Role of Cellular Permeability Alterations and Pectic and Cellulolytic Enzymes in the Maceration of Carnation Tissue by Pseudomonas caryophylli and Corynebacterium sp.

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ABSTRACT

Growth of *Corynebacterium* sp. and activity of endopolygalacturonate *trans*-eliminase (endo-PGTE) were greater in detached carnation leaf tissue simultaneously inoculated with *Pseudomonas caryophylli* and *Corynebacterium* sp. than in tissue inoculated with *Corynebacterium* sp. alone. Tissue was macerated by culture filtrates of *Corynebacterium* sp. which contained both endo-PGTE and cellulase. Maceration paralleled the endo-PGTE activity of culture filtrates, but was apparently unrelated to cellulolytic activity. Bathing solutions from leaf tissue inoculated with *P. caryophylli* contained more amino nitrogen, phosphorus, and potassium and supported greater growth of *Corynebacterium* sp. than did those from noninoculated tissue or tissue inoculated with *Corynebacterium* sp. Growth of *Corynebacterium* sp. in a basal medium containing glucose was dependent on a supply of amino nitrogen and growth in synthetic media, and in bathing solutions from *P. caryophylli*-infected tissue was related to the amino nitrogen content. It is suggested that enhanced growth of *Corynebacterium* sp. in tissue simultaneously inoculated with *P. caryophylli* and *Corynebacterium* sp. occurs because *P. caryophylli* causes an increase in the cellular permeability of the tissue, resulting in the release of nutrients from the plant cells which permit growth of *Corynebacterium* sp. Growth of *Corynebacterium* sp. is accompanied by synthesis of endo-PGTE which macerates carnation tissue. Phytopathology 61:476-483.

Previous studies indicated that rapid wilting, basal stem rot, and root rot of carnation plants occurred when cuttings were root-inoculated with *Pseudomonas caryophylli* and a species of *Corynebacterium* which was isolated from basal soft rot tissue of carnation plants infected by *P. caryophylli* (3, 4). 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, detached carnation leaf tissue was rapidly macerated when simultaneously inoculated with both bacteria, but not when inoculated with either bacterium alone. Rapid maceration occurred when leaf tissue was first inoculated with *P. caryophylli* and 24 hr later with *Corynebacterium* sp., but was delayed when the sequence of inoculations was reversed. Tissue maceration in soft rot diseases of plants has been associated with the production of pectic enzymes by pathogens (2). While synthesis of pectic or cellulolytic enzymes was not demonstrated for the pathogen, *Corynebacterium* sp. produced endopolygalacturonate *trans*-eliminase (endo-PGTE) and cellulase (5). More recently, we have shown that *P. caryophylli*, but not *Corynebacterium* sp., altered the cellular permeability of carnation leaf tissue, resulting in a release of intracellular constituents from the plant cells (6). The objective of this investigation was to determine the role of cellular permeability alterations and pectic and cellulolytic enzymes in the enhanced maceration of carnation tissue simultaneously inoculated with *P. caryophylli* and *Corynebacterium* sp.

MATERIALS AND METHODS.—*Inoculum.*—In addition to the isolates of *Pseudomonas caryophylli* and *Corynebacterium* sp. used in previous studies (3, 4, 5, 6), *Pseudomonas syringae* (isolate PS27), *Pseudomonas marginalis* (isolate PM14), *Erwinia carotovora* (isolate EC14), *Bacillus polymyxa*, and *Pseudomonas fluorescens* also were used. All bacteria were grown in nutrient broth plus 1.0% glucose at 30 C for 24 hr in a Metabolyte water bath shaker (120 strokes/min). The cultures were centrifuged at 5,000 *g* for 30 min, and the pellets washed twice with sterile distilled water. Water suspensions of washed cells containing approx 10^6 cells/ml were used as inoculum.

Inoculation tests.—Leaf samples of carnation were obtained as previously described (3, 4); each sample weighed 2 g except as otherwise stated. The samples were placed into 125-ml Erlenmeyer flasks containing 20 ml of sterile distilled water as the bathing solution, and were inoculated by adding 1 ml of the appropriate bacterial inoculum to the bathing solution. One ml of sterile distiled water was added to control flasks. When leaf samples were inoculated with two bacteria simultaneously, 1 ml of each bacterial inoculum was added to the bathing solution. All inoculated and noninoculated leaf samples were incubated at 30 C in the Metabolyte shaker (120 strokes/min).

Growth of P. caryophylli or Corynebacterium sp. in media containing inorganic or amino nitrogen.—A nitrogen-free basal medium, supplemented with 1.0% glucose and various nitrogenous compounds, was used to determine the ability of *P. caryophylli* and *Corynebacterium* sp. to utilize inorganic and amino nitrogen. The basal medium contained K_2HPO_4 (1.0 g), KCl (0.2 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $ZnSO_4 \cdot 7H_2O$ (0.8 mg), $FeCl_2 \cdot 4H_2O$ (0.25 mg), $MnSO_4 \cdot H_2O$ (0.18 mg), distilled water (1,000 ml), and 1 N HCl (4 ml). One g of either casein hydrolysate (Oxoid, 6.5% amino nitrogen), KNO_3 or $(NH_4)_2SO_4$ was added to 1 liter of basal medium. An amino acid medium was prepared by adding 100 mg of each of several amino acids to one liter of basal medium. The amino acids used were L-lysine-HCl, L-proline, L-alanine, L-aspartic acid, glycine, L-threonine, L-leucine, L-valine, L-tryptophan, L-isoleucine, L-serine, L-methionine, L-arginine-HCl, L-phenylalanine, L-cysteine-HCl, L-histidine-HCl, and L-glutamic acid. The pH of all media was adjusted to 7.0 when necessary, and 38-ml quantities were dispensed into 125-ml Erlenmeyer flasks. The media were autoclaved at 121 C for 15 min, and 2 ml of a filter-sterilized 20% solution of glucose were added to each flask. The media were inoculated with either *P. caryophylli* or *Corynebacterium* sp. by adding 0.5 ml of the appropriate inoculum to the flasks. The flasks were incubated at 30 C in the Metabolyte shaker (120 strokes/min). At 48 hr after inoculation, 5 ml of each culture were centrifuged at 5,000 *g* for 30 min and the pellet was washed twice with sterile distilled water and resuspended in 5 ml of distilled water. The absorbance of the suspension at 620 nm was determined, and absorbance values were converted to dry wt from standard curves prepared for each bacterium.

The effect of various concentrations of amino nitrogen on growth of *Corynebacterium* sp. was also determined. Casein hydrolysate was added to the basal medium to give final concentrations of 50 μg/ml, 100 μg/ml, and 500 μg/ml of amino nitrogen. Portions of the media (38 ml) were dispensed into 125-ml Erlenmeyer flasks and autoclaved at 121 C for 15 min; then 2 ml of the glucose solution were added. The media were inoculated with 0.5 ml of *Corynebacterium* sp. inoculum and incubated at 30 C in the Metabolyte shaker. At 12, 24, 36, and 48 hr after inoculation, 5 ml of each culture were removed and growth was determined as above.

Maceration of carnation tissue by culture filtrates of Corynebacterium sp. which contain cellulase and endo-PGTE.—Five g of sodium polypectate were dissolved in 1 liter of nutrient broth (Difco) at 70 C. The pH of the medium was adjusted to 7.0 with 1 N NaOH; then 200-ml quantities of the medium were dispensed into 500-ml Erlenmeyer flasks. The medium was autoclaved for 20 min at 121 C, and 1 ml of *Corynebacterium* sp. inoculum was added to each flask. At 72 hr after incubation at 30 C in the Metabolyte shaker (120 strokes/min), the cultures were centrifuged at 5,000 *g* for 30 min and the culture filtrates were combined and freeze-dried. The freeze-dried material was stored at 6 C over calcium chloride. A suspension containing 2.8 g of the freeze-dried material in 100 ml of distilled water was centrifuged at 10,000 *g* for 15 min at 6 C and the culture filtrate was collected and used as a source of endo-PGTE and cellulase for maceration tests. The cellulolytic and endo-PGTE activity and ability to macerate carnation tissue at various pH levels was determined for the culture filtrate. The pH levels were maintained with 0.1 M acetate buffer (below pH 6.0), [tris (hydroxymethyl) amino methane]-HCl buffer 0.1 M phosphate buffer (pH 6.0-7.0), and 0.1 M Tris-(pH 8.0-9.0). Cellulase and endo-PGTE activities were determined viscometrically in Fenske-Ostwald viscometers (size 300) at 30 C. The reaction mixture for each endo-PGTE assay contained 3 ml of 1.2% sodium polypectate (Lot 14505, K + K Laboratories, Plainview, N.Y.) in buffer, 2 ml of distilled water, and 1 ml of culture filtrate. The reaction mixture for the determination of cellulase activity was the same as that described for endo-PGTE assays except that 3 ml of 1.2% carboxymethylcellulose (CMC, type 7-MP, Cellulose Gum, Lot 41952, Hercules Powder Co., Delaware, Md.) in buffer served as substrate. Activity of both enzymes was expressed as relative activity units (16). The reaction mixture for maceration tests contained 5 g of carnation leaf pieces (1.0 × 0.3 cm), 10 ml of culture filtrate, and 10 ml of buffer, and was contained in a 125-ml Erlenmeyer flask. The flasks were incubated on a reciprocating shaker (140 strokes/min) at 28 ± 2 C, and maceration was determined by the chlorophyll method (4). The effect of calcium chloride and EDTA on maceration of carnation leaf tissue at pH 8.6 was also determined. Calcium chloride or EDTA was added to reaction mixture for maceration tests to give final concentrations of 10^{-3} M and 3×10^{-5} M, respectively. Reaction mixtures without calcium chloride or EDTA and reaction mixtures containing culture filtrate, autoclaved for 15 min at 121 C, were used as controls. The flasks were incubated on the reciprocating shaker, and maceration was determined at 0, 15, 30, 45, and 60 min.

RESULTS. — *Production of endopolygalacturonate trans-eliminase (endo-PGTE) and cellulase and maceration of carnation tissue.*—At 12, 24, 36 and 48 hr after inoculating leaf samples with *P. caryophylli* alone, *Corynebacterium* sp. alone, or both bacteria, each leaf sample was homogenized with its bathing solution in a mortar. The tissue homogenate was filtered through four layers of cheesecloth, and the filtrate centrifuged at 14,000 *g* for 20 min at 6 C. Cellulase and endo-PGTE activity of the supernatant was determined viscometrically at pH 6.0 and 8.6, respectively, by the procedures described above. Maceration of leaf samples was also determined.

The endo-PGTE activity of supernatants from non-inoculated tissue, tissue inoculated with *P. caryophylli* alone, or tissue inoculated with *Corynebacterium* sp. alone was negligible. In contrast, endo-PGTE activity was detected in extracts from tissue inoculated with

TABLE 1. Maceration, synthesis of endopolygalacturonate *trans*-eliminase (endo-PGTE) and cellulase in carnation leaf samples after simultaneous inoculation with *Pseudomonas caryophylli* and *Corynebacterium* sp.

Hr after inoculation	Enzymatic activity[a]		
	Cellulase	endo-PGTE	Maceration[b]
12	0	0	0
24	0	33	0
36	12.5	1,428	12.2
48	66.6	3,360	38.5

[a] Enzymatic activity was expressed as relative activity units which represent the reciprocal of the time in min × 10^3 required for a 50% loss in the viscosity of a reaction mixture (16).

[b] Maceration was determined by the chlorophyll method (4) and expressed as μg chlorophyll/ml.

both bacteria at 24, 36, and 48 hr after inoculation (Table 1). The activity increased from 33 relative activity units at 24 hr after inoculation to 3,360 relative activity units at 48 hr after inoculation. Cellulase activity was detected only at 36 and 48 hr after inoculation, and was less than that observed for endo-PGTE. As was previously reported (4), maceration occurred only at 36 and 48 hr after inoculation when leaf tissue was inoculated with both bacteria. The data suggest that maceration was associated with the occurrence of significant levels of endo-PGTE and cellulase in this tissue.

Populations of P. caryophylli and Corynebacterium sp. in carnation leaf samples.—The number of cells of *P. caryophylli* and *Corynebacterium* sp. in carnation leaf samples inoculated with *P. caryophylli* alone, *Corynebacterium* sp. alone, or with both bacteria was determined at 12, 24, 36, and 48 hr after inoculation. At 12 and 24 hr after inoculation, each leaf sample was removed from the bathing solution and washed with 50 ml of a 20% solution of commercial Clorox (5.25% sodium hypochlorite) and rinsed with three changes of sterile distilled water. Each sample was then transferred to a sterilized mortar and homogenized with 20 ml of sterile distilled water. The number of bacteria in the tissue homogenate was ascertained by plating serial dilutions with potato-dextrose agar (PDA). When tissue was inoculated with both bacteria, the serial dilutions from leaf samples were plated with PDA and with PDA containing 20 μg/ml of penicillin G (1,585 units/mg, Nutritional Biochemicals Corp., Cleveland, Ohio). The population of each bacterium in dilutions was determined with these media because growth of *Corynebacterium* sp. was inhibited by penicillin. Consequently, all colonies which developed on the medium containing penicillin were attributed to *P. caryophylli*, while colonies which developed in the absence of penicillin were attributed to both bacteria. Dilution plates were incubated at 30 C for 72 hr. Leaf samples inoculated with both bacteria were macerated at 36 and 48 hr after inoculation. This made it impossible to distinguish between bacterial cells inside the leaf samples and those in the bathing solutions; consequently, each inoculated leaf sample at these time periods was homogenized with its bathing solution, and the total bacterial population of both leaf sample and bathing solution was determined.

At 12 and 24 hr after inoculation, the population of *Corynebacterium* sp. was considerably greater in leaf samples inoculated with both bacteria than in leaf samples inoculated with this organism alone (Table 2). In contrast, the population of *P. caryophylli* in leaf samples inoculated with both bacteria was only slightly greater than that in samples inoculated with the pathogen alone. These results suggest that in leaf samples inoculated with both bacteria, growth of *Corynebacterium* sp. is increased by the presence of *P. caryophylli* whereas growth of *P. caryophylli* is only slightly affected by *Corynebacterium* sp. At 36 and 48 hr after inoculation, the population of *P. caryophylli* in leaf samples and bathing solutions, inoculated with this organism alone, was greater than in samples and bathing solutions inoculated with both bacteria. On the contrary, the population of *Corynebacterium* sp. was greater when samples and bathing solutions were inoculated with both bacteria than when they were inoculated with *Corynebacterium* sp. alone.

TABLE 2. Population of *Pseudomonas caryophylli* and *Corynebacterium* sp. in carnation leaf samples at 12 and 24 hr after inoculation and in both leaf samples and bathing solutions at 36 and 48 hr after inoculation with either *P. caryophylli* alone, *Corynebacterium* sp. alone, or with both bacteria

	No. cells $\times 10^6$			
	P. caryophylli		*Corynebacterium* sp.	
Hr after inoculation	P.c.[a]	P.c. + C.sp.[b]	C.sp.[c]	P.c. + C.sp.
12[d]	3.18	3.90	0.00012	0.37
24[d]	13.60	15.70	0.00078	8.60
36[e]	29.00	26.00	1.12	1,180.00
48[e]	146.00	42.60	1.43	1,360.00

[a] P.c. = inoculated with *P. caryophylli* alone.
[b] P.c. + C.sp. = inoculated with *P. caryophylli* and *Corynebacterium* sp.
[c] C.sp. = inoculated with *Corynebacterium* sp. alone.
[d] Bacterial population of leaf sample only.
[e] Bacterial population of leaf sample and bathing solution.

Accumulation of amino nitrogen, reducing sugar, and various inorganic elements in bathing solutions of carnation leaf samples.—At 12, 24, 36, and 48 hr after inoculating leaf samples (5 g) with *P. caryophylli* or *Corynebacterium* sp., the bathing solutions were centrifuged at 5,000 *g* for 30 min. The supernatant was filtered through a Nalgene membrane filter (0.2 μ), and the filtrate was analyzed for amino nitrogen by the method of Moore & Stein (11), with L-leucine as a standard, for reducing sugar with dinitrosalicylic acid (10), and for Na, K, Ca, Mg, Zn, Fe, Mn, and P with a Photoelectric spectrograph (Model 29000, Appl. Res. Lab., Pasadena, Calif.). There was little detectable difference in the concentration of phosphorus or potassium in bathing solutions of carnation leaf samples inoculated with *Corynebacterium* sp. or noninoculated samples (Fig. 1). In contrast, the concentration of these elements in bathing solutions of leaf samples inoculated with *P. caryophylli* had markedly increased at 36 and 48 hr after inoculation. The concentration of phosphorus and potassium in bathing solutions at 48 hr after inoculation was approx 8 and 20 times greater, respectively, for samples inoculated with *P. caryophylli* than for samples inoculated with *Corynebacterium* sp. or noninoculated samples. There was no significant difference in the concentrations of Ca, Na, Fe, Mg, Zn, or Mn in bathing solutions of inoculated or noninoculated leaf samples. While amino nitrogen was scarcely detectable in bathing solutions of leaf samples inoculated with *Corynebacterium* sp. and noninoculated samples, the concentration of amino nitrogen in bathing solutions of samples inoculated with *P. caryophylli* had successively increased at 24, 36, and 48 hr after inoculation. When carnation leaf samples were inoculated with *P. fluorescens,* amino nitrogen was not detected in bathing solutions at 12, 24, 36, or 48 hr after inoculation. When *P. syringae* was used as inoculum, however, amino nitrogen was not detectable at 12 and 24 hr after inoculation, but the bathing solutions contained 2 μg/ml and 9 μg/ml at 36 and 48 hr after inoculation, respectively. Thus, *P. caryophylli* caused a greater accumulation of amino nitrogen in bathing solutions of leaf samples than did *P. fluorescens* or *P. syringae.*

The concentration of amino nitrogen, reducing sugar, and protein in noninoculated leaf samples or samples inoculated with P. caryophylli or Corynebacterium sp.—At 48 hr after inoculation the bathing solution was removed, and 50 ml of 95% ethanol were added to each inoculated and noninoculated leaf sample. The flasks were closed with rubber stoppers to prevent loss of ethanol, and were incubated at 60 C in the Metabolyte shaker. The ethanol extract was collected after 4 hr and evaporated to dryness at 40 C with a flash evaporator and the residue was suspended in 10 ml of distilled water. The suspension was centrifuged at 5,000 *g* for 30 min, the supernatant was shaken with an equal volume of chloroform, and the upper aqueous phase analyzed for amino nitrogen and reducing sugar. After alcohol extraction, the tissue was ground with 100 ml of phosphate buffer (0.1 M, pH 6.8) in a Waring Blendor at high speed for 2 min. The tissue homogenate was squeezed through several layers of cheesecloth, and the filtrate centrifuged at 12,000 *g* for 30 min. The supernatant was analyzed for protein by the Folin method (9).

Leaf samples inoculated with *P. caryophylli* contained considerably less amino nitrogen at 48 hr after inoculation than did samples inoculated with *Corynebacterium* sp. or noninoculated samples (Table 3); in contrast, the protein content of all samples was similar. The insignificant differences in the protein content of these samples suggest that the amino nitrogen observed in bathing solutions of leaf samples inoculated with *P. caryophylli* (Fig. 1) was derived from the amino nitrogen pool of the tissue and not from the degradation of proteins. Although there was less reducing sugar in leaf samples inoculated with *P. caryophylli* than in samples inoculated with *Corynebacterium* sp. or noninoculated samples at 48 hr after inoculation, results of tests for reducing sugar in bathing solutions of all samples were negative. The absence of an accumulation of reducing sugar in bathing solutions of samples inoculated with *P. caryophylli* alone, indicate that the decreased sugar concentration in this tissue was due to utilization of reducing sugar by the bacterium. This suggestion is supported by previous studies which showed that *P. caryophylli* caused a rapid depletion of the reducing sugar content of a carnation extract medium (5).

TABLE 3. Comparison of concentrations of amino nitrogen, reducing sugar, and protein in noninoculated carnation leaf samples and samples 48 hr after inoculation with *Pseudomonas caryophylli* or *Corynebacterium* sp.

	Concentration[a] (mg/g fresh wt)		
	Noninoculated	*Corynebacterium* sp.	*P. caryophylli*
Amino nitrogen[b]	1.13 ± .11	1.01 ± .10	0.029 ± .005
Reducing sugar[c]	2.27 ± .07	2.23 ± .06	0.61 ± .09
Protein[d]	6.69 ± .16	6.64 ± .18	6.40 ± .12

[a] Mean of five determinations ± standard error of the mean.
[b] Amino nitrogen was determined with ninhydrin (11) and expressed as mg of L-leucine equivalents/g.
[c] Reducing sugar was determined by the dinitrosalicylic acid method (10) and expressed as mg glucose equivalents/g.
[d] Protein was determined by the Folin method (9) and expressed as mg bovine serum albumin equivalents/g.

Relation of inorganic and amino nitrogen to growth and synthesis of endo-PGTE by Corynebacterium sp.—At 12, 24, 36, and 48 hr after inoculation, the bathing solutions from leaf samples (5 g) inoculated with *P. caryophylli* and noninoculated samples were centrifuged at 5,000 *g* and filtered through a Nalgene membrane filter (0.2 μ), and the amino nitrogen content of the filtrate was determined. The remainder of the filtrate was adjusted to pH 7.0 with 1 N NaOH, and 20-ml quantities were placed into 125-ml Erlenmeyer flasks. After autoclaving at 121 C for 15 min, filter-sterilized glucose solution was added to give a final concentration of 10 mg/ml, and the filtrates were inoculated with 0.1 ml of *Corynebacterium* sp. inoculum. After incubation at 30 C in the Metabolyte shaker for 48 hr, the cultures were centrifuged at 5,000 *g* for 30 min. The endo-PGTE activity of the supernatant at pH 8.6 was determined viscometrically. The pellet was washed twice with distilled water and resuspended

TABLE 4. Growth of *Corynebacterium* sp. and production of endopolygalacturonate *trans*-eliminase (endo-PGTE) in bathing solutions at 48 hr after inoculation

	Time (hr) at which bathing solutions were collected from carnation leaf samples after inoculation with *Pseudomonas caryophylli* or from noninoculated samples							
	12		24		36		48	
	N[a]	P.c.[b]	N	P.c.	N	P.c.	N	P.c.
Growth[c]	0.002	0.005	0.005	0.15	0.006	0.23	0.006	0.42
Endo-PGTE[d]	0.0	0.0	0.0	186.5	0.0	520.0	0.0	860.0

[a] N = noninoculated.
[b] P.c. = inoculated with *P. caryophylli.*
[c] Growth was expressed as mg (dry wt)/ml.
[d] Endo-PGTE activity was expressed as relative activity units (16).

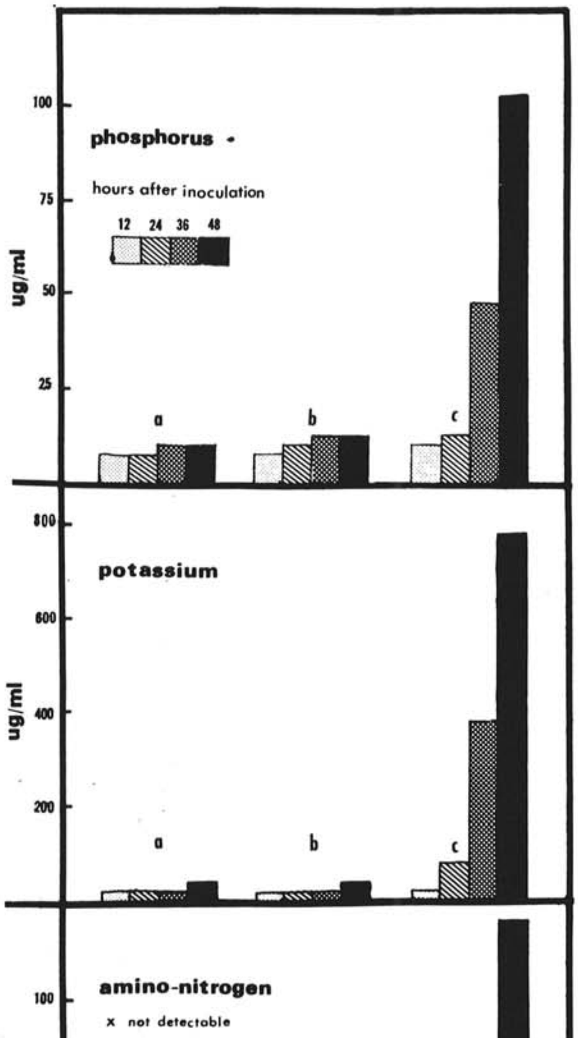

Fig. 1. Changes in the concentration of phosphorus, potassium, and amino nitrogen in bathing solutions from (a) noninoculated carnation leaf samples; samples inoculated with (b) *Corynebacterium* sp.; or (c) *Pseudomonas caryophylli* at 12, 24, 36, and 48 hr after inoculation.

in 20 ml distilled water and absorbance determined at 620 nm. Absorbance readings were converted to dry wt from a standard curve.

Growth of *Corynebacterium* sp. was greater at 48 hr in bathing solutions of leaf samples collected at 24, 36, and 48 hr after inoculation with *P. caryophylli* than in bathing solutions of noninoculated samples (Table 4). While endo-PGTE activity was not detected when *Corynebacterium* sp. grew in bathing solutions of noninoculated leaf samples, activity was detected in bathing solutions of samples collected 24, 36 and 48 hr after inoculation with *P. caryophylli.* The amino nitrogen concentrations of these bathing solutions were similar to those previously reported for bathing solutions of noninoculated leaf samples and samples inoculated with *P. caryophylli* (Fig. 1); therefore, the results suggest that the increased growth of *Corynebacterium* sp. and subsequent endo-PGTE synthesis in the bathing solution of leaf samples inoculated with *P. caryophylli* is due to the increased amino nitrogen content of the bathing solutions. *P. caryophylli* grew in the basal medium containing glucose and either amino or inorganic nitrogen, but growth of the bacterium in the medium containing amino nitrogen was markedly greater than in media with inorganic nitrogen (Table 5). On the contrary, *Corynebacterium* sp. grew only with amino nitrogen. Growth of both bacteria was greater with a mixture of amino acids than with casein hydrolysate as source of amino nitrogen. The growth of *Corynebacterium* sp. at 24, 36, and 48 hr after inoculation was increased by increasing the concentration of amino nitrogen in the basal medium (Fig. 2). These results support previous conclusions that increased growth of *Corynebacterium* sp. in bathing solutions from tissue inoculated with *P. caryophylli* is due to the higher concentration of amino nitrogen.

The effect of amino acids and glucose on the maceration of carnation leaf tissue by Corynebacterium sp.—Leaf samples were placed into 125-ml Erlenmeyer flasks containing 20 ml of a sterilized solution of either glucose (1%), the amino acid medium, or the amino acid medium plus glucose (1%), and were inoculated with *Corynebacterium* sp. In other flasks, leaf samples were placed into 20 ml of sterile distilled water and were simultaneously inoculated with both *P. caryophylli* and *Corynebacterium* sp. All flasks were incubated on the Metabolyte shaker, and maceration was determined at 12, 24, 36, 48, and 60 hr after inoculation. Maceration was detected for leaf samples incubated with the

TABLE 5. Growth of *Pseudomonas caryophylli* and *Corynebacterium* sp. in a basal medium containing glucose and various sources of nitrogen 48 hr after inoculation

Addition to basal medium	Growth (mg dry wt/40 ml) *P. caryophylli*	*Corynebacterium* sp.
Glucose	0.0	0.0
Glucose + $(NH_4)_2SO_4$	24.12	0.0
Glucose + KNO_3	62.34	0.0
Glucose + Casein hydrolysate	105.32	16.41
Glucose + amino acids	128.60	36.80

amino acid medium plus glucose at 24 hr after inoculation (Fig. 3), while samples incubated with glucose alone or amino acid medium alone commenced to macerate at 48 hr after inoculation. Maceration was detected for samples inoculated with both bacteria at 36 hr after inoculation. These results suggest that car-

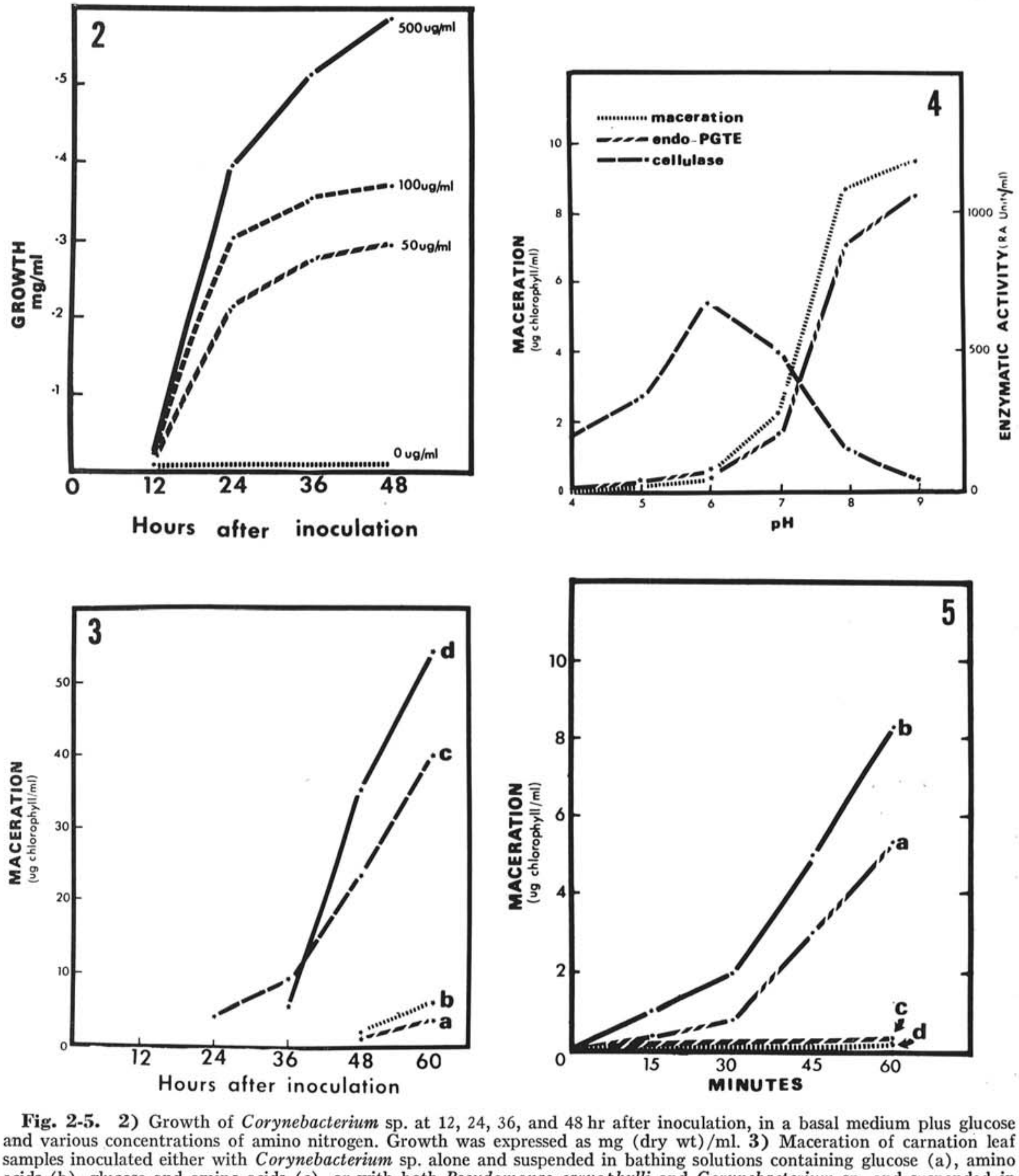

Fig. 2-5. **2)** Growth of *Corynebacterium* sp. at 12, 24, 36, and 48 hr after inoculation, in a basal medium plus glucose and various concentrations of amino nitrogen. Growth was expressed as mg (dry wt)/ml. **3)** Maceration of carnation leaf samples inoculated either with *Corynebacterium* sp. alone and suspended in bathing solutions containing glucose (a), amino acids (b), glucose and amino acids (c), or with both *Pseudomonas caryophylli* and *Corynebacterium* sp. and suspended in sterile distilled water (d). **4)** The effect of pH on the cellulolytic and endopolygalacturonate *trans*-eliminase (endo-PGTE) activity and macerating ability of culture filtrates from *Corynebacterium* sp. **5)** Maceration of carnation tissue by culture filtrates which contain cellulase and endopolygalacturonate *trans*-eliminase of *Corynebacterium* sp. Reaction mixtures contained 5 g of carnation leaf tissue, 10 ml of 0.1 M Tris-HCl buffer (pH 8.6), and 10 ml of culture filtrate (a), and was regulated to contain either 10^{-3} M $CaCl_2$ (b) or 3×10^{-5} M EDTA (c). Culture filtrate autoclaved for 15 min at 121 C was used as control (d). Reaction mixtures were incubated at 28 ± 2 C on a reciprocating shaker (140 strokes/min) and maceration was determined by the chlorophyll method (4).

nation leaf tissue can be rapidly macerated by *Corynebacterium* sp. alone if the nutrients required for the growth of this bacterium are available.

Maceration of carnation tissue by culture filtrates of Corynebacterium sp.—Carnation leaf tissue was macerated by culture filtrates of *Corynebacterium* sp. which contained both cellulase and endo-PGTE activity (Fig. 4). Maceration of tissue paralleled the endo-PGTE activity of the culture filtrate, but was apparently not related to their cellulolytic activity. Previous studies showed that the activity of *Corynebacterium* sp. endo-PGTE was stimulated by calcium chloride and inhibited by EDTA (3). In the present study, tissue maceration was also stimulated by calcium chloride and inhibited by EDTA (Fig. 5). The results of these studies suggest that the endo-PGTE of *Corynebacterium* sp. is the macerating agent in carnation tissue inoculated with *P. caryophylli* and *Corynebacterium* sp.

Maceration of carnation leaf tissue by various combinations of bacteria.—Carnation leaf samples were inoculated with either *P. caryophylli*, *P. syringae*, or *P. fluorescens*, and then with either *P. marginalis*, *B. polymyxa*, *E. carotovora*, or *Corynebacterium* sp. Leaf samples inoculated with each bacterium singly were used as controls. The inoculated samples were incubated in the Metabolyte shaker, and maceration was determined visually. At 48 hr after inoculation, carnation leaf samples were macerated only when inoculated simultaneously with *P. caryophylli* and either *Corynebacterium* sp. or *B. polymyxa*. The reason for an absence of rapid tissue maceration in other inoculated leaf samples was not determined.

Discussion.—Our previous studies showed that carnation leaf tissue is rapidly macerated when simultaneously inoculated with *Pseudomonas caryophylli* and *Corynebacterium* sp., but not when inoculated with either bacterium alone (3, 4). In addition, *Corynebacterium* sp. produced an endopolygalacturonate *trans*-eliminase (endo-PGTE) and a cellulase, while synthesis of pectic or cellulolytic enzymes was not demonstrated for *P. caryophylli* (5). In this study, growth of *Corynebacterium* sp. and synthesis of endo-PGTE were considerably greater in carnation tissue inoculated with both bacteria than in tissue inoculated with *Corynebacterium* sp. alone (Tables 1, 2). Several investigators have demonstrated that pectic *trans*-eliminases are primary agents of tissue maceration in diseased plants (2, 7, 8, 12, 15). Culture filtrates of *Corynebacterium* sp., containing both endo-PGTE- and cellulase, macerated carnation leaf tissue (Fig. 4). Maceration paralleled the endo-PGTE activity of culture filtrates, but was apparently unrelated to the cellulolytic activity. Moreover, maceration was stimulated by calcium chloride and inhibited by EDTA (Fig. 5). Calcium chloride stimulates and EDTA inhibits the activity of the endo-PGTE of *Corynebacterium* sp. (3) and several other *trans*-eliminases (2, 13, 14). Therefore, it is concluded that the endo-PGTE of *Corynebacterium* sp. is the primary agent in the maceration of carnation leaf tissue.

Previous studies indicated that the permeability of carnation leaf cell membranes was increased when tissue was inoculated with *P. caryophylli*, but not when inoculated with *Corynebacterium* sp. (6). Apparently, cellular permeability alterations result in the release of nutrients from plant cells which promote growth of *Corynebacterium* sp. in carnation leaf tissue inoculated with both bacteria. This suggestion is supported by several lines of evidence. Carnation leaf tissue, inoculated with *P. caryophylli*, contained considerably less amino nitrogen and reducing sugar than was contained in tissue inoculated with *Corynebacterium* sp. or noninoculated tissue at 48 hr after inoculation (Table 3). In addition, bathing solutions from *P. caryophylli*-infected tissue contained more amino nitrogen, phosphorus, and potassium, and supported greater growth of *Corynebacterium* sp. than did bathing solutions from noninoculated tissue (Fig. 1, Table 4). *Corynebacterium* sp. requires amino nitrogen for growth in a basal medium supplemented with glucose (Table 5), and growth of the organism is related to the amino nitrogen concentration of the medium (Fig. 2). Consequently, the amino nitrogen and reducing sugar released from plant cells inoculated with *P. caryophylli* would appear to be very essential nutritional requirements for growth of *Corynebacterium* sp.

The data presented in these studies suggest that carnation leaf tissue is rapidly macerated when inoculated with *P. caryophylli* and *Corynebacterium* sp., because *P. caryophylli* alters the cellular permeability of the leaf tissue; this results in the release of nutrients which stimulate growth of *Corynebacterium* sp. and subsequent synthesis of endo-PGTE, which macerates carnation tissue. The suggestion is supported by the occurrence of rapid tissue maceration by *Corynebacterium* sp. alone, when amino nitrogen and glucose are supplied in the bathing solutions (Fig. 3). The evidence indicates that although *Corynebacterium* sp. has the potential to macerate carnation tissue, the unavailability of nutrients for growth limits this potential when tissue is inoculated with this bacterium alone. In addition, the inability of *Corynebacterium* sp. to macerate carnation tissue simultaneously inoculated with *P. fluorescens* or *P. syringae* may be a reflection of the inability of these bacteria to release sufficient nutrients from carnation leaf cells for growth of *Corynebacterium* sp. and, subsequently, synthesis of endo-PGTE. This suggestion is partially supported by the low concentration of amino nitrogen detected in bathing solutions of tissue inoculated with these bacteria.

Endopolygalacturonate *trans*-eliminase production has been reported for *E. carotovora* (12, 14, 15), *B. polymyxa* (14), *P. marginalis* (13), and *Corynebacterium* sp. (5). Only *B. polymyxa* and *Corynebacterium* sp. caused the rapid maceration of carnation tissue simultaneously inoculated with *P. caryophylli*. This differential ability of the bacteria to rapidly macerate carnation tissue may be due to their ability to grow and produce pectic enzymes under the experimental conditions.

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