Synergism between Pseudomonas caryophylli and a Species of Corynebacterium

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ABSTRACT

Corynebacterium sp. and *Pseudomonas caryophylli* were grown separately or together in various synthetic and semisynthetic media. When grown alone in media containing free sugars, *Corynebacterium* sp. produced acids which apparently restricted its growth. Growth of *Corynebacterium* sp. was greater when associated with *P. caryophylli* than when grown alone in these media, and acid accumulation was not detected. Growth of *P. caryophylli* was enhanced when associated with *Corynebacterium* sp., except in media where glucose or sodium polypectate served as the sole carbon source.

Corynebacterium sp. produced extracellular pectic and cellulolytic enzymes, but these enzymes were not detected in supernatants of fragmented cells of *P. caryophylli* or in culture filtrates of this pathogen. Cellulase synthesis was repressed in media containing glucose or sucrose. Cellulolytic activity could not be detected in a carnation extract medium inoculated with *Corynebacterium* sp. alone, but was detected when the same medium was inoculated with both bacteria simultaneously. Synthesis of cellulase in media inoculated with both bacteria was due to a rapid depletion of the reducing sugar content and derepression of cellulase biosynthesis by *Corynebacterium* sp. The pectic enzyme produced by *Corynebacterium* sp. was characterized as an endo-polygalacturonate *trans*-eliminase. Phytopathology 60:1046-1051.

We reported that rapid wilting, basal stem rot, and root rot of carnation plants occurred when cuttings were root-inoculated with *Pseudomonas caryophylli* and with a species of *Corynebacterium* which was isolated from basal soft rot tissue of carnation plants infected by *P. caryophylli* (3). Slow development of wilting and a slight discoloration of roots occurred when plants were inoculated with *P. caryophylli* alone; plants were not visibly affected when inoculated with *Corynebacterium* sp. alone. Furthermore, carnation leaf tissue was rapidly macerated when inoculated with both *P. caryophylli* and *Corynebacterium* sp. Gehring (7) reported that *P. caryophylli* does not produce pectic enzymes, and Nelson & Dickey (19) could not demonstrate tissue degradation in tissue sections from carnation stems infected by *P. caryophylli* alone. Therefore, an investigation was initiated to determine the possible in vitro interactions of *P. caryophylli* and *Corynebacterium* sp. and their relation to the maceration of carnation leaf tissue and the production of the basal stem rot and root rot symptoms. A preliminary report of these studies has been made (2).

MATERIALS AND METHODS.—*Organisms and media.*—A virulent isolate (B-1) of *P. caryophylli* and the previously described *Corynebacterium* sp. were used throughout and were maintained on potato-dextrose agar (PDA) (3).

Anhydrous dextrose (Mallinkrodt Chemical Works), sodium polypectate (Lot 14505, K + K Laboratories, New York), pectin N.F. (No. 3442 Sunkist Growers Inc., Ontario, California), or carboxymethylcellulose (CMC Type 7-MP, Cellulose Gum, Lot 41952, Hercules Powder Co., Wilmington, Delaware) were incorporated into a basal medium at 5.0 g/liter. The basal medium contained $NH_4H_2PO_4$ (1.0 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), KCl (0.2 g), casein hydrolysate (Difco) (2.0 g), yeast extract (Difco) (1.0 g), 1.0 ml of a solution containing $ZnSO_4 \cdot 7H_2O$ (80 mg), $FeCl_2 \cdot 6H_2O$ (25 mg) and $MnSO_4 \cdot H_2O$ (18 mg) in 100 ml of distilled water, and 1,000 ml of distilled water. The medium described by Husain & Kelman (14) which contains 1.2% sucrose was also used. A carnation extract medium was prepared by grinding 100 g of fresh carnation stems with 400 ml of distilled water in a blender at high speed for 10 min. The suspension was filtered through four layers of cheesecloth, and the filtrate was diluted to 1 liter by the addition of distilled water. All media were adjusted to pH 7.0 with 1 N NaOH, and 25 ml portions were dispensed in 125-ml Erlenmeyer flasks. The media were autoclaved for 20 min at 121 C, and the pH was readjusted to 7.0 when necessary.

Media were inoculated with either *P. caryophylli* alone, *Corynebacterium* sp. alone, or both. The bacterial suspensions were prepared by washing cells from 24-hr-old PDA slant cultures with sterile distilled water, and absorbance at 620 nm was adjusted to 0.20 for *P. caryophylli* and 0.24 for *Corynebacterium* sp. (approximately 10^6 cells/ml). Inoculations were made by adding 0.1 ml of either suspension to the various media, when the organisms were grown alone, or 0.1 ml of each suspension when grown together. Flasks were incubated at 30 ± 4 C on a reciprocating shaker operating at 120 excursions/min.

Growth determinations.—Growth of the bacteria in the various media was determined by the dilution plate method. Only nutrient agar (Difco) was used when the bacteria were grown alone, whereas, both nutrient agar and a modification of Gehring's pectin medium (7) were used when the two bacteria were grown together. The basal layer for the modified pectin medium contained $(NH_4)_2SO_4$ (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), Ca$(NO_3)_2$ (0.1 g), $CaCl_2 \cdot 2H_2O$ (5.0 g), 1 N NaOH (2.0 ml), 25 ml bromthymol blue (BTB) solution (2.4 g

of BTB powder in 234 ml of distilled water plus 16 ml of 0.01 N NaOH), agar (18.0 g), distilled water (1,000 ml). The mixture was dissolved by heating, and 120-ml portions were dispensed in 250 ml Erlenmeyer flasks which were autoclaved for 20 min at 121 C. After cooling, approximately 10 ml were poured into sterile petri plates and allowed to solidify. The upper layer was prepared by mixing 20 g of pectin N.F., 400 ml of hot distilled water, 24 ml of BTB solution, and 30 ml 1 N NaOH in a Waring Blendor for 5 min. The mixture was removed and thoroughly mixed with 600 ml of hot distilled water. Nine-ml quantities were dispensed in tubes, and these were autoclaved for 5 min at 121 C. The dilution plates were made by adding 1 ml of the appropriate dilution to 9 ml of the pectin medium. The tube was held on a Vortex Jr. mixer for a few sec, and the mixture was then poured onto the basal layer. All dilutions were made in duplicate and incubated at 27 C.

The simultaneous use of nutrient agar and modified pectin medium provided a method of determining the growth of one organism in the presence of the other. *Corynebacterium* sp. grew on the modified pectin medium in 24-36 hr and colonies were surrounded by characteristic opaque zones of calcium pectate while *P. caryophylli* produced visible colonies in 48-60 hr without zones. The difference in colony counts on the modified medium after 36 hr and on nutrient agar after 48 hr was attributed to *P. caryophylli*.

Enzyme assay methods.—All inoculated and noninoculated media were centrifuged at 10,000 g for 20 min at 4 C, and the supernatant fluids were filtered through 0.22-μ Millipore filters and were assayed directly for enzyme activity. The pH of culture filtrates was always adjusted to that of the substrate prior to assay. The pH of the substrates was adjusted with 0.1 M acetate buffer (below pH 6.0), 0.1 M phosphate buffer (pH 6.0-7.0), or 0.1 M Tris [tris (hydroxymethyl) amino methane]-HCl buffer (pH 7.0-9.0). Culture filtrates autoclaved for 20 min at 121 C were used as enzyme controls. All assays were made at 30 C.

Three methods were used to determine the pectolytic activity of culture filtrates: (i) increase in reducing groups of reaction mixtures containing sodium polypectate as estimated by the dinitrosalicylic acid (DNS) method of Miller (16). The reaction mixtures contained 4.0 ml of 1.2% buffered solution of sodium polypectate and 1.0 ml of culture filtrate. Enzyme activity was expressed as mg of anhydrous galacturonic acid equivalents/ml reaction mixture per hr. (ii) The loss in viscosity of 1.2% buffered solution (pH 8.5) of sodium polypectate or pectin N.F. as determined in Fenske-Ostwald viscometers (size 300) at 30 C (1). The reaction mixtures contained 6.0 ml of buffered solution and 1.0 ml of culture filtrate. Enzyme activity was expressed as per cent loss in viscosity as a function of time. (iii) *Trans*-eliminase activity as estimated by the thiobarbituric acid method (23).

Cellulolytic enzyme activity was assayed by: (i) the liberation of reducing groups in reaction mixtures as determined by the DNS method (16). The reaction mixtures contained 4 ml of a 1.2% buffered solution of CMC and 1 ml of culture filtrate. Enzyme activity was expressed as mg equivalents of anhydrous glucose/ml reaction mixture per hr. (ii) The loss in viscosity of a reaction mixture containing 6 ml of 1.2% buffered solution of CMC (pH 6.0) and 1 ml of culture filtrate as determined in Fenske-Ostwald viscometers. Results were expressed as per cent loss in viscosity as a function of time.

Intracellular enzymes.—*P. caryophylli* was grown in sodium polypectate or carboxymethylcellulose medium for 24 hr. The cells were removed from the culture by centrifugation at 6,000 g for 30 min and resuspended in phosphate buffer (0.1 M, pH 7.0). A cell suspension (OD, 0.8 at 620 nm) was disrupted with a Fisher Ultrasonic Probe (Model BP2, Blackstone Ultrasonics Inc.) for 15 min at maximum intensity. Approximately 90% of the cells were broken by this treatment. The suspension was centrifuged at 25,000 g for 30 min at 4 C and the supernatant fluid was assayed for pectolytic and cellulolytic enzymes.

RESULTS.—*Growth and pH changes in various media.*—The growth of *P. caryophylli* was slightly increased when grown with *Corynebacterium* sp. except in glucose and sodium polypectate media (Table 1). Growth of the pathogen was greatest in media containing glucose. The pH was only slightly altered in media inoculated with the pathogen alone, but media inoculated with both organisms had final pH values above 7.5, except for the glucose and sucrose media which had pH values of 6.4 and 6.5, respectively.

Growth of *Corynebacterium* sp. was greater in glucose, sucrose, and carnation extract media inoculated with both organisms than when inoculated with *Corynebacterium* sp. alone. In addition, the pH of these media was 4.0, 4.2, and 4.5, respectively, when inoculated with *Corynebacterium* sp. alone and 6.4, 6.5, and 8.4, respectively, when inoculated with both bacteria. These results indicated that the growth of *Corynebacterium* sp. was reduced by low pH in the environment.

Pectic enzymes.—Cell-free extracts of *P. caryophylli* did not exhibit pectolytic enzyme activity. Pectolytic activity was not detected in culture filtrates from various media inoculated with the pathogen, but was detected in media inoculated with *Corynebacterium* sp. alone or *Corynebacterium* sp. and *P. caryophylli* (Table 2). The pectolytic activity of culture filtrates from glucose, sucrose, and carnation extract media was considerably less when inoculated with *Corynebacterium* sp. alone than when inoculated with both bacteria, while the activity of culture filtrates from CMC, sodium polypectate, and pectin N.F. media were similar. It was apparent that there was a correlation between the growth of *Corynebacterium* sp. (Table 1) and the pectolytic activity of the various media (Table 2). This suggested that the pectic enzyme present in filtrates of cultures inoculated with both bacteria was produced by *Corynebacterium* sp.

The pectic enzyme produced by *Corynebacterium* sp. had an optimum pH of 8.6. The products of the reaction with sodium polypectate had an absorption maximum at 230 nm, and they reacted with thiobarbituric acid to form a red chromogen which absorbed

TABLE 1. Number of viable cells of *Pseudomonas caryophylli* and *Corynebacterium* sp. and pH of various media 48 hr after inoculation with *P. caryophylli* alone, *Corynebacterium* sp. alone, or *P. caryophylli* and *Corynebacterium* sp.

| | Log. no. of viable cells/ml[b] | | | | pH of medium[c] | | |
| | *P. caryophylli* | | *Corynebacterium* sp. | | | | |
Medium[a]	P.c.[d]	P.c. + C.sp.	C.sp.[e]	P.c. + C.sp.	P.c.	C.sp.	P.c. + C.sp.
Glucose	8.75	8.23	4.71	8.23	6.4	4.0	6.4
Sucrose	6.82	7.41	4.53	7.78	6.5	4.2	6.5
CMC	6.20	6.89	7.25	7.31	7.2	7.2	8.0
Sodium polypectate	6.65	6.41	7.34	7.62	7.3	6.9	8.4
Pectin N.F.	6.12	6.80	7.58	7.86	7.4	7.6	7.8
Carnation extract	6.28	7.02	4.12	6.87	7.2	4.5	8.4

[a] Glucose, carboxymethylcellulose (CMC), sodium polypectate and pectin N.F. were incorporated into the basal medium (see text) at 0.5%. Sucrose (1.2%) was incorporated into Husain & Kelman's medium (14). The carnation extract medium was prepared as described in the text.

[b] Initial population of each organism was approximately 4.0×10^3 (log 3.60) cells/ml in all media.

[c] All media were adjusted to an initial pH of approximately 7.0.

[d] P.c. = *P. caryophylli*.

[e] C.sp. = *Corynebacterium* sp.

maximally at 548 nm. These data suggested the cleavage of the substrate by a *trans*-eliminase (1).

The enzyme was more reactive with sodium polypectate than with pectin N.F. In viscometric assays, reductions in viscosity in 4.8 min of 50% and 3% were obtained with sodium polypectate and pectin N.F., respectively. A 50% reduction in the viscosity of a solution of sodium polypectate was accompanied by the release of only 0.025% of the potential reducing groups. Based on these results, the enzyme appeared to be an endopolygalacturonate *trans*-eliminase (endo-PGTE) (1).

Cellulolytic enzymes.—Cellulolytic activity could not be detected in culture filtrates from any of the various media inoculated with *P. caryophylli* (Table 3) or in cell-free extracts of this pathogen. Culture filtrates from glucose or sucrose media inoculated with *Corynebacterium* sp. alone or with both bacteria and from carnation extract medium inoculated with *Corynebac-*

terium sp. alone did not exhibit cellulase activity. In contrast, cellulolytic activity with an optimum pH at 6.0 was readily detected in culture filtrates of all other media.

The results suggested repression of cellulase biosynthesis by sugar (9, 10, 11, 17, 22). The reducing sugar concn of the carnation extract medium was more rapidly reduced at 24 hr after inoculation with both bacteria than when inoculated with either organism alone (Fig. 1). The reducing sugar concn in the medium inoculated with *Corynebacterium* sp. remained relatively constant after 24 hr, but *P. caryophylli* caused a continuous decrease.

During the first 24 hr after inoculation, *Corynebacterium* sp. populations increased rapidly, but there was also a rapid reduction in reducing sugar (Fig. 2). Cellulolytic activity was low at 12 hr and showed only a slight increase at 24 hr. Between 24 and 48 hr after inoculation, the growth of *Corynebacterium* sp. showed

TABLE 2. Pectolytic activity[a] of culture filtrates from various media 48 hr after inoculation with *Pseudomonas caryophylli* alone, *Corynebacterium* sp. alone, or *P. caryophylli* and *Corynebacterium* sp.

| | mg equivalents D-galacturonic acid/ml per hr | | |
Medium[b]	*P. caryophylli*	*Corynebacterium* sp.	*P. caryophylli* + *Corynebacterium* sp.
Glucose	0.00	0.03	0.08
Sucrose	0.00	0.05	0.11
CMC	0.00	0.12	0.13
Sodium polypectate	0.00	0.13	0.14
Pectin N.F.	0.00	0.13	0.15
Carnation extract	0.00	0.03	0.14

[a] Determined by the reducing group analysis with dinitrosalicylic acid according to Miller (16). Reaction mixtures contained 4.0 ml of 1.2% sodium polypectate in 0.1 M Tris-HCl buffer (pH 8.6) and 1.0 ml of culture filtrate. Incubation was for 2 hr at 30 C.

[b] The media were as described in Table 1.

TABLE 3. Cellulolytic activity[a] of culture filtrates from various media 48 hr after inoculation with *Pseudomonas caryophylli* alone, *Corynebacterium* sp. alone, or *P. caryophylli* and *Corynebacterium* sp.

| | mg equivalents of glucose/ml per hr | | |
Medium[b]	*P. caryophylli*	*Corynebacterium* sp.	*P. caryophylli* + *Corynebacterium* sp.
Glucose	0.00	0.00	0.00
Sucrose	0.00	0.00	0.00
CMC	0.00	0.22	0.26
Sodium polypectate	0.00	0.22	0.23
Pectin N.F.	0.00	0.24	0.26
Carnation extract	0.00	0.00	0.22

[a] Cellulolytic activity determined by the reducing group assay, using dinitrosalicylic acid (16). Reaction mixtures contained 4.0 ml of 1.2% CMC in 0.1 M phosphate buffer (pH 6.0) and 1 ml of culture filtrate. Incubation was for 2 hr at 30 C.
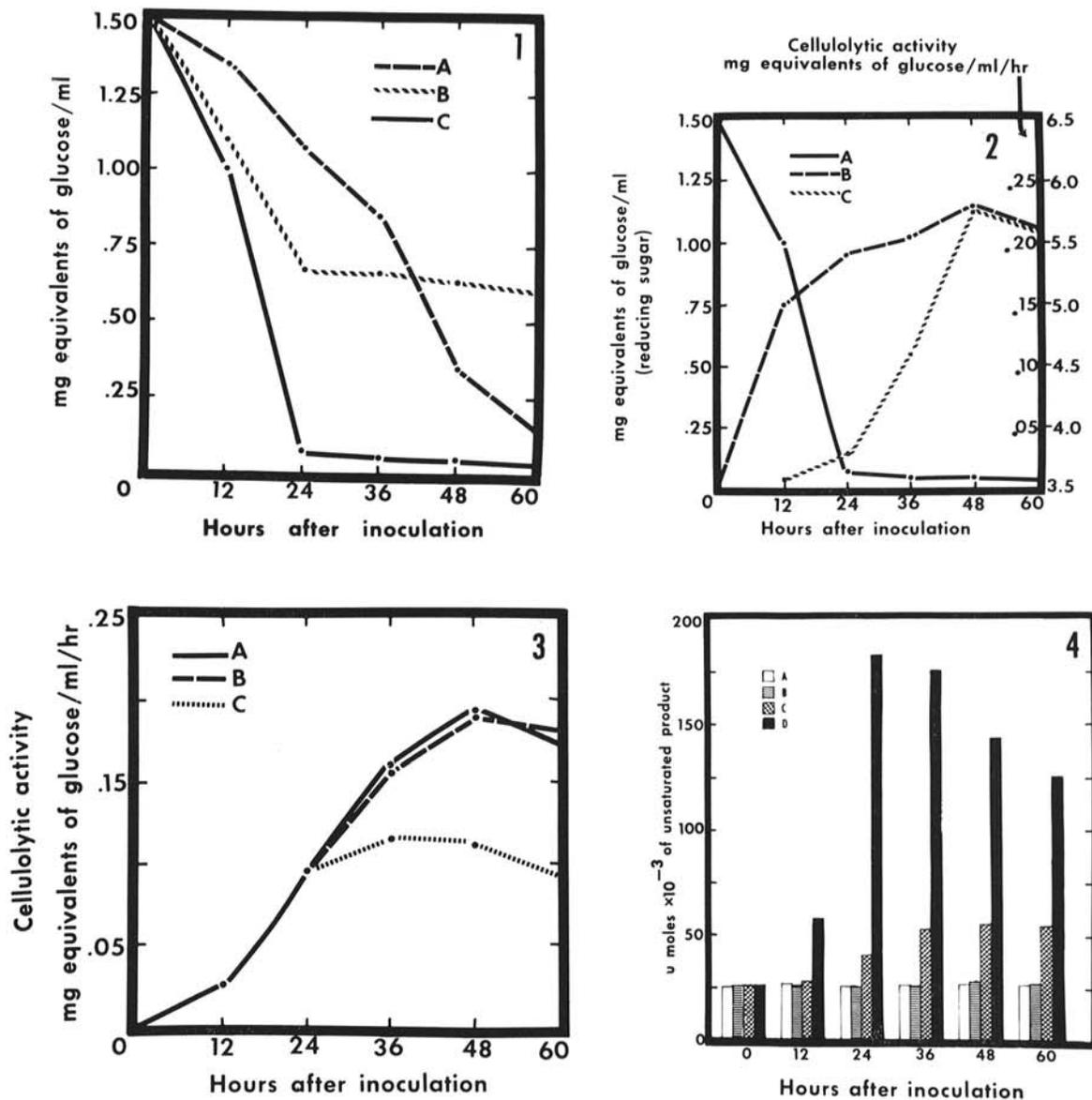
[b] The media were as described in Table 1.

Fig. 1-4. 1) Concentration of reducing sugars in culture filtrates from carnation extract medium 0 to 60 hr after inoculation with (A) *Pseudomonas caryophylli* alone; (B) *Corynebacterium* sp. alone; or (C) *P. caryophylli* and *Corynebacterium* sp. 2) Reducing sugar concentration (A); numbers of viable cells of *Corynebacterium* sp. (B); and cellulolytic activity (C); 0 to 60 hr after inoculation of carnation extract medium with *P. caryophylli* and *Corynebacterium* sp. 3) The effect of glucose on the cellulolytic activity of carboxymethylcellulose medium inoculated with *P. caryophylli* and *Corynebacterium* sp. At 24 hr after inoculation, sterile distilled water was added to the control (A) and glucose solutions were added to give final concentrations of 0.1 mg/ml (B) or 0.6 mg/ml (C). 4) Concentration of unsaturated product from the degradation of pectic substances in carnation extract medium 0 to 60 hr after inoculation with (A) noninoculated; (B) *P. caryophylli* alone; (C) *Corynebacterium* sp. alone; or (D) *P. caryophylli* and *Corynebacterium* sp. The absorbance at 548 nm was converted to μmoles of unsaturated product from a standard curve prepared for 2-keto-3-deoxygluconic acid (23).

a more gradual increase, the concn of reducing sugars was very low, and there was a rapid increase in cellulase activity.

The inverse relationship between cellulase activity and reducing sugar concn indicated a derepression of cellulase synthesis (9, 10, 22). Glucose inhibited cellulase synthesis at 0.6 mg/ml but not at 0.1 mg/ml (Fig. 3). This indicated that the concn of reducing sugar in

the carnation extract medium prior to 24 hr (Fig. 1) could repress cellulase production; however, in medium inoculated with both bacteria, the concn (approximately 0.1 mg/ml) at 24 hr and thereafter, could not.

Degradation of pectic substances in carnation extract medium.—The concn of compounds in the carnation extract medium which reacted with thiobarbituric acid to give a product with max absorption at 548 nm re-

mained relatively constant when the medium was inoculated with *P. caryophylli* (Fig. 4). In contrast, these compounds increased in media inoculated with *Corynebacterium* sp. alone or with both organisms, but the concn in medium with both organisms decreased after 24 hr. The concn was greater in media inoculated with both organisms than in media inoculated with *Corynebacterium* sp. alone. These results are similar to those obtained for pectolytic activity of culture filtrates from carnation extract medium (Table 2).

DISCUSSION.—Several interactions occur when *P. caryophylli* and *Corynebacterium* sp. are grown together in vitro. The data indicate that the inhibition of the growth of *Corynebacterium* sp. may have been due to the accumulation of acid(s) in the medium (Table 1). The mechanism responsible for the absence of acid accumulation in media inoculated with both organisms was not determined. However, several investigators (4) have shown that utilization of acids by one organism may result in the enhanced growth of another.

Cellulase production was not demonstrated for *P. caryophylli* in this study, although Gehring (7) reported that the organism produced cellulase in a CMC medium. The absence of cellulase production by the virulent isolate of the pathogen used in our investigation may indicate that cellulase production in vitro is an unstable character. *Corynebacterium* sp. cellulase was repressed in media containing glucose or sucrose (Table 3). Glucose repression of cellulase biosynthesis has been reported for several phytopathogenic organisms (9, 12, 13). The production of cellulase in carnation extract medium inoculated with both bacteria can be attributed to the utilization of the reducing sugars of the medium and subsequent derepression of cellulase of *Corynebacterium* sp. (Fig. 2).

The production of cellulase by the association of these bacteria in the carnation extract medium is a case of synergism as defined by Burkholder (5) because the enzyme was not produced by either bacterium growing alone. Although *P. caryophylli* could not be demonstrated to produce pectolytic enzymes, the increased growth of *Corynebacterium* sp. when associated with *P. caryophylli* resulted in increased pectolytic activity of culture filtrates (Table 2). This enhanced production of pectolytic enzyme could be considered as an example of synergism only if Burkholder's definition (5) is broadened.

The results of this study may be related to the enhanced maceration of carnation tissue previously reported (3). Pectic *trans*-eliminases are important factors in tissue maceration (1, 6, 15, 21). These pectic enzymes are produced by several bacteria which cause tissue maceration and soft rot development in plants (18, 20, 21). It seems likely, therefore, that the endo-PGTE of *Corynebacterium* sp. is involved in the maceration of carnation tissue. The role of *P. caryophylli* in the rapid maceration of carnation tissue is less obvious. We have suggested that *P. caryophylli* provides an environment favorable for the growth of *Corynebacterium* sp. (3) and this was substantiated by the in vitro studies. The exact mechanism by which

P. caryophylli provides this environment in vivo was not determined. Holtzmann & Thomas (8) reported that wilting of carnation plants infected by *P. caryophylli* may be induced by a toxin. What role, if any, a toxin may play in tissue maceration and soft rot development was not investigated in our studies.

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