates were transferred to PDA plates free of any onion extract, fungal growth resumed, indicating the inhibitory compound is fungistatic rather than fungicidal to mycelium of P. terrestris. Assay tests involving root extract from SWG showed that inhibition of the fungus was also confined to two fractions. However, extract from this susceptible cultivar resulted in only slight inhibition and did not completely arrest fungal growth. The inhibitory compound was found to exist in the fleshy bulb portion of Nebuka and shown to be confined to two fractions. Extracts from bulbs of Nebuka exhibited the same effects on fungal growth as did root extract. P. terrestris does not attack living bulb tissue of either cultivar.

In attempts to further characterize the inhibitory compound, preparative TLC and normal TLC were employed. The active fraction from Nebuka root extract was used to streak a preparative TLC plate. When the plate was developed and viewed under ultraviolet light, a fluorescent band was observed about 8 cm above the original

streak. Bioassay tests showed that fungal inhibition was confined to the band which fluoresced when exposed to ultraviolet light. This was true also for bulb extract from Nebuka.

The inhibitory fraction of extracts from roots and bulbs of Nebuka was separated on TLC plates, and subsequently exposed to iodine vapors. In each case five separate spots were clearly defined, with Rf values of 0.24, 0.44, 0.74, 0.79 and 0.89. When the plates were divided into bands and assayed for activity, it was shown that fungal inhibition was associated with the spot with Rf value of 0.44.

Portions of the material eluted from each spot were monitored on a spectrophotometer. No specific absorbance peaks were recorded in any of the extracts suggesting impurities may be masking maximum absorption of the various constitutents.

Chromatograms were sprayed with suggested reagents used to detect sugars, amino acids, phenolic compounds, steroids, carboxylic acids and sulfur-containing compounds. The spot associated with fungal inhibition (Rf of 0.44) reacted positively only to ninhydrin and tetrazotized benzidine to form a claret-maroon color indicating a possible amino-acid-phenolic complex. Similar reactions were observed on TLC plates spotted with extract from onion cultivars which are known to be susceptible to P. terrestris. Additional reagents specific for detection of phenolic compounds were used to verify these findings. The spot involved in inhibition reacted

positively to ammoniacal silver nitrate to form a dark gray color; a strong positive reaction was observed with tetrazotized benzidine and a very slight reaction with diazotized sulphanilic acid.

Roux and Maiks (52) noted that ammoniacal silver nitrate is reduced very slowly by mono- and meta-hydroxyphenols. They also showed that the color of the hydroxyphenol on the chromatogram depends on the concentration and the nature of the reducing group. Catechol groups for example may produce either black, gray or brown colors depending on the concentration. They also noted that the benzidine reagent is one of the most selective available. Chromatograms spotted with the inhibitory fraction from Nebuka roots and sprayed with this reagent (benzidine) resulted in a claret-maroon color suggesting that a combination of catechol and phloroglucinol or pyrogallol in catechins may be involved in the reaction.

Ferric chloride-potassium ferricyanide reacted positively with all spots on the chromatogram. This reagent is very sensitive to phenolic compounds of all types (54).

The inhibitory compound may be a phenolic amine such as tyrosine or dihydroxyphenylalanine in which case the molecules would be both ninhydrin and phenolic positive. It is also possible that the amino acid or acids may occur independently of the phenolic compound although it appears that they have similar Rf values under the conditions used in studying the onion root extract. Using two dimensional

chromatography and proper solvents, one may find that the amino acid complex moves independently of the phenolic compound indicating no association between the two molecules. If the amino-phenolic complex is separable, additional assay tests would be required to determine the portion of the molecule responsible for fungal inhibition. However, if the amino-phenolic complex is linked, upon breakage of this linkage one may find that the fungistatic activity exhibited prior to separation has been reduced. It is possible also that the amino acid in combination with the phenolic compound resulted in increased fungistatic properties. Clark et al. (8) have shown that in corn, amino acid-chlorogenic acid combinations resulted in compounds highly toxic to the growth of Helminthosporum carbonum. They attributed this toxicity in part to the ease of oxidation to quinone and in part to inability of the fungus to detoxify the oxidation product.

Quantitative estimation of total phenols showed that the phenolic content in highly resistant cultivars was greater than that found in susceptible ones. Little differences in phenolic content could be detected in cultivars which varied in degrees of susceptibility to the pink root fungus.

The accumulation of phenolic compounds in the host as a response to penetration by a pathogen has been shown to be a widespread phenomenon (1, 2, 10, 13, 29, 30, 39, 51, 68). However, not many cases have been found where resistance is attributed to

preformed phenolic compounds in healthy plant tissue. Probably the best known example is the work conducted by Link et al. (43) and Link and Walker (44) who showed that preformed phenolics in pigmented onions are responsible for resistance to onion smudge. Another example can be cited in the review by Frakas and Kiraly (15) in which it was shown that the seed coat of pea seeds of varieties resistant to root diseases contained higher amounts of phenolics compounds.

Johnson and Schaal (31) reported that the periderm layer from uninfected potatoes resistant to Streptomyces scabes was found to contain a higher phenolic content than susceptible ones. Lee and LeTourneau (42), using both chromatographic procedures and quantitative estimation of phenols, showed that roots of potatoes resistant to Verticillium wilt contained higher amounts of chlorogenic acid.

In a few cases, the activity of phenol oxidizing enzymes in healthy tissue may indicate the degree of resistance. Umaerus (69) showed that resistance of potato plants to Phytophthora infestans could be correlated with peroxidase activity.

In these studies, it was shown that strong fungal inhibition was characteristic of root extracts from the highly resistant cultivar Nebuka. This is also the cultivar that yielded the highest concentration of phenolic material in quantitative tests.

Chromatograms spotted with extracts from YSS, SWG, and Nebuka all reacted similarly when sprayed with various indicator

reagents used in visualization, and all separated in a similar fashion resulting in the same number of spots with similar Rf values. Yet strong inhibition of P. terrestris was associated only with Nebuka, particularly the material eluted from the spot with an Rf value of 0.44. The fact that strong fungal inhibition is associated only with Nebuka suggests that if the compound or compounds responsible for resistance in this cultivar exist in susceptible ones, the molecular configuration or constituents involved is such that the toxic effect is reduced. However, differences in phenolic content could not be detected in cultivars of varying susceptibility sufficient to account for the degree of susceptibility. These results suggest that the higher total phenolic content in Nebuka roots may in part explain resistance to the pink root fungus. Similar results were obtained in assay tests involving two strains of P. terrestris.

This could be a valuable tool in testing breeding lines for resistance to infection by P. terrestris, since it would eliminate dependence on field tests which are influenced by the vagaries of weather, soils, etc. However, detailed studies would be needed to determine whether inhibition of mycelial growth in the laboratory is always paralleled by resistance to infection in the field. The fact that expression of resistance could be influenced by environment, pathogenicity of the strains of P. terrestris, level of inoculum, and possibly nutrition must be kept in mind.

Attempts to demonstrate the presence of the inhibitor being

leached or exuded from Nebuka roots failed. Perhaps the inhibitory compound is being exuded but in amounts not detectable by the techniques employed. The possibility of a substance being liberated from roots of Nebuka which is fungistatic to P. terrestris should not be eliminated as a defense mechanism. Development of more sensitive techniques for detection of inhibitory compounds may show that such a mechanism is operative.

Cavallito and Bailey (5) showed Allium species to contain alkythiolosulphintes, a compound which possesses antibiotic properties. If compounds of this type are being leached or exuded from roots into the soil rhizosphere in sufficient concentrations, they might function as a protective shield against potential pathogens. However, Coley-Smith et al. (9) pointed out that no evidence has been shown to indicate a reduction of bacterial numbers in soil in association with Allium species.

SUMMARY

1. Preliminary tests showed that mycelial growth of P. terrestris was inhibited by extracts from roots of the resistant cultivar Nebuka but not by extracts from the susceptible cultivar SWG.
2. The effects on mycelial growth of P. terrestris was shown to be fungistatic rather than fungicidal.
3. The inhibitory compound occurs in both infected and non-infected roots and bulbs indicating that invasion by the pathogen is not required for their production.
4. Root and bulb extracts from Nebuka were passed through a Bio-Gel P-10 column and 25 fractions, 10 ml each, were collected. Bioassay tests showed that the inhibitory compound was confined to two fractions.
5. Thin layer chromatography indicated that the inhibitory compound had an Rf of 0.44 and reacted positively to ninhydrin, ammoniacal silver nitrate, tetrazotized benzidine and diazotized sulphanilic acid suggesting an amino-phenolic compound or complex.
6. Quantitative estimation of total phenols of several onion cultivars ranging from highly resistant to completely susceptible showed that resistant cultivars contained more total phenols than susceptible ones.

7. The effectiveness of the inhibitory compound is lost upon autoclaving for 15 min at 120 C and 15 psi or under exposure to extreme acid or base conditions.

8. Attempts were made to detect whether or not the healthy roots of the resistant cultivar Nebuka liberated an inhibitory substance. No evidence was obtained for the release of an inhibitor of fungal growth.

9. Volatile oils liberated by Nebuka and SWG onion extracts had little effect on mycelial growth of P. terrestris.

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