Production of Basal Soft Rot Symptom and Maceration of Carnation Tissue by Pseudomonas caryophylli and Corynebacterium species

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ABSTRACT

A species of *Corynebacterium* was commonly isolated from the basal soft rot tissue of carnation plants infected by *Pseudomonas caryophylli*. Carnation plants root-inoculated with *P. caryophylli* and, 1 week later, with *Corynebacterium* sp. wilted more rapidly than plants inoculated with *P. caryophylli* alone or simultaneously with the two organisms. When wilting occurred, all plants inoculated first with *P. caryophylli* and later with *Corynebacterium* sp. developed extensive root discoloration and rotting, while plants inoculated simultaneously with the two bacteria exhibited less extensive root rot; only a few plants inoculated with *P. caryophylli* alone showed any discernible root discoloration. *Corynebacterium* sp. alone caused no visible effect on the plants.

Carnation leaf tissue was rapidly macerated when incubated in phosphate buffer (pH 7.0, 0.1 M) containing both *P. caryophylli* and *Corynebacterium* sp.; maceration commenced 30 hr after inoculation and reached a maximum value at 66 hr. The initiation and amount of maceration was affected by the inoculum concentration of cells of *Corynebacterium* sp. but not by *P. caryophylli*. Maceration was more rapid when tissue was inoculated with *P. caryophylli* and later inoculated with *Corynebacterium* sp. than when the sequence of inoculation was reversed. Tissue incubated with *Corynebacterium* sp. alone was slightly macerated 72 hr after inoculation, while tissue incubated with *P. caryophylli* alone was not macerated after 7 days. Phytopathology 60:1040-1045.

Numerous studies indicate that nonpathogenic bacteria may reduce symptom expression and disease development in infected plants (19), but few reports indicate enhanced pathogenesis as a result of the association of nonpathogenic bacteria with pathogens. Burkholder & Guterman (8) and White & McCulloch (23) demonstrated synergism between pathogenic and nonpathogenic bacteria in a bacterial disease of English Ivy (*Hedera helix*). The former authors reported that simultaneous inoculation of the pathogen and nonpathogen into leaf wounds resulted in accelerated symptom development and an increase in lesion size. They concluded that the nonpathogenic organism had an accelerating effect on the pathogen as well as on the disease complex.

Soft rot of roots and base of the stem is one of the symptoms characteristic of bacterial wilt of carnation (*Dianthus caryophyllus* L.) caused by *Pseudomonas caryophylli*. Burkholder (7) reported that many secondary organisms were associated with the rotted roots. Nelson & Dickey (20) reported that basal rot occurred in 87% of wilted carnations that had been artificially inoculated, and that root discoloration and disintegration occurred in all infected plants. They also noted that, in addition to the pathogen, unidentified bacteria occurred in the basal soft rot tissue, and they questioned the significance of these bacteria in the wilting of plants. More recently, Dickey & Nelson (11) found that carnation shoots wilted more rapidly when they were inoculated simultaneously with *P. caryophylli* and an unidentified bacterium isolated from a wilted carnation plant infected by *P. caryophylli*. Therefore, it was deemed advisable to investigate the relative importance of these bacteria in the breakdown of carnation tissue. A preliminary report of this work has been published (5).

MATERIALS AND METHODS.—*Identification of the bacterium.*—Unidentified bacteria were isolated from the basal soft rot tissue of wilted plants previously inoculated with *P. caryophylli*. One of the bacteria most commonly isolated from the basal soft rot tissue was selected for further study. A single-cell isolate was obtained by the method of DeVay & Schnathorst (10) and maintained on potato-dextrose agar (PDA) which contained 1.0% dextrose (pH 7.0). The morphological and physiological characteristics were determined by methods described in the Manual of Microbiological Methods (22) except as otherwise stated. Carbon sources were added to the basal medium of Ayers et al. (2). A 1.6% alcoholic solution of bromthymol blue (BTB) was used as the pH indicator. The method of Hugh & Leifson (16) was used to differentiate between oxidative and fermentative utilization of sugars. Metachromatic granules were demonstrated by the methylene blue technique (9). Cells grown on nutrient agar (Difco) containing 2 μg/ml $MnCl_2$ were stained with malachite green to determine production of endospores. Bacterium was incubated at 27 C except as otherwise stated. Test for hypersensitive reaction of tobacco leaves (*Nicotiana tabacum* L.) infiltrated with the bacterium was made using the method of Klement et al. (18).

Inoculation tests.—Carnation cuttings were rooted in steam-treated rooting medium under intermittent mist. Rooted cuttings were inoculated by clipping uniform root systems to 3.0 cm from the base of the stem and submerging the injured roots into a suspension of bacterial cells for 30 min. The bacterial suspensions were prepared by washing cells of *P. caryophylli* (isolate B-1) or of the unidentified bacterial isolate from PDA slant cultures which had been incubated at 27 C for 24 hr. Suspensions of each organism

were made in sterile distilled water and adjusted to an absorbance of 0.5 at 620 nm with a Spectronic 20 colorimeter. Mixed inoculum, containing both bacteria, was prepared so the concn of cells of each organism was comparable to that in inoculum containing only one bacterium. Inoculated cuttings were planted in 4-inch clay pots containing a steam-treated mixture of soil, perlite, sand, and peat moss. The cuttings were placed in a controlled environment chamber which was maintained at day and night temp of 27 and 21 C, and relative humidities of 81 and 73%, respectively. The light intensity was 2,000 ft-c during a 14-hr photoperiod. When root-inoculated plants were inoculated 1 week later, 50 ml of the bacterial suspension were poured onto the soil in the pots, followed by immediate watering of the plants. The plants were observed each day for the development of typical wilt symptoms. When a plant wilted, it was removed from the pot and was examined for discoloration and rotting of the roots and basal stem.

Maceration tests.—Young leaf tissue of Improved White Sim carnation plants was used because it is relatively free of bacteria as compared with stem tissue (11). The three pairs of fully expanded leaves immediately below the apical region were removed from uniform carnation plants, washed in tap water, and dried with paper towels. Three pieces of leaf tissue were removed from the central portion of each leaf by making four transverse cuts 1.0 cm apart. Two-g samples of leaf pieces were randomly selected and placed in sterile plastic petri plates. Twenty ml of 20% solution of commercial Clorox (5.25% sodium hypochlorite) containing one drop of Triton X100 were added to each sample, and the petri plate was rotated vigorously for 2 min. The tissue was washed with five changes of sterile distilled water. Each sample was transferred aseptically to a 125-ml Erlenmeyer flask containing 18.0 ml of sterilized phosphate buffer (pH 7.0, 0.1 M). The tissue was inoculated immediately with *P. caryophylli* alone, the unidentified bacterial isolate alone, or both bacteria. The inoculum of each organism was prepared by washing cells from 24-hr-old PDA slant cultures with sterile distilled water. The inocula contained approximately 10^6 cells/ml. One ml of either inoculum was added to the flasks when the organisms were grown alone, and 1 ml of each inoculum when they were grown together. The total volume of liquid in each flask was adjusted to 20 ml by the addition of sterile distilled water when necessary. The control flasks contained 2 g of tissue, 18 ml of buffer, and 2 ml of sterile distilled water. The flasks were incubated at room temp (30 ± 4 C) on a reciprocating shaker at 120 excursions/min.

Maceration was determined by a modification of the method described by Zaitlin & Coltrin (25), who reported that maceration of tobacco leaf tissue resulted in the release of single cells and that the chlorophyll content of these cells was a valid index of maceration. Maceration of carnation leaf tissue by bacteria in our studies also resulted in the release of single cells. At various time intervals, two 1.0-ml samples were each mixed with 4.0 ml of 80% acetone. The mixture was thoroughly agitated by a Vortex mixer for 1 min. The bacteria and cell debris were removed by centrifugation at 3,000 *g* for 10 min, and the absorbance of the supernatant was determined at 645 nm and 663 nm with a Beckman DU-2 spectrophotometer. The total chlorophyll content of the sample was calculated by Arnon's formula (1) as μg chlorophyll/ml at a designated time.

RESULTS.—*Generic identity of the unidentified bacterium.*—Cells from 24-hr-old PDA slant cultures are nonacid fast, gram-positive rods, 1.0-1.5 × 5.0-8.0 μ. When cultures were incubated for 1 week, gram-stained cells showed clavate swellings at the ends, considerable pleomorphism and a granular appearance. Granules stained gram-positive, but the cell walls varied in their gram reaction and positive and negative regions were observed in the same cell. Three-week-old cultures contained cells which were completely gram-negative. Metachromatic granules were evident in cells at all stages of development but were more apparent in cells from old cultures. Rods of considerable length, e.g., 30 μ, were observed when the bacterium was grown in undisturbed nutrient broth (Difco). Endospores were not observed. Rudimentary branching occurred rarely.

Colonies on PDA are white, small, circular, and convex with undulate margins. On nutrient agar (Difco), colonies are translucent and grow less rapidly than on PDA. Growth in nutrient broth (Difco) was good and sedimentation of the cell mass occurred as the culture aged. Tests for motility using motility agar and hanging drop techniques were positive.

Fermentative utilization of glucose, sucrose, and lactose occurred by 48 hr. Both acid and gas were produced from these sugars. Acid but no gas was produced from cellobiose after 21 days. Five days after inoculation, acid was produced from inulin; there was no evidence of gas production by 30 days. On triple sugar agar, a yellow slant and butt were produced indicating utilization of sucrose and lactose by the organism. On endo agar, small colorless colonies were produced but there was no change in the color of the medium 6 days after inoculation. Pectin gel (15) was rapidly liquefied, but gelatin was not. Nitrate was reduced to nitrite in 48 hr. Tests for lysine decarboxylase (14) were negative. A solid curd had formed in skim milk 21 days after inoculation, but there was no clearing of the medium. The catalase and methyl-red tests were positive and the Voges-Proskauer test was negative. Proteolytic activity was not detected at 30 days when an alkaline egg medium was used. Growth in indole medium was poor and a negative indole reaction was obtained. Although the bacterium remained viable, slight acid but no gas was produced from sodium tartrate (0.2%) at 30 days. Growth in nutrient broth containing 1 or 3% NaCl was good, but there was no growth with 5 or 7% NaCl. The bacterium caused a hypersensitive reaction in tobacco leaves.

The morphological characteristics and some physiological reactions indicate that this bacterium belongs to the genus *Corynebacterium* Lehmann & Neumann (6) when considered in *sensu lato* as described by Jensen (17). This is based primarily on the fact that the bacterium is a pleomorphic nonacid-fast, pre-

dominantly gram-positive rod with clavate swellings at the ends of the cells, exhibits rudimentary branching, does not produce endospores, and contains metachromatic granules in the cells. The motility of the organism tends to relate it closely to soil and phytopathogenic coryneform bacteria and less closely to species of *Corynebacterium* in *sensu stricto* (17). Although a species of *Corynebacterium* with characteristics described herein does not appear in Bergey's Manual of Determinative Bacteriology (6), a specific epithet will not be designated at this time.

Inoculation tests.—Rooted cuttings which were root-inoculated with *P. caryophylli* and inoculated 1 week later with *Corynebacterium* sp. developed wilt symptoms more rapidly than plants inoculated with the pathogen alone or with the two organisms simultaneously (Table 1). The roots of cuttings that rapidly wilted were extensively discolored and rotted when wilting occurred (Fig. 1). Cuttings inoculated simultaneously with both organisms exhibited less discoloration and rotting of the roots. Some of the plants inoculated with the pathogen alone showed only slight root discoloration at the time of wilting. Inoculation with *Corynebacterium* sp. alone had no visible effect on the cuttings.

Maceration of carnation tissue.—Carnation leaf tissue inoculated simultaneously with *P. caryophylli* and *Corynebacterium* sp. visually appeared to be completely macerated 60 hr after inoculation (Fig. 2). Tissue inoculated with *Corynebacterium* sp. alone was slightly macerated after 72 hr, whereas noninoculated tissue and tissue inoculated with *P. caryophylli* alone were not macerated 7 days after inoculation.

The maceration of leaf tissue as determined by the modified Zaitlin and Coltrin technique (25) is shown in Fig. 3. Tissue inoculated simultaneously with both bacteria commenced to macerate about 30 hr after inoculation, and maceration continued to increase until 66 hr (Fig. 3-A). Tissue inoculated with *Corynebacterium* sp. showed no maceration until 60 hr after inoculation (Fig. 3-D). Although a small amount of chlorophyll was detected for tissue inoculated with *P. caryophylli* and noninoculated tissue, no maceration was observed. These results are analogous to those made by visual observations (Fig. 2).

When carnation leaf tissue was inoculated first with *P. caryophylli* and 24 hr later with *Corynebacterium* sp. (Fig. 3-B), the rate of maceration was similar to that when the tissue was inoculated simultaneously with both organisms. However, when tissue was inoculated with *Corynebacterium* sp. and 24 hr later with *P. caryophylli,* initiation of maceration was delayed and the initial rate was reduced (Fig. 3-C). These results suggested that an interaction between the host

TABLE 1. Number of days required for the occurrence of wilting in Improved White Sim carnations root-inoculated with *Pseudomonas caryophylli* or *P. caryophylli* and *Corynebacterium* sp.

	No. days between inoculation and wilting[a]		
Experiment	*P. caryophylli*	*P. caryophylli* + *Corynebacterium* sp. simultaneously	*P. caryophylli* and *Corynebacterium* sp. 1 week later
1	32 ± 2	29 ± 2	21 ± 1
2	38 ± 4	36 ± 4	24 ± 3
3	37 ± 2	37 ± 4	23 ± 2

[a] Each value is the average for 6 plants.

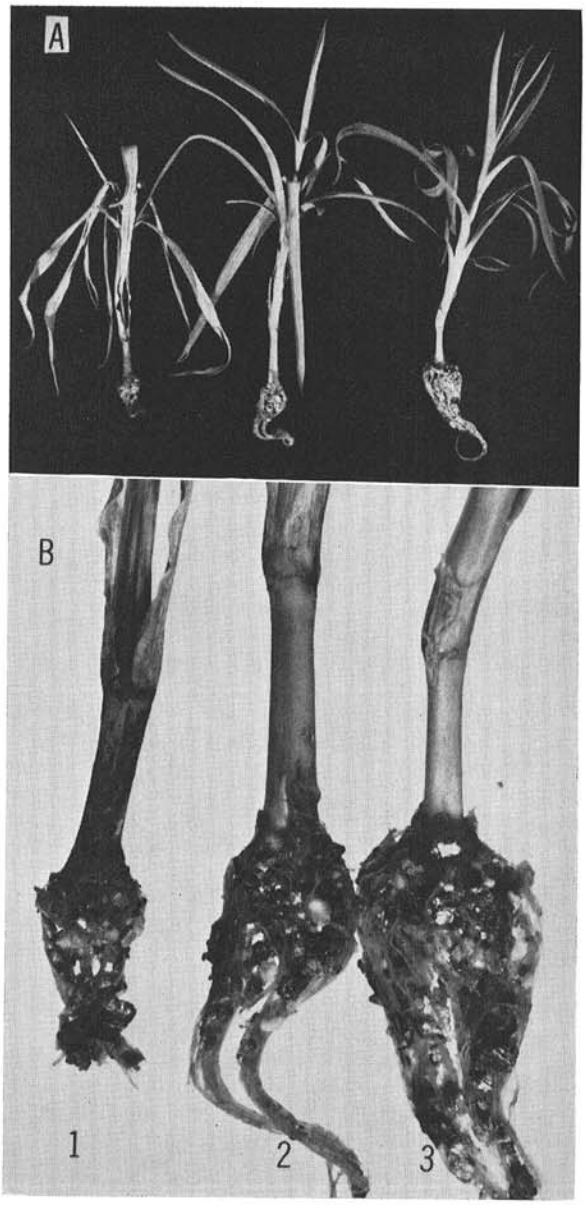

Fig. 1. Symptoms on Improved White Sim carnation plants inoculated with (1) *Pseudomonas caryophylli* and 1 week later with *Corynebacterium* sp.; (2) *P. caryophylli* alone; (3) noninoculated. **A)** Entire plants and **B)** base of same plants showing (left to right) wilting with rotted roots and basal stem, wilting with slight discoloration of roots, and no wilting with nonaffected roots and basal stem.

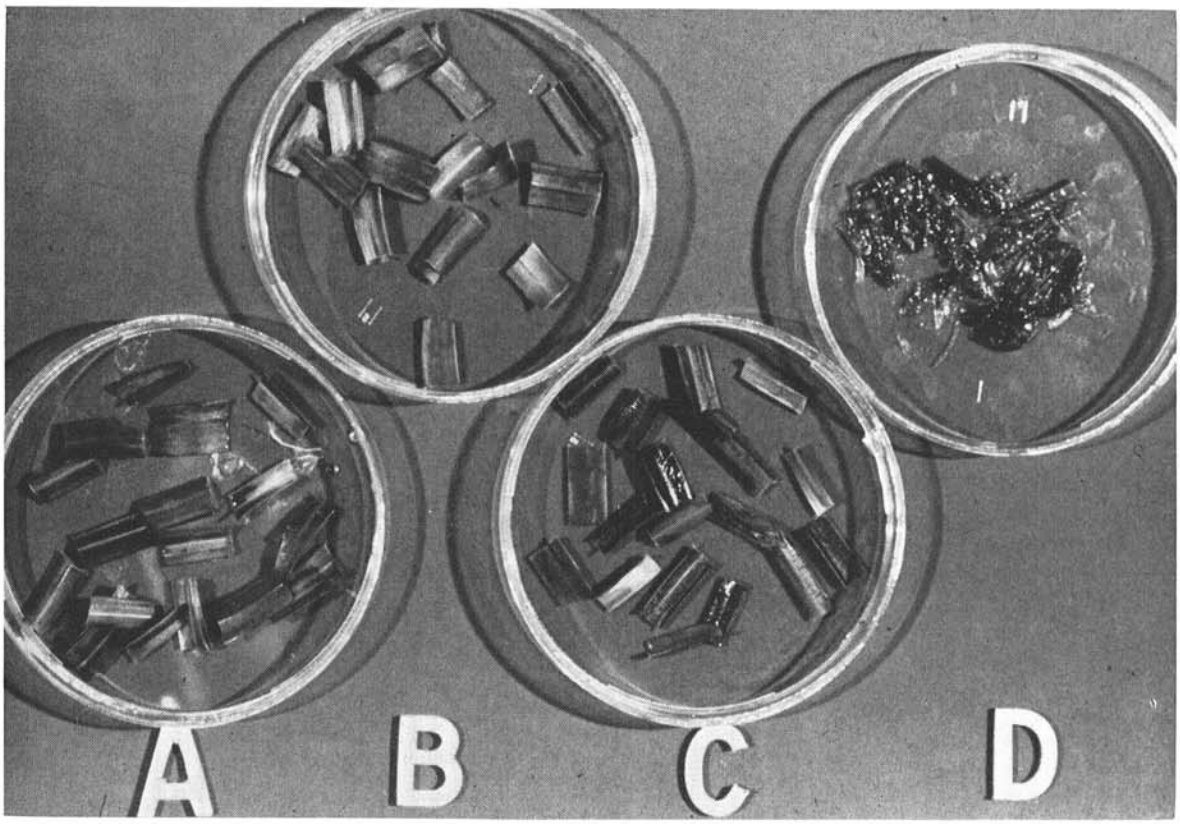

Fig. 2. Effect of *Pseudomonas caryophylli* and *Corynebacterium* sp. on carnation leaf tissue in phosphate buffer (pH 7.0, 0.1 M), 60 hr after inoculation with **A)** noninoculated; **B)** *Corynebacterium* sp.; **C)** *P. caryophylli;* **D)** *P. caryophylli* and *Corynebacterium* sp. Note maceration of leaf tissue inoculated with both bacteria (D).

tissue and *P. caryophylli* was a prerequisite for rapid maceration.

The amount and time of initiation of maceration of leaf tissue incubated with both bacteria was not influenced by variation in the inoculum concentration of *P. caryophylli* in the presence of a constant concn of cells of *Corynebacterium* sp. (Fig. 4-D). However, a reduction in the number of cells of *Corynebacterium* sp. in the inoculum delayed the initiation and reduced the amount of maceration at 30, 36, and 42 hr after inoculation (Fig. 4-A, B, C). At 48 hr there was little difference in the amount of maceration for different ratios of the bacteria. The results indicated that maceration was affected by the concn of cells of *Corynebacterium* sp. in the inoculum.

Less variation between results of chlorophyll assay for replicate treatments occurred in the early stages of maceration (e.g., 30-54 hr after inoculation) than in later stages (54-66 hr). The maximum standard deviation for samples collected during the later stages was 3.5. The variation during the later stages was in part due to the difficulty of pipetting a homogeneous and representative sample of the fluid which had become viscous.

Discussion.—Tissue maceration and soft rot development in infected plants has often been associated with the production of pectic enzymes by pathogens during pathogenesis (3, 4, 24). Gehring (15) reported that *P. caryophylli* does not produce pectic enzymes. Histopathological evidence indicates that there is no degradation of pectic substances in carnation plants infected by *P. caryophylli* alone (21). Our results suggest that *P. caryophylli* is not solely responsible for the basal soft rot symptom that is often associated with bacterial wilt of carnations. The species of *Corynebacterium* used in these studies may be only one of several microorganisms which can interact with the pathogen and the host to cause the soft rot of infected plants.

Evidence for maceration of plant tissue due to the association of microorganisms has not been reported frequently. However, Elarosi (12, 13) found that when potato tubers were first inoculated with *Rhizoctonia solani* and then with *Fusarium solani* there was enhanced rotting of the tubers. The phenomenon did not occur when the sequence of inoculation was reversed. Elarosi also found that pectic enzymes of both pathogens were involved in the process (13).

The rapid maceration of carnation leaf tissue only in the presence of both bacteria (Fig. 2) indicates that both organisms are involved in physiological processes contributory to maceration. The occurrence of rapid maceration when tissue is first inoculated with *P. caryophylli* and then with *Corynebacterium* sp. (Fig.

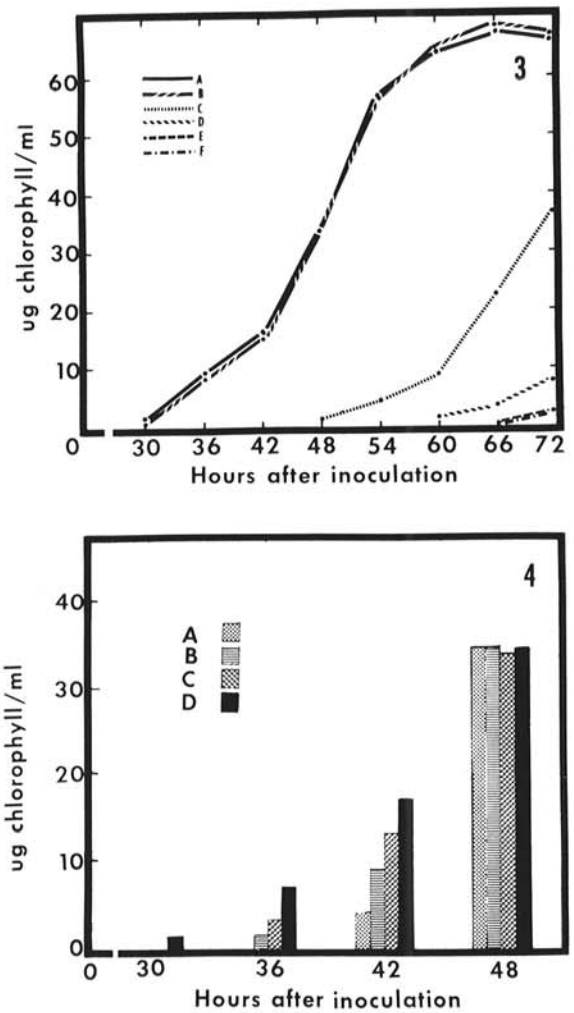

Fig. 3-4. 3) Maceration of carnation leaf tissue, measured by chlorophyll assay, 0 to 72 hr after inoculation with (A) *Pseudomonas caryophylli* and *Corynebacterium* sp. simultaneously; (B) *P. caryophylli* and 24 hr later with *Corynebacterium* sp.; (C) *Corynebacterium* sp. and 24 hr later with *P. caryophylli*; (D) *Corynebacterium* sp.; (E) *P. caryophylli*; (F) noninoculated. Concn of cells of each bacterium in all inocula = approximately 10^6/ml. **4)** Effect of the concn of *Pseudomonas caryophylli*: *Corynebacterium* sp. cells in the inoculum on maceration of carnation leaf tissue, as measured by chlorophyll assay. (A) 1.0:0.50; (B) 1.0:0.66; (C) 1.0:0.83; (D) 1.0, or 0.83, or 0.66, or 0.50:1.0. 1.0 = 10^6 cells/ml.

3) and the marked delay in the initiation of maceration when the sequence of inoculation is reversed suggests that the pathogen-plant tissue interaction is a prerequisite for the rapid tissue maceration. The initial amount of maceration varies with the inoculum concn of *Corynebacterium* sp., but apparently is not related to the inoculum concn of the pathogen (Fig. 4). This suggests that *Corynebacterium* sp. is the biotic agent in the maceration process. It seems likely that the interaction between *P. caryophylli* and the carnation tissue results in an environment favorable for the proliferation of *Corynebacterium* sp. which then causes maceration. This suggestion is supported by the occurrence of basal soft rot only when plants are inoculated first with *P. caryophylli* and then with *Corynebacterium* sp.

LITERATURE CITED

1. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. Plant Physiol. 24:1-15.
2. Ayers, S. H., P. Rupp, & W. T. Johnson. 1919. A study of the alkali-forming bacteria in milk. USDA Bull. 782:1-39.
3. Bateman, D. F., & R. L. Millar. 1966. Pectic enzymes in tissue degradation. Annu. Rev. Phytopathol. 4:119-146.
4. Bateman, D. F. 1968. The enzymatic maceration of plant tissue. Netherland J. Plant Pathol. 74 (Suppl.): 67-80.
5. Brathwaite, C. W. D., & R. S. Dickey. 1969. Enhanced maceration of carnation tissue by the association of a species of *Corynebacterium* and *Pseudomonas caryophylli*. Phytopathology 59:398 (Abstr.).
6. Breed, R. S., E. G. D. Murray, & N. R. Smith. 1957. Bergey's Manual of determinative bacteriology. The Williams & Wilkins Co., Baltimore, Md. 1095 p.
7. Burkholder, W. H. 1942. Three bacterial plant pathogens: *Phytomonas caryophylli* sp. n., *Phytomonas aliicola* sp. n., and *Phytomonas manihotis* (Arthaud-Berthet et Bondar) Viegas. Phytopathology 32:141-149.
8. Burkholder, W. H., & C. E. F. Guterman. 1932. Synergism in a bacterial disease of *Hedera helix*. Phytopathology 22:781-784.
9. Clark, F. E., & R. B. Mitchell. 1942. Cell inclusions of globiforme and related types of soil microorganisms. J. Bacteriol. 44:529-532.
10. DeVay, J. E., & W. C. Schnathorst. 1963. Single-cell isolation and preservation of bacterial cultures. Nature 199:775-777.
11. Dickey, R. S., & P. E. Nelson. 1970. *Pseudomonas caryophylli* in carnation. IV. Unidentified bacteria isolated from carnation. Phytopathology 60:647-653.
12. Elarosi, H. 1957. Fungal Associations I. Synergistic relation between *Rhizoctonia solani* and *Fusarium solani* in causing a potato tuber rot. Ann. Bot. 21: 555-586.
13. Elarosi, H. 1958. Fungal Associations III. The role of pectic enzymes on the synergistic relation between *Rhizoctonia solani* and *Fusarium solani* in the rotting of potato tubers. Ann. Bot. 22:399-416.
14. Falkow, S. 1958. Activity of lysine decarboxylase as an aid in the identification of *Salmonellae* and *Shigellae*. Amer. J. Clin. Pathol. 29:598-600.
15. Gehring, F. 1962. Untersuchungen über den Infektionsverlauf einer durch *Pectobacterium parthenii* (Starr) Hellmers var. *dianthicola* Hellmers verursachten Nelkenbakteriose sowie über enzymatische Eigenschaften dieses Bakteriums im Vergleich mit *Pseudomonas caryophylli* (Burkholder) Starr et Burkholder und einigen typischen Nassfäuleerregern. Phytopathol. Z. 43:383-407.
16. Hugh, R., & E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative bacteria. J. Bacteriol. 66:24-26.
17. Jensen, H. L. 1952. The coryneform bacteria. Annu. Rev. Microbiol. 6:77-90.
18. Klement, A., G. L. Farkas, & L. Lovrekovich. 1964. Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopathology 54:474-477.
19. Leben, C. 1965. Epiphytic microorganisms in relation to plant disease. Annu. Rev. Phytopathol. 3:209-230.
20. Nelson, P. E., & R. S. Dickey. 1963. Reaction of

twenty-one commercial carnation varieties to *Pseudomonas caryophylli*. Phytopathology 53:320-324.
21. NELSON, P. E., & R. S. DICKEY. 1966. *Pseudomonas caryophylli* in carnation II. Histological studies of infected plants. Phytopathology 56:154-163.
22. SOCIETY OF AMERICAN BACTERIOLOGISTS. Manual of Microbiological methods. McGraw-Hill Book Co., New York. 1957.
23. WHITE, R. P., & LUCIA MCCULLOCH. 1934. A bacterial disease of *Hedera helix*. J. Agr. Res. 48:807-815.
24. WOOD, R. K. S. 1960. Pectic and cellulolytic enzymes in plant disease. Annu. Rev. Plant Physiol. 11:299-322.
25. ZAITLIN, J., & D. COLTRIN. 1964. Use of pectic enzymes in a study of the nature of intercellular cement of tobacco leaf tissue. Plant Physiol. 39:91-95.