Permeability Alterations in Detached Carnation Leaf Tissue Inoculated with *Pseudomonas caryophylli* and *Corynebacterium* sp.

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Number 7 in a series of studies on *P. caryophylli* in carnation.

Accepted for publication 1 October 1970.

ABSTRACT

Radioactive phosphate (^{32}P) was incorporated into the phosphate compounds of detached carnation leaf tissue when this tissue was vacuum-infiltrated with a solution of $K_2H^{32}PO_4$. Infiltrated leaf tissue was placed into flasks containing sterile distilled water as the bathing solution, and was inoculated with either *Pseudomonas caryophylli* or *Corynebacterium* sp. Four phosphate fractions were obtained by partitioning the phosphate compounds of inoculated and noninoculated tissue. These contained, respectively, soluble esters and inorganic phosphate, ribonucleic acids, deoxyribonucleic acids, and phospholipid and phosphoprotein. At 48 hr after inoculation, the radioactivity of the phosphate fractions obtained from tissue inoculated with *P. caryophylli* was less than that of fractions from noninoculated tissue or tissue inoculated with *Corynebacterium* sp.

An accumulation of ^{32}P in bathing solutions occurred 12 hr after inoculating tissue with *P. caryophylli* but not with *Corynebacterium* sp. Approximately 10.3% and 53.2% of the ^{32}P initially present in the tissue was detected in bathing solutions at 24 and 48 hr after inoculation with *P. caryophylli*. Accumulation of ^{32}P in bathing solutions was inhibited by inhibiting growth of *P. caryophylli* at 12 hr after inoculation but not at 24 hr after inoculation. Mercaptoethanol and sodium *p*-chloromercuribenzoate caused a loss of ^{32}P from infiltrated tissue, while extracts from *P. caryophylli*-infected tissue did not. It was concluded that *P. caryophylli* caused an increase in the cellular permeability of detached carnation leaf tissue, while *Corynebacterium* sp. did not. Phytopathology 61:317-321.

Previous studies indicated that detached carnation (*Dianthus caryophyllus* L.) leaf tissue was rapidly macerated when simultaneously inoculated with *Pseudomonas caryophylli,* causal agent of bacterial wilt of carnation, and a species of *Corynebacterium,* but not when inoculated with either organism alone (2). Maceration occurred more rapidly when leaf tissue was first inoculated with *P. caryophylli* and 24 hr later with *Corynebacterium* sp. than when the sequence of inoculations was reversed. Maceration of plant tissue has often been associated with the production of pectic enzymes by pathogens (1). While *Corynebacterium* sp. produced endopolygalacturonate *trans*-eliminase, synthesis of pectic enzymes was not demonstrated for *P. caryophylli* (3). It was concluded from these studies that *Corynebacterium* sp. produced the agent responsible for maceration of carnation tissue inoculated with both bacteria. The role of *P. caryophylli* in the process remained obscure. It has been demonstrated that phytopathogenic bacteria alter the permeability of plant cell membranes, while saprophytic bacteria do not (4, 7). This study was undertaken to determine the effect of *P. caryophylli* and *Corynebacterium* sp. on the cellular permeability of detached carnation leaf tissue as an approach to the elucidation of the mechanism of rapid tissue maceration in carnation inoculated with both bacteria.

MATERIALS AND METHODS.—*Pseudomonas caryophylli* (isolate B-1) and *Corynebacterium* sp. were grown in nutrient broth (Difco) plus 1.0% glucose. Broth cultures were incubated for 24 hr at 30 C in a Metabolyte water bath shaker operated at 120 strokes/min. The cultures were centrifuged at 5,000 *g* for 30 min, and the pellets were washed twice with sterile distilled water. Water suspensions of washed cells containing approx 10^6 cells/ml were used as inoculum. The three pairs of fully expanded leaves immediately below the apical region of uniform carnation cuttings (*Dianthus caryophyllus* 'White Pikes Peak CSU') were collected and leaf pieces, 2 cm long, were cut from their central portions and washed as previously described (2). Twenty g of washed leaf pieces were immersed in 200 ml of a filter-sterilized solution of $K_2H^{32}PO_4$ (0.12 μc/ml, specific activity 20 mc/mM) in a 500-ml sterilized beaker. The beaker was placed in a vacuum desiccator which was evacuated for 10 min (Hg reading = 60 mm). The phosphate solution was infiltrated into the leaf pieces by slowly reducing the vacuum. The residual phosphate solution was removed from the beaker, and the tissue was washed twice with 200 ml of sterile distilled water. The leaf pieces were placed on paper towels in a sterile transfer chamber (28 ± 2 C, 700 ft-c) for 4 hr, after which evaporation of the liquid from the surface and intercellular spaces appeared to be complete. Leaf samples were composed of 2 g of randomly selected leaf pieces. The amount of ^{32}P contained in leaf samples was determined. Each of four 2-g leaf samples was ground with 10 ml of 10 N HCl. The tissue homogenate was filtered through four layers of cheesecloth, and the filtrate was centrifuged at 5,000 *g* for 30 min. One-milliliter samples of the supernatant were placed in steel planchets and dried at room temp. Radioactivity of all samples was determined with a gas flow detector (Nuclear Chicago, Model 470) as counts per min (cpm).

Leaf samples of ^{32}P-infiltrated leaf pieces were placed into 125-ml Erlenmeyer flasks that contained 20 ml of sterile distilled water as bathing solution, and

were inoculated with either *P. caryophylli* or *Corynebacterium* sp. by adding 1 ml of the appropriate inoculum to the bathing solution. One ml of sterile distilled water was added for leaf samples not inoculated with bacteria. All inoculated and noninoculated samples were immediately incubated at 30 C in the Metabolyte shaker operating at 120 strokes/min. At 30 min, 12, 24, 36, and 48 hr after inoculation, two 1-ml samples of the bathing solution were removed from each leaf sample and placed into steel planchets, dried, and their radioactivity was determined. The compounds containing phosphate in leaf samples were partitioned into four fractions by the method of Loughman & Martin (10) as modified by Loughman & Russell (11). Each sample was extracted with 10 ml of the appropriate solvent at each stage in the extraction, and the radioactivity of 1-ml samples of the extract was determined.

The effect of sodium *p*-chloromercuribenzoate (PCMB) or mercaptoethanol on the loss of ^{32}P from leaf samples was determined by placing ^{32}P-infiltrated leaf samples into 20 ml of a solution of PCMB or mercaptoethanol contained in sterilized 125-ml Erlenmeyer flasks. The flasks were incubated at 30 C in the Metabolyte shaker (120 strokes/min), and 1-ml samples of the solution were removed and assayed for radioactivity at 12, 24, 36, and 48 hr.

The effect of tetracycline hydrochloride on the loss of ^{32}P from carnation tissue inoculated with *P. caryophylli* was also determined. One ml of a filter-sterilized solution of tetracycline hydrochloride (2 mg/ml, 980 mcg/mg, Nutritional Biochemicals Corp., Cleveland, Ohio) was added to the bathing solution of different leaf samples at 12 and 24 hr after inoculation. After the addition of tetracycline hydrochloride, the flasks were immediately placed into the vacuum desiccator and the antibiotic was infiltrated into the leaf pieces as described above. The flasks were returned to the shaker, and the radioactivity of 1-ml samples of the bathing solution of all leaf samples was determined 12, 24, 36, and 48 hr after inoculation.

Water extracts of *P. caryophylli*-infected tissue were prepared by blending four leaf samples which had not been infiltrated with ^{32}P and their bathing solutions, 48 hr after inoculation with *P. caryophylli,* 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 15,000 *g* for 30 min at 6 C. The supernatant was filtered through Nalgene membrane filters (0.2 μ), and 10 ml of filtrate were placed into sterilized 125-ml Erlenmeyer flasks. Leaf samples, previously infiltrated with ^{32}P, were added to filtrate autoclaved at 121 C for 15 min, to nonautoclaved filtrate, and to flasks containing 10 ml of sterile distilled water. The leaf samples in water were either inoculated with *P. caryophylli* or noninoculated. The radioactivity of the filtrate or bathing solution was determined at 30 min, 24 and 48 hr after incubation at 30 C in the Metabolyte shaker.

Populations of *P. caryophylli* in inoculated leaf samples were determined by the dilution plate method. At 12, 24, 36, and 48 hr after inoculation, noninfiltrated leaf samples were 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 transferred to a sterilized mortar and thoroughly ground with 10 ml of sterile distilled water. The number of bacteria in the tissue homogenate was ascertained by plating serial dilutions with potato-dextrose agar. In another series, 1 ml of the tetracycline hydrochloride solution was added to the bathing solution of leaf samples at 12 and 24 hr after inoculation. The antibiotic was vacuum-infiltrated into the tissue, and the bacterial population of the tissue samples was determined at 12, 24, 36, and 48 hr after inoculation.

RESULTS.—Determination of ^{32}P uptake by several 2-*g* samples of carnation leaf pieces showed that when these samples were completely submerged and infiltrated slowly with the phosphate solution, the activity of ^{32}P in each sample was relatively constant (17,241 ± 213 cpm/ml). The radioactivity of the bathing solutions of both inoculated and noninoculated leaf samples of ^{32}P-infiltrated tissue was about 550 cpm/ml at 30 min after inoculation (Fig. 1). This activity was probably derived from residual ^{32}P washed from the surface and intercellular spaces of the leaf tissue. The activity of the bathing solutions of leaf samples inoculated with *Corynebacterium* sp. or noninoculated samples decreased during incubation and was 96 and 82 cpm/ml, respectively, at 48 hr after inoculation. This decreased radioactivity was very possibly due to absorption of ^{32}P by the leaf tissue. In contrast, bathing solutions of leaf samples inoculated with *P. caryophylli* showed increased radioactivity. The activity of the bathing solution at 24, 36, and 48 hr after inoculation was 1,145, 2,696 and 4,459 cpm/ml, respectively.

The percentage of radioactive phosphate in the DNA fraction and in the phospholipid and phosphoprotein fraction showed slight variation for both noninoculated leaf samples and samples inoculated with *Corynebacterium* sp. at 24 and 48 hr after inoculation (Table 1). The radioactivity of the soluble esters and inorganic phosphate fraction for these samples, however, had decreased between 24 and 48 hr after inoculation. This decreased radioactivity was probably related to the increase in the radioactivity of the ribonucleic acid fraction of the samples. At 24 hr after inoculation with *P. caryophylli,* the radioactivity of the ribonucleic acid fraction of leaf samples was slightly greater, and that of the soluble esters and inorganic phosphate fraction less, than was obtained for samples inoculated with *Corynebacterium* sp. or noninoculated samples; however, the radioactivity of all fractions extracted from samples inoculated with *P. caryophylli* had markedly decreased. The most significant decrease occurred in the soluble esters and inorganic phosphate fraction, which had decreased from 52.1% at 24 hr to 24.9% at 48 hr after inoculation.

The foregoing results suggest that *P. caryophylli* apparently caused an alteration in the permeability of carnation tissue resulting in a loss of intracellular constituents. To further investigate this phenomenon, the effect of sodium *p*-chloromercuribenzoate (PCMB) and mercaptoethanol on loss of radioactive phosphate was determined because these compounds are considered to

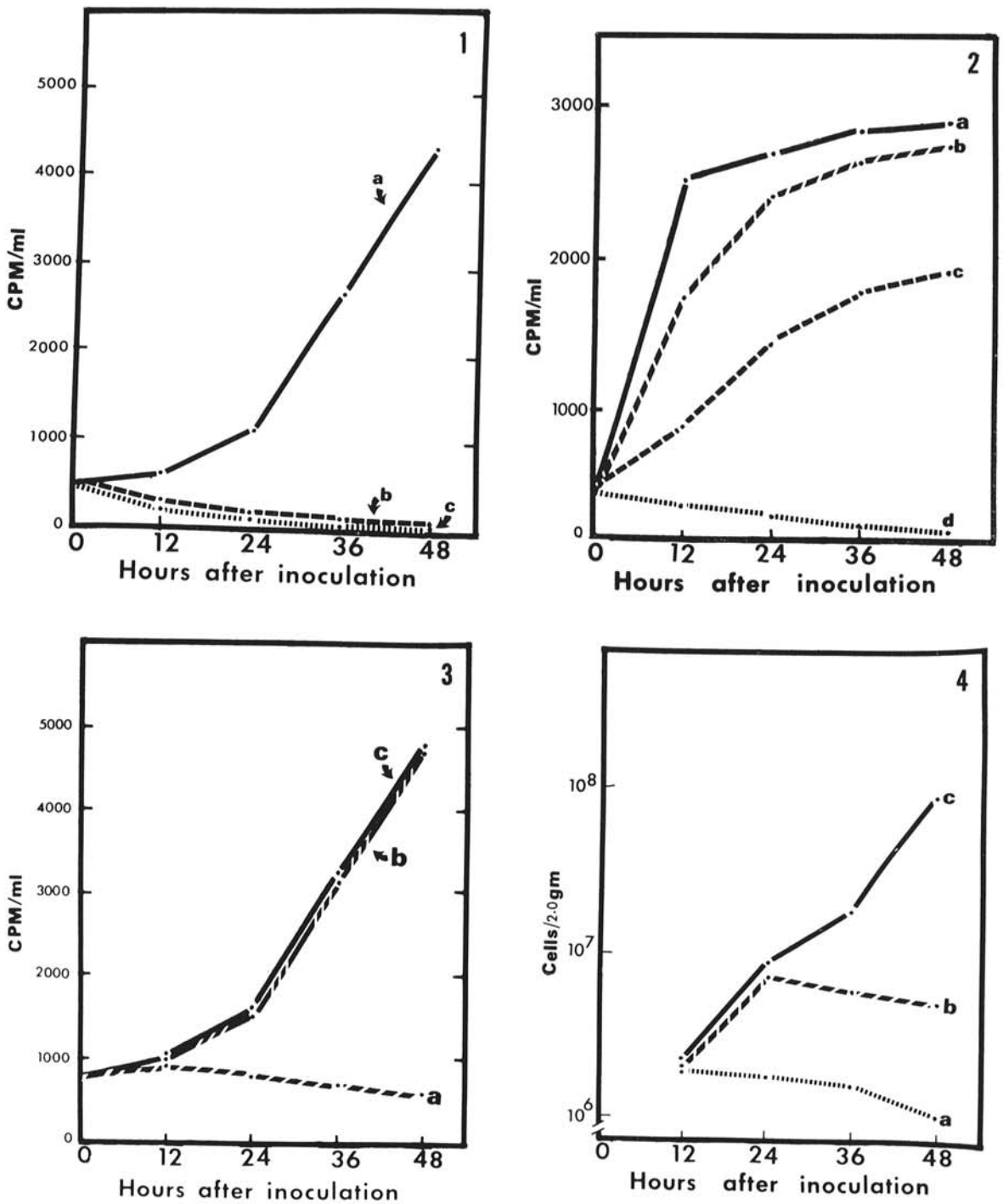

Fig. 1-4. **1)** Radioactivity of bathing solutions of ^{32}P-infiltrated carnation leaf samples at 0.5, 12, 24, 36, and 48 hr after inoculation with (a) *Pseudomonas caryophylli*; (b) *Corynebacterium* sp.; or (c) noninoculated. **2)** The effect of sodium *p*-chloromercuribenzoate (PCMB) and mercaptoethanol on the loss of ^{32}P from ^{32}P-infiltrated carnation leaf samples treated with (a) mercaptoethanol (0.1 M); (b) PCMB (0.05 M); (c) PCMB (0.005 M); (d) distilled water. **3)** Loss of ^{32}P from ^{32}P-infiltrated carnation leaf samples inoculated with *P. caryophylli* and infiltrated with tetracycline hydrochloride solution at (a) 12 hr after inoculation; (b) 24 hr after inoculation; (c) no tetracycline hydrochloride added. Results for noninoculated samples were similar to those shown for noninoculated samples in Fig. 1-c. **4)** Growth of *P. caryophylli* in carnation leaf samples infiltrated with tetracycline hydrochloride solution at (a) 12 hr after inoculation; (b) 24 hr after inoculation; (c) no tetracycline hydrochloride solution added.

TABLE 1. Distribution of ^{32}P in bathing solutions and among four phosphate fractions from noninoculated carnation (*Dianthus caryophyllus*) leaf samples or leaf samples at 24 and 48 hr after inoculation with *Pseudomonas caryophylli* or *Corynebacterium* sp.

Phosphate fraction or bathing solution	Hr after inoculation					
	Noninoculated		*Corynebacterium sp.*		*P. caryophylli*	
	24	48	24	48	24	48
1 Soluble esters and inorganic phosphate	67.3[a]	56.0	68.8	58.5	52.1	24.9
2 Ribonucleic acids	14.1	22.2	13.5	20.5	16.2	10.4
3 Deoxyribonucleic acids	8.2	10.4	8.8	10.2	9.9	6.2
4 Phospholipid and phosphoprotein	10.4	11.4	8.9	10.8	11.5	5.3
Bathing solution	0.0	0.0	0.0	0.0	10.3	53.2

[a] Percentage of total quantity of ^{32}P absorbed by each leaf sample when infiltrated with $K_2H^{32}PO_4$ prior to placement in bathing solution and the addition of inoculum. Each value is the mean of four determinations. The least significant difference (5% level) for various fractions are (1) 2.3; (2) 1.9; (3) 1.6; (4) 1.2.

cause deleterious effects in cells by altering membrane permeability (6, 7, 14). Both PCMB and mercaptoethanol caused a rapid loss of radioactive phosphate from carnation leaf tissue (Fig. 2).

The loss of radioactive phosphate from tissue inoculated with *P. caryophylli* was inhibited by tetracycline hydrochloride at 12 hr after inoculation, but was unaffected by similar treatment at 24 hr after inoculation (Fig. 3). Because tetracycline hydrochloride inhibited growth of *P. caryophylli* at both 12 and 24 hr after inoculation (Fig. 4), the results suggest that loss of radioactive phosphate from tissue is dependent on growth of *P. caryophylli* at 12 hr but not at 24 hr after inoculation. Water extracts of *P. caryophylli*-infected tissue did not cause a loss of radioactive phosphate from tissue (Table 2), and consequently it was concluded that these extracts did not contain factors which induce loss of ^{32}P. In addition, uptake of ^{32}P from the extracts did not occur. This inhibition of ^{32}P uptake may be attributed to ionic competition, owing to the higher concn of unlabelled phosphate ions in the extracts.

DISCUSSION.—The results of this study indicate that radioactive phosphate (^{32}P) can be incorporated into intracellular phosphate compounds when carnation leaf tissue is vacuum-infiltrated with a solution containing this isotope. The mechanism by which phosphate was accumulated intracellularly was not determined; however, it is generally agreed that uptake of phosphate by plant cells is metabolically dependent (9, 13, 15). When leaf tissue previously infiltrated with ^{32}P was inoculated with *P. caryophylli*, there was a loss of ^{32}P from the tissue (Fig. 1); inoculation with *Corynebacterium* sp. did not result in a loss of phosphate. Mercaptoethanol and PCMB also caused a rapid loss of phosphate from carnation tissue (Fig. 2), and since these compounds have been demonstrated to cause an alteration in the permeability of plant cell membranes (6, 14), it was concluded that loss of ^{32}P from tissue inoculated with *P. caryophylli* is due to an alteration in cell membrane permeability.

Alteration in the cellular permeability of host tissue has been reported for several host-pathogen combinations (17). Several recent studies have indicated that phytopathogenic bacteria alter the cellular permeability of plant tissue, while saprophytic bacteria do not (4, 7). Williams & Keen (16) have demonstrated an association between cellular permeability alteration in cucumber and pathogenesis by *P. lachrymans*. Cook & Stall (5) have also shown that *Xanthomonas vesicatoria* causes permeability changes in both susceptible and resistant peppers. Although the exact role of cellular permeability alterations in pathogenesis is not clear (17), Luke et al. (12) speculated that permeability alterations may be the mode of action of certain "wilt inducing" pathogens. Thus, our data may be relevant to an elucidation of the mechanism of wilting in carnation plants inoculated with *P. caryophylli*.

The mechanism by which *P. caryophylli* induces permeability alterations in carnation tissue remains obscure. The ability to inhibit loss of ^{32}P from tissue by inhibiting growth of *P. caryophylli* at 12 hr after inoculation (Fig. 3, 4), and the inability to do the same at 24 hr after inoculation, suggest that permeability alterations in carnation tissue are induced by growth of *P. caryophylli* prior to 24 hr after inoculation but become independent of growth of the bacterium thereafter. In addition, extracts from *P. caryophylli*-infected tissue

TABLE 2. Radioactivity of ^{32}P-infiltrated carnation (*Dianthus caryophyllus*) leaf samples at 0.5, 24 and 48 hr after treatment with water extracts from leaf samples inoculated with *Pseudomonas caryophylli*

Hr	Radioactivity (cpm/ml)			
	Water extract[a]		Controls[b]	
	Nonautoclaved	Autoclaved	*P. caryophylli*	Noninoculated
0.5	1095	1153	1101	1071
24	1070	1084	1593	600
48	1004	1057	4179	231

[a] The extract was prepared 48 hr after inoculation with *P. caryophylli* as described in the text. Portion of the extract was autoclaved for 15 min at 121 C. The reaction mixture contained 2 g of ^{32}P-infiltrated leaf tissue and 10 ml of extract and was incubated at 30 C.

[b] Samples of ^{32}P-infiltrated leaf tissue were placed into sterile distilled water and either were inoculated with *P. caryophylli* or were noninoculated.

did not cause a loss of ^{32}P from tissue (Table 2). These results suggest that the living bacterium initiates the permeability phenomenon between 12 and 24 hr after inoculation, but the continuation of the process is dependent on host cell reactions and not on continuous bacterial growth. A similar phenomenon has been observed by Klement & Goodman (8). More recently, Burkowicz & Goodman (4) reported that a "threshold" level of bacterial population is necessary for the induction of permeability alterations in apple tissue inoculated with *Erwinia amylovora*. It may be that in our system a threshold bacterial population is reached between 12 and 24 hr after inoculation, permeability alterations are induced, and subsequently the permeability phenomenon is independent of bacterial growth. The relation of cellular permeability alterations to the maceration of carnation tissue simultaneously inoculated with *P. caryophylli* and *Corynebacterium* sp. will be presented elsewhere.

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