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Diseased leaf lyophilization: a method for long-term prevention of loss of virulence in phytopathogenic bacteria

Y. BASHAN & Y. OKON* *Department of Plant Genetics, The Weizmann Institute of Science, Rehovot, 76 100, Israel and * Department of Plant Pathology and Microbiology, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot, Israel*

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A technique for long-term preservation of phytopathogenic bacteria is described. It is based on selective multiplication of the bacterial pathogen in host tissue, disinfection of leaf surfaces to reduce contamination, lyophilization of leaves, and storage under dry conditions at −80°C. With this technique, the pathogenicity of large numbers of the pathogens *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria* was maintained for 4 years.

Most of the methods used for preservation of phytopathogenic bacteria are similar to those employed for other bacteria and were recently reviewed by Sleesman (1982) and Kirsop (1985). They include frequent isolation; transfer to fresh medium; preservation in lyophilized skim milk or other substances; storage at moderately low or very low temperatures; drying on beads, stones, silica gel particles, agar discs dried over $CaCl_2$ or on other materials such as soil, sand, paper discs, or cellulose fibres; drying in bacterial exudates; vacuum drying without freezing; storage in sterile, distilled, and tap water; preservation in host tissue either by drying or freezing; and storage on mineral oil overlay.

Sleesman (1982) proposed that an optimal preservation method might involve desiccation followed by long-term storage at low or ultra-low temperatures. The proposed method should involve use of high cell concentrations harvested at their greatest level of desiccation resistance, rapid desiccation, protective medium, controlled residual moisture, controlled storage atmosphere and appropriate protection during rehydration. The technique should be simple, inexpensive, equipment- and labour-efficient, provide for easy recovery, and offer optimum flexibility.

Information on commonly used lyophilization procedures is available (Lapage *et al.* 1970). Martin (1964) has pointed out that the major advantages of lyophilization include prevention of contamination by storage in vacuum sealed containers, long-term survival of most bacteria and the unimportance of storage temperatures. Roughly 95% of all known bacteria can be preserved by this method. Major disadvantages of lyophilization include low cell recovery and the possible induction of mutations during storage (Goodman 1975).

One of the most important practical problems encountered in every phytobacteriological laboratory is that of maintaining the pathogenicity of the collection and avoiding any change in features related to pathogenicity, such as production of toxins, sensitivity to bacteriophages, and degree of virulence or aggressiveness. Maintenance of viable cultures on media slants is practical for limited collections and for short periods. Often, however, these cultures must be discarded because of a loss of virulence. Lyophilization of pure cultures in skim milk (Goodman 1975) can help to solve this problem, but this method is time consuming, especially when the number of cultures is large. Thus, protocols of methods suggested for preservation of phytopa-

thogenic bacteria include, in addition to survival tests, data on pathogenicity after several years of storage.

Xanthomonas campestris pv. *vesicatoria* (XCV) is known to display geographical and host plant variation (Dye 1962). When grown on nutrient slants, the bacterial life span is short (10–15 d), and this is a crucial limiting factor in its preservation and mail delivery.

This report describes a procedure for the maintenance and recovery of large numbers of two bacterial pathogens without loss of pathogenicity after long storage.

Materials and Methods

ORGANISMS

Pseudomonas syringae pv. *tomato* (PST)(WT-1) and *Xanthomonas campestris* pv. *vesicatoria* (XCV)(R-3) isolated from infected plants in 1977 and 1979, respectively, were used for a 4 year storage experiment. Compatible hosts used were: tomato, *Lycopersicon esculentum* cv. 'VF-198' for PST and pepper, *Capsicum annuum* cv. 'Ma'or' for XCV.

GROWTH MEDIA

The medium used for recovery of XCV from diseased pepper plants was composed of Nutrient Broth (Difco) supplemented with (g/l): sucrose, 5; $CaCl_2$, 1·5 and sodium deoxycholate, 0·2 (the latter added aseptically after autoclaving) (NSD) (Diab *et al.* 1982). King-B medium (King *et al.* 1954) supplemented with (mg/l): basic fuchsin, 9 and triphenyltetrazolium chloride (Sigma), 1·4 (KFT) was used for recovery of PST from diseased tomato plants (Sharon *et al.* 1982).

STORAGE BY SUBCULTURING

Five isolates of XCV and four of PST were used for virulence tests throughout this study. Bacteria were transferred to new agar slopes (NSD or KFT) every 10 to 30 d (16–36 times), and pathogenicity tests were carried out after every two transfers. Pathogenicity tests of colonies were conducted using four plants per isolate (4–6 true leaves). Each bacterial isolate was grown in the respective liquid medium for 24 h, harvested by centrifugation at 12000 g for 10 min, double washed in sterile saline (0·85% NaCl) and diluted in saline to a final concentration of 10^7–10^8 cfu/ml. Plants were sprayed with a suspension of each isolate and maintained for 5–7 d in humid chambers (approximately 100% r.h. with free water on the leaf surfaces; 30° ± 2°C for XCV and 22° ± 2°C for PST, natural light) until the appearance of symptoms. The disease index was then measured using the four mature upper leaves of each plant. The index scale was: 0, no symptoms; 1, 2–5 specks or scabs, together or spread over the leaf; 2, 6–10 specks or scabs; 3, more than 11 specks or scabs on each leaf. The number of lesions per leaf was counted separately and the mean of the four leaves determined the disease index of the plant (Fallik *et al.* 1983).

STORAGE BY LYOPHILIZATION

The same bacterial isolates as above were used. Lyophilization was as follows. Compatible host plants were inoculated as previously described (Bashan *et al.* 1978; Diab *et al.* 1982). After the appearance of severe symptoms (disease index of 2·85 for tomato plants and 3·0 for pepper plants), 250 g of detached leaves were collected from each diseased host. The leaves were thoroughly washed in 3–5 l of sterile tap water, dried for 1 h on sterile filter paper in a laminar flow hood, and surface disinfected by the method described by Sharon *et al.* (1982). Excess water remaining on the leaves after the final washing was removed by vacuum pumping (0·5 Torr) using a small Pasteur pipette. The leaves were transferred into sterile glass centrifugation tubes and pressed by hand using a sterile glass piston. The leaves were frozen in two stages, first by dipping the tubes in solid carbon dioxide suspended in technical alcohol, and then by storage at −80°C for 24 h. The leaves in the tubes were lyophilized for 2 d to absolute dryness (less than 0·5% moisture), and immediately milled in a sterilized homogenizer. The powder was kept in hermetically sealed glass containers containing silica gel at the bottom to prevent condensation on the walls of the glass containers. The silica gel was renewed after each opening. The containers were held at −80°C in an ice-free refrigerator for 4 years.

The pathogens were recovered from the lyophilized dry leaves using the liquid enrichment method developed for the detection of PST and XCV in seeds (Bashan & Assouline

1983). Samples of leaf powder (0·5 g) were removed from the glass containers and milled under sterile conditions into a very fine powder. Portions (0·1 g) were transferred to 50 ml of the appropriate liquid medium. The 250 ml Erlenmeyer flasks were vigorously shaken (250 strokes/min) at 22° ± 2°C for 3 h or 30° ± 2°C for 4 h. The homogenates were decimally diluted in the respective medium and 0·1 ml amounts were spread with a glass rod on the surface of solid NSD medium for XCV or solid KFT medium for PST. The inoculated plates were incubated at 22° ± 2°C for 72 h (for PST) or 30° ± 2°C for 72 h (for XCV) (Bashan & Assouline 1983). Pathogenicity tests for each isolate were as described above.

Table 1. Morphological and physiological tests carried out for testing *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* upon recovery from the lyophilized preparations

P. syringae pv. *tomato*		*X. campestris* pv. *vesicatoria*	
Characteristic	Reaction	Characteristic	Reaction
Aerobic[a]	+	Aerobic[e]	+
Gram staining[a]	−	Gram staining[e]	−
Green fluorescent pigment[a]	+	Yellow carotenoid formation[g]	+
Oxidase[a]	−	Growth at 40°C[e]	+
Acid production from:		Acid production from:	
glucose, fructose, galactose, mannose, sucrose, glycerol[a]	+	sucrose, maltose, glycerol, mannose, starch[e]	−
lactose, maltose, cellobiose, starch[a]	−	Use of: sucrose, maltose, glycerol, mannose, starch[e]	+
Use of organic acids:			
acetate, citrate, malate, succinate[a]	+		
benzoate, lactate, oxalate, tartarate[a]	−		
Production of indole or H_2S[a]	−		
Amino acid utilization:		Amino acid utilization:	
asparagine, glutamine, glycine, lysine, serine, threonine[c]	+	asparagine, glutamine[f]	+
alanine, arginine, aspartate; leucine, cystin, methionine, histidine, phenylalamine, proline, tryptophan, tryosine, valine[c]	−		
Pathogenicity towards tomato[h]	+	Pathogenicity towards pepper[h]	+
Pathogenicity towards pepper[h]	−	Pathogenicity towards tomato[h]	−
Induction of HR in pepper[a]	+	Induction of HR in cucumber[a]	+
Induction of hydrolytic activities in tomato plants:		Induction of hydrolytic activities in pepper plants:	
proteases[c]	+	proteases[i]	+
polygalacturonase[b]	+	polygalacturonase[i]	+
cutinases[b]	+	cellulase[i]	−
cellulase[b]	−		
asparaginase and glutaminase[c]	+		
Induction of oxidative enzymes in tomato plants:		Induction of oxidative enzymes in pepper plants:	
peroxidase[d]	+	peroxidase[i]	+
polyphenoloxidase[d]	+	polyphenol oxidase[i]	+
catalase[d]	−		
Ammonia production in tomato plants[c]	+	Ethylene induction in pepper plants[f]	+
Ethylene production in tomato, pepper, cucumber[f]	−	Ethylene induction in tomato and cucumber plants[f]	−

[a] According to Wilkie & Dye (1974).
[b] According to Bashan *et al.* (1985).
[c] According to Bashan *et al.* (1986a).
[d] According to Bashan *et al.* (1986b).
[e] According to Gardner & Kendrick (1921).
[f] According to Ben David *et al.* (1986).
[g] According to Starr & Stephens (1964).
[h] According to Bashan *et al.* (1978).
[i] Ben David, Bashan & Okon, unpublished data.
HR, Hypersensitivity reaction.

Morphological and physiological tests were carried out on isolates of both bacterial species using the methods of Wilkie & Dye (1974; Bashan *et al.* 1985, 1986a,b) for PST and Gardner & Kendrick (1921) and Ben-David *et al.* (1986) and unpublished data for XCV and consisted of the tests summarized in Table 1. Specific carotenoid production of XCV was tested according to Starr & Stephens (1964).

Results

SUBCULTURING GROWTH AND LEVEL OF PATHOGENICITY OF ISOLATES

Transfer and storage of XCV and PST in fresh agar medium for long periods led to a decrease in the pathogenicity of both bacteria. Colonies of XCV grew very well on agar medium but became avirulent after 6 months or more. The decrease in pathogenicity of PST was less drastic, with a loss of a third of its virulence towards tomato plants during this period (Table 2).

LYOPHILIZATION, BACTERIAL COUNTS AND RECOVERY AND ADVANTAGES OF THE TECHNIQUE

The two pathogens were counted in stored lyophilized leaf preparations. It was found that 23·3% of the XCV population survived after 1 month and 14·2% after 50 months, whereas 33·2% and 21·3% of the PST population survived after 1 month and 50 months, respectively (Table 3).

Table 2. Mean decrease in virulence of five isolates of *Xanthomonas campestris* pv. *vesicatoria* (XCV) and four isolates of *Pseudomonas syringae* pv. *tomato* (PST) after several transfers into fresh growth media*

Time after isolation from plants (months)	No. of transfers into fresh medium†		Disease index‡ units (0–3)	
	XCV	PST	XCV	PST
1	3	1	3·0	2·81 ± 0·13
2	6	2	3·0	2·86 ± 0·24
3	9	3	2·45 ± 0·17	2·86 ± 0·24
6	18	6	0·6 ± 0·23	2·35 ± 0·09
8	24	8	0	1·96 ± 0·11
12	36	12	0	1·86 ± 0·26
16	ND	16	ND	1·81 ± 0·12

* Maintained at room temperature.
† XCV was transferred every 10 d. PST was transferred every 30 d.
‡ Mean of five isolates for XCV and four for PST each tested on 10 plants.
ND, Not determined.

Table 3. Mean of colony-forming units of five isolates of *Xanthomonas campestris* pv. *vesicatoria* (XCV) and four isolates of *Pseudomonas syringae* pv. *tomato* (PST) in dried leaves and disease severity in pathogenicity tests of leaf lyophilized isolates after various periods of time

Time after lyophilization (months)	cfu of pathogens/g dry leaf ‖		Disease index units (0–3)	
	XCV	PST	XCV	PST
0*	$4·3 \pm 3·1 \times 10^6$	$3·1 \pm 2·7 \times 10^7$	3·0	2·87 ± 0·05
1	$9·6 \pm 0·3 \times 10^5$	$1·03 \pm 0·2 \times 10^7$	2·88 ± 0·08	2·96 ± 0·02
2–14†	$7·4 \pm 1·2 \times 10^5$	$8·44 \pm 2·1 \times 10^6$	2·76 ± 0·12	2·71 ± 0·14
18	$6·2 \pm 2·6 \times 10^5$	$7·96 \pm 0·4 \times 10^6$	3·0	2·91 ± 0·02
40‡	$5·8 \pm 1·4 \times 10^5$	$6·4 \pm 1·7 \times 10^6$	3·0	2·86 ± 0·04
50§	$6·1 \pm 2·7 \times 10^5$	$6·6 \pm 2·1 \times 10^6$	2·8 ± 0·22	2·78 ± 0·14

* Before lyophilization.
† Isolation was carried out monthly.
‡ Seventeen replicates.
§ Eight replicates.
‖ Five replicates.

Techniques for liquid enrichment (Bashan & Assouline 1983) of bacteria from leaves gave good recovery of bacteria from lyophilized leaves. The surface disinfection process carried out before lyophilization (Sharon *et al.* 1982) eliminated most of the saprophytes and left only the pathogenic strains that proliferated inside plant tissue. By using diagnostic media, saprophytes did not contaminate the isolated pathogen colonies. *Xanthomonas campestris* pv. *vesicatoria* and PST isolated from lyophilized leaves fully maintained their original pathogenicity. No changes were found in morphological and physiological features of the isolates during the storage period (Table 1).

Discussion

One obvious disadvantage of preserving phytopathogenic bacteria by the commonly used methods of drying or freezing of plant materials (Sleesman & Leben 1978) is that, upon recovery, the desired bacterial pathogen may be accompanied by contaminants. The technique presented in this study overcomes this difficulty and it also has an advantage of high survival level of cells in the dried leaves compared to 0·1% survival of cells stored in the commonly used skim milk lyophilized preparation (Goodman 1975).

The physiological basis of high pathogen survival in lyophilized leaves is not yet known. Perhaps bacteria are protected inside the tissues in micro-colonies under hypobiotic conditions (Leben 1981), or are subjected to less lyophilization stress than when in pure cultures. The protective effect of leaf tissue may be nutritional or related to exudates resulting from the host–pathogen interaction.

The results presented in this study suggest that the proposed technique is reliable, simple, space-efficient, inexpensive and fulfills many of the requirements cited by Sleesman (1982) for an ideal preservation technique. Only two phytopathogenic bacterial species were tested by this technique. The technique is probably suitable, however, and should be tested for the storage of other bacterial pathogens in host tissue.

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