# Application of the Scholander pressure bomb to studies on endophytic bacteria of plants

**J. Hallmann, J.W. Kloepper, and R. Rodríguez-Kábana**

**Abstract**: The Scholander pressure bomb system, which expresses vascular plant sap, was compared with the trituration method, in which roots are surface disinfested and triturated, for recovery of endophytic bacteria. The two methods were compared for recovery of indigenous and introduced endophytes from roots of several plant genera. The pressure bomb method was acceptable for routine recovery of eudophytes from cotton (*Gossypium hirsutum*), soybean (*Glycine max*), and bean (*Phaseolus vulgaris*), but owing to tissue collapse under pressure, the method did not work reliably for cucumber (*Cucumis sativa*) or tomato (*Lycopersicon esculentum*) seedlings. High bacterial densities on the root surface, experimentally obtained by dipping cotton roots into a suspension of *Enterobacter asburiae* JM22 immediately prior to processing, did not affect the population densities of recovered indigenous endophytic bacteria by the pressure bomb technique but resulted in increased bacterial densities for the trituration method. Internal populations of JM22 following application as a seed treatment were statistically equivalent with the trituration and pressure bomb techniques. Analysis of taxonomic diversity of a group of indigenous endophytes recovered with the trituration and pressure bomb techniques indicated some differences between the two groups. The total number of bacterial genera and species recovered was greater using the pressure bomb method. Gram-positive species, such as *Bacillus* spp., were more frequently isolated with the trituration method than with the pressure bomb method. *Agrobacterium radiobacter* and less common species were more often isolated using the pressure bomb technique. *Pseudomonas* spp. and *Phyllobacterium* spp. were recovered with equal frequencies using both techniques. These results suggest that the two techniques sample two different internal habitats available for colonization by endophytic bacteria, i.e., the trituration method recovering mainly endophytes residing in the root cortex and the pressure bomb method detecting vascular colonists. A combination of both methods is recommended for understanding the full pattern of internal plant colonization by endophytic baeteria.

*Key words*: endophytic bacteria, Scholander pressure bomb, isolation method, cotton.

**Résumé** : Le système de bombe pressurisée de Scholander pour l'extraction de la sève des plantes vasculaires a été comparé à la méthode de trituration, dans laquelle les racines sont soumises à une désinfection de surface et triturées, pour la récupération de bactéries endophytes indigènes et introduites dans les racines de plusieurs genres de plantes. La méthode de bombe pressurisée s'est révélée acceptable pour la récupération routinière des endophytes du coton (*Gossypium hirsutum*), du soja (*Glycine max*) et du haricot (*Phaseolus vulgaris*), mais elle ne s'est pas avérée fiable pour les plantules de concombre (*Cucumis sativa*) ou de la tomate (*Lycopersicon esculentum*) parce que la pression écrasait les tissus. Des densités élevées de bactéries à la surface des racines ont été obtenues en plongeant des racines de coton dans une suspension d'*Enterobacter asburiae* JM22 avant de les soumettre aux traitements. La technique de bombe pressurisée n'a pas affecté les densités de populations de bactéries endophytes indigènes recouvrées, mais la méthode de trituration a conduit à des densités bactériennes augmentées. Par suite de l'application de JM22 à des graines de semence, les populations internes de cette souche ont été statistiquement équivalentes pour les deux méthodes. Uue analyse de la diversité taxonomique d'un groupe d'endophytes indigènes recouvrés par les deux méthodes a révélé certaines différences entre les deux méthodes. Le nombre total de genres et d'espèces de bactéries recouvrées a été supérieur par la métbode de bombe pressurisée. Des espèces gram-positives, telles que *Bacillus* spp., ont été plus fréquemment isolées par la méthode de trituration. L'*Agrobacterium radiobacter* et des espèces moins communes ont été plus souvent isolées par la méthode de bombe pressurisée. Les deux techniques ont récupéré en fréquences égales les *Pseudomonas* spp. et *Phyllobacterium* spp. Ces résultats suggèrent que ces techniques échantillonnent deux habitats internes différents disponibles pour la colonisation des endophytes : la trituration récupère surtout les endophytes du cortex racinaire et la méthode de bombe pressurisée ceux des tissus vasculaires. Une combinaison des deux méthodes est recommandée pour comprendre le pattern eomplet de la colonisation interne des plantes par les endophytes bactériens.

*Mots clés* : bactéries endophytes, bombe pressurisée de Sholander, méthodes d'isolement, coton.
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## Introduction

Endophytic microorganisms are ubiquitous in plant species (Bell et al. 1995; McInroy and Kloepper 1995). Standard techniques for recovering endophytic bacteria from roots usually include pretreatment with surface disinfestants such as NaOCl or 75% ethanol, several washes in sterile water, sonication in buffer solutions, or a combination of these methods (Kloepper and Beauchamp 1992; Mishaghi and Donndelinger 1990). The plant tissue is then triturated with a mortar and pestle and the macerate is plated on agar. The lethal effect of any surface disinfestant may extend to the interior root tissue and kill endophytic bacteria. Endophytic bacteria colonize a continuum of internal root tissues from the vascular stele, through the cortex, to the epidermis and associated rhizosphere (Old and Nicolson 1978; Kloepper et al. 1992); hence, slight variations in the concentration and incubation time of surface disinfestation may affect the number and type of bacteria isolated. Contradictory reports on the isolation frequency of endophytic bacteria from within the same plant species might partly be explained by differences in the isolation procedure. It would, therefore, be advantageous to bypass pretreatment with strong disinfestants and isolate endophytic bacteria directly from internal root tissues.

A vacuum extraction method was successfully shown to recover endophytic bacteria from grapevine (Bell et al. 1995) and citrus trees (Gardner et al. 1982); however, the procedure required woody stems since softer herbaceous stems collapse under vacuum. An alternative approach would be the extraction of plant sap by pressure using a Scholander pressure bomb (Scholander et al. 1965). For this technique, a plant sample is inserted into a pressure chamber, and when pressure is released, plant sap can be collected at the cut surface. The plant sap consists of fluid from the vascular vessels and the adjacent apoplast, which is a major colonization site for endophytic bacteria (Mahaffee et al. 1996; Quadt-Hallmann and Kloepper 1996). To our knowledge, this extraction method has not previously been applied to roots of herbaceous plants. The objectives of this study were to test the effectiveness of the Scholander pressure bomb in the recovery of endophytic bacteria from roots and to compare this method with the standard trituration technique for estimating population densities and taxonomic diversity of indigenous endophytes in cotton plants.

## Methods

### Bacterial strain

*Enterobacter asburiae* JM22 was originally isolated from within healthy cotton tissue (Musson et al. 1995) and shown to colonize the internal root and stem tissues of bean, cotton, and cucumber (Mahaffee et al. 1996; Quadt-Hallmann and Kloepper 1996). For this study, strain JM22 was stored at −80°C in tryptic soy broth (TSB) (Difco, Detroit, Mich.) containing 20% glycerol and grown on tryptic soy agar (TSA) (Difco, Detroit, Mich.) at 28°C for 24 h prior to use.

### Isolation of endophytic bacteria

Cotton (*Gossypium hirsutum* L. cv. Rowden) seeds were surface disinfested by mixing for 5 min in 1.05% NsOCl (20% household bleach), rinsed three times in sterile distilled water, and then planted in pasteurized sand or field soil under greenhouse conditions. Pasteurized sand was used for experiments designed to detect introduced *E. asburiae* JM22, while field soil was used in experiments designed to detect indigenous bacteria. Twice the number of required plants were planted to compensate for losses due to incomplete surface disinfestation, as indicated by the disinfestation control (described below). Root systems were washed with tap water and root fresh weight was recorded prior to further processing by the trituration or pressure bomb methods.

For the trituration method, roots were surface disinfested in 1.05% NaOCl for 60 s, rinsed three times for 5 min in sterile 0.01 M potassium phosphate buffer (pH 7.0) (PB), and then pressed onto TSA as a disinfestation control. Roots were triturated with a mortar and pestle in PB, serially diluted, and plated onto 1/20th strength TSA. Quantification of population densities of endophytic bacteria was done using only those samples for which the disinfestation controls lacked any bacterial growth after incubation at 28°C for 24 h.

For the pressure bomb method, roots with an attached stem length of 1.5 cm were were placed directly, without surface disinfestation, into a modified Scholander pressure bomb (Scholander et al. 1965) consisting of a 20 cm high × 8 cm diameter metal cylinder. The root system was inserted into the pressure chamber with the cut end of the stem exiting through the hole in the cover. The stem was cut using aseptic conditions after swabbing the stem with 75% ethanol, which was previously found to provide complete disinfestation of cotton stems (A. Quadt-Hallmann, unpublished). The root base below the tubing was sealed with a rubber sealing gasket. For softer plant tissue, additional sealing with silicone helped to prevent pressure leakage. Approximately 1.0 cm of the remaining stem base was then removed aseptically to reduce the potential of contamination through the stem cutting process. Commercial nitrogen was released into the pressure chamber (average increase of approximately 0.1 MPa/30 s) with maximum pressure not exceeding 1.7 MPa to avoid plant cell disruption. Sap appearing at the cut surface of the stem was collected with a sterile pasteur pipette (30 μL) and streaked directly onto 1/20th strength TSA. The pressure necessary to collect 30 μL plant sap varied between 1.0 and 1.7 MPa. After incubation at 28°C for 48 h, bacterial colonies were counted. Population densities were expressed as log(CFU/g root fresh weight).

### Bacterial diversity

To study bacterial diversity, TSA plates with bacterial densities less than 500 CFU/plate were selected. To ensure a random and representative sample of bacteria was taken, the centre of each plate was marked with a triangular segment containing approximately 40 bacterial colonies. Starting from the centre of the segment, each bacterial colony within the segment was subcultured on TSA. Isolates were restreaked to ensure purity and stored in full-strength TSB plus 20% glycerol at −80°C. The first 25 isolates of each sample were identified using fatty acid methyl ester (FAME) analysis (Sasser 1990). Extraction procedures and gas chromatography were as described by McInroy and Kloepper (1995). The fatty acid profile of each isolate was identified using the Microbial Identification System Aerobe Library. Depending on the similarity index (SI), the isolates were identified at the species level ($SI > 0.400$), genus level ($SI = 0.200-0.400$), or described as unidentified ($SI < 0.200$).

Bacterial diversity within each treatment was expressed by four biodiversity indices at the genus level as described by Ludwig and Reynolds (1988), namely richness (total number of genera), Hill's diversity number $N1$ (modified by Shannon), Hill's diversity number $N2$ (modified by Simpson), and a modified Hill's ratio (evenness index $E5$). Increasing number size indicates a higher genera richness (more bacterial genera per treatment), a more diverse bacterial spectrum (maximum diversity is reached when all genera are represented by the same number of individuals with $N2$ more than

$N1$, considering very abundant genera), or greater evenness (maximum when all the genera in a sample are equally abundant). The data were statistically analyzed using SAS software for analysis of variance (ANOVA)(SAS Institute, Inc., Cary, N.C.).

### Comparison of methods for recovery of an introduced endophytic bacterial species

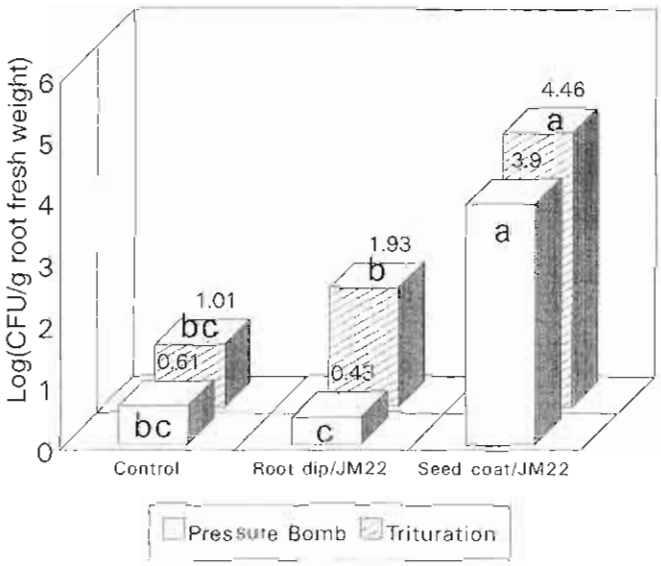
An experiment was designed to compare the population densities, recovered with the pressure bomb and the trituration methods, of endophytes in cotton seedlings that were either noninoculated or inoculated with the endophyte *E. asburiae* JM22. An additional objective of the experiment was to determine if high bacterial populations on the root surface affect recovery of endophytes with either method. The experiment was a randomized complete block with three treatments: the noninoculated control, seed treatment with JM22, and seedlings (produced from noninoculated seed) dipped into a suspension of JM22 ($8.0 \times 10^8$ CFU/mL) immediately before processing by the trituration or pressure bomb techniques. Seed treatment with JM22 was used because it was previously shown to be a practical delivery system for introducing endophytes into cotton plants (Musson et al. 1995). Each treatment was replicated six times. Plants were harvested 5 weeks after planting; the root system was gently washed under tap water and the root fresh weight was recorded. Bacterial counts were expressed as log values for each sample and mean population density as log(CFU/g root fresh weight) was calculated using the general linear models procedure in PC SAS. Samples with bacterial recovery below the detection limits (log(CFU/g root fresh weight) = 1.95 and 1.77 for trituration pressure bomb techniques, respectively) were scored as zero. When significant $F$ values resulted, the least significant difference (LSD) at $P = 0.05$ was calculated. The experiment was conducted twice.

### Comparison of methods for recovery of indigenous endophytic bacteria

An experiment was designed to compare populations of indigenous endophytes recovered with each method (trituration and Scholander pressure bomb) and to test the hypothesis that the two methods would recover two distinct communities of indigenous endophytes, i.e., cortical colonists with the trituration method and vascular colonists with the pressure bomb method. Cotton seeds (cv. Rowden) were planted in 1000-$cm^3$ cylindrical plastic pots containing sandy loam field soil and sand (1:1, v/v). After germination, the plants were thinned to one plant per pot and fertilized biweekly with 20 mL soluble 20-20-20 (N, P, K) fertilizer solution (Peter's fertilizer, Scott-Sierra, Maryvilles, Ohio). After growth for 5 weeks, roots from four replicate plants were processed as described above for the trituration and pressure bomb techniques. Total CFU per gram root fresh weight were recorded after bacterial growth for 48 h at 28°C on 1/20th strength TSA. Mean population densities (log(CFU/g root fresh weight)) were calculated using SAS. Identification of bacterial isolates was restricted to TSA plates with less than 500 CFU/plate, and single colonies were subcultured onto TSA as above. Bacterial diversity of identified isolates was conducted as described above. The experiment was conducted twice.

The feasibility of using the Scholander pressure bomb technique for recovery of indigenous endophytes on crops other than cotton was tested. Plants tested included soybean (*Glycine max* cv. Davis), bean (*Phaseolus vulgaris* cv. Kentucky Wonder), cucumber (*Cucumis sativa* cv. SMR 58), and tomato (*Lycopersicon esculentum* Mill. cv. Rutgers). All plants were grown in field soil in the greenhouse for 5–6 weeks prior to processing of roots for sampling with the Scholander pressure bomb as described above. Information was recorded on whether tissues from the plant types collapsed in the pressure bomb, if sap could be expressed, and if bacteria could be isolated from the sap after plating onto TSA.

**Fig. 1.** Recovery of bacteria from cotton roots of various treatments following extraction by the Scholander pressure bomb technique and trituration. Treatments with the same letter were not significantly different at $P < 0.05$.




## Results

### Comparison of methods for recovery of an introduced endophytic bacterial species

The greatest population densities of endophytes were recovered in treatments where JM22 was applied to seeds. No significant differences were found between the trituration (log(CFU/g root fresh weight) = 3.90) and pressure bomb (log(CFU/g root fresh weight) = 4.46) techniques (Fig. 1). Dipping of roots in a suspension of JM22 prior to processing (to mimic conditions of high population densities on the root surface) had no significant effect on endophyte populations recovered by the pressure bomb technique (log(CFU/g root fresh weight) = 0.43) when compared with the control (log(CFU/g root fresh weight) = 0.61) (Fig. 1). Statistically equivalent population densities of endophytic bacteria were recovered with the trituration and pressure bomb methods for the control and treatment with JM22 applied as a seed treatment. When high populations of bacteria were present on the root surface (roots dipped into a suspension of JM22), the recovered population density of endophytes was significantly greater with the trituration technique than the pressure bomb method.

### Comparison of methods for recovery of indigenous endophytic bacteria

The trituration method recovered significantly higher populations of indigenous endophytes compared with the pressure bomb method ($P = 0.05$), with means from two experiments of log(CFU/g root fresh weight) = 4.1 and 3.5 for the trituration and pressure bomb methods, respectively. The results of the identification of endophytic bacteria (Table 1) indicate that the frequency of recovery of taxa varied within and between each treatment/technique. In some cases, individual cotton plants were colonized by only one or two species (representing >80% of the isolates identified), while in other cases, up to 12 species of bacteria were recovered per

**Table 1.** Comparison of endophytic bacteria extracted from cotton roots by trituration of surface-disinfested tissue and by the Scholander pressure bomb technique.

| | Number of isolates recovered for various replications[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Trituration | | | | | Pressure bomb | | | | |
| Bacterial species | 1 | 2 | 3 | 4 | Σ | 1 | 2 | 3 | 4 | Σ |
| **Gram positive** | | | | | | | | | | |
| *Bacillus* | | | | | | | | | | |
| *B. cereus* | 3 | | | | 3 | | | | | 0 |
| *B. laterosporus* | | | 3 | | 3 | | | | | 0 |
| *B. megaterium* | | | 4 | | 4 | | 1 | | | 1 |
| *B. pumilus* | | | 2 | | 2 | | | | | 0 |
| *B. sphaericus* | 1 | | | | 1 | | | | | 0 |
| *B. subtilis* | | | 1 | | 1 | | | | | 0 |
| Total | 4 | 0 | 10 | 0 | 14 | 0 | 1 | 0 | 0 | 1 |
| *Clavibacter michiganensis* | | | | | 0 | | 1 | | | 1 |
| *Comamonas acidovorans* | | | | 2 | 2 | 3 | | 1 | 3 | 7 |
| *Paracoccus denitrificans* | | | | | 0 | | 1 | | | 1 |
| Subtotal | 4 | 0 | 10 | 2 | 16 | 3 | 3 | 1 | 3 | 10 |
| **Gram negative** | | | | | | | | | | |
| *Acidovorax delafieldii* | | | | | 0 | | 1 | | | 1 |
| *Agrobacterium* | | | | | | | | | | |
| *A. radiobacter* | | 3 | 6 | 6 | 15 | 1 | 5 | 22 | 2 | 30 |
| *A. rubi* | | | | | 0 | | | | 1 | 1 |
| *Brevundimonas vesicularis* | | | | 13 | 13 | | | | | 0 |
| *Burkholderia* | | | | | | | | | | |
| *B. cepacia* | | | | | 0 | | 1 | | | 1 |
| *B. pickettii* | | | | | 0 | | | | 1 | 1 |
| *Chryseobacterium* | | | | | | | | | | |
| *C. indologenes* | | | 9 | | 9 | | | | 1 | 1 |
| *C. meningosepticum* | | | | | 0 | | | | 1 | 1 |
| *Enterobacter taylorae* | | | | | 0 | | | | 2 | 2 |
| *Phyllobacterium* | | | | | | | | | | |
| *P. myrsinacearum* | | 1 | | | 1 | | | | | 0 |
| *R. rubiacearum* | | 21 | | 4 | 25 | 1 | 14 | 1 | 4 | 20 |
| *Pseudomonas* | | | | | | | | | | |
| *P. chlororaphis* | 10 | | | | 10 | | | | | 0 |
| *P. fluoreseens* | 4 | | | | 4 | | | | 3 | 3 |
| *P. putida* | 7 | | | | 7 | 20 | | | 4 | 24 |
| *Salmonella choleraesuis* | | | | | 0 | | | | 1 | 1 |
| *Variovorax paradoxus* | | | | | 0 | | | 1 | 2 | 3 |
| *Xanthobacter agilis* | | | | | 0 | | 1 | | | 1 |
| Subtotal | 21 | 25 | 15 | 23 | 84 | 22 | 22 | 24 | 22 | 90 |
| Total | 25 | 25 | 25 | 25 | 100 | 25 | 25 | 25 | 25 | 100 |
| Number of species | | | | | 15 | | | | | 19 |
| Number of genera | | | | | 7 | | | | | 14 |

[a]Values for 1, 2, 3, and 4 replications, including the sum (Σ) are given.

plant. Sixteen percent of all the bacteria isolated by trituration were gram positive, with *Bacillus* spp. as the principal genus. By contrast, gram-positive taxa represented only 10% of the total bacteria recovered with the pressure bomb method. No significant differences could be seen in the number of gram-negative bacteria recovered using either isolation technique. The most common isolates recovered belonged to the former *Pseudomonas* group (*Brevundimonas*, *Burkholderia*, *Pseudomonas*) with 34% for the trituration method and 29% for the pressure bomb technique, followed by *Phyllobacterium* spp. with 25 and 20%, and *Agrobacterium radiobacter* with 15 and 30% frequency of the total population, respectively. However, the pressure bomb method recovered a higher number of not commonly detected genera and concomittantly resulted in higher indices for bacterial richness and diversity: richness (number of

genera) = 5.1 for the pressure bomb method and 2.2 for the trituration method; diversity $N1$ = 3.3 for the pressure bomb method and 2.1 for the trituration method; diversity $N2$ = 3.1 for the pressure bomb method and 2.1 for the trituration method. The evenness index showed no difference for the two extraction procedures (data not shown).

When the feasibility of using the Scholander pressure bomb technique was tested on plants other than cotton, the results varied with plant type. With cucumber and tomato, tissues sometimes collapsed under pressure, resulting in no extraction of sap. However, with soybean and bean, tissue collapse rarely occurred, sap was expressed, and indigenous endophytic bacteria were recoverable on TSA.

## Discussion

The Scholander pressure bomb was successfully used to isolate endophytic bacteria from roots. With the techniques and plant systems used in this study, the frequency of plant samples with surface contamination of the stem portion (that part which extended from the pressure chamber) was low and no greater than for the trituration method. High bacterial densities on the root surface, experimentally achieved by dipping the roots in a suspension of JM22, did not affect overall bacterial recovery by the pressure bomb method, suggesting that only endophytic bacteria were recovered using this technique. The pressure applied was well below the permanent wilting point for cotton of −3.8 MPa (Slatyer 1957) and considered not to have caused damage of the root tissue, which could have resulted in rapid bacterial passage throughout the tissue. By contrast, the trituration technique did not completely disinfest the root surface when high populations of JM22 were present on the root. Under these conditions, three times as many samples had to be surface-disinfested for sufficient samples to be processed. Samples from treatments dipped in a suspension of JM22 immediately prior to processing for recovery of endophytes and passing the disinfestation control still showed higher bacterial densities than samples not dipped in JM22. We consider this to be due to the survival of bacterial cells of JM22 within collapsed root epidermal cells and other similar protected niches along the root surface (Quadt-Hallmann and Kloepper 1996).

When the endophytic bacterial densities estimated by the Scholander pressure bomb method are compared with those estimated by the trituration method, the results varied between the experiments with introduced JM22 and indigenous endophytes. Using seed treatment of cotton with JM22 planted into pasteurized soil (i.e., conditions under which the predominant recovered endophyte should be JM22), the mean log(CFU/g root fresh weight) was statistically equivalent for the two methods (Fig. 1). However, when seeds were planted in nonpasteurized field soil without seed treatment, the estimated population density of indigenous endophytes was significantly larger using the trituration method than using the Scholander pressure bomb method. The total population density of $1.2 \times 10^3 - 9.3 \times 10^4$ CFU/g root fresh weight for indigenous endophytes is within the range reported for cotton (Mishagbi and Donndelinger 1990; Musson et al. 1995).

Our finding of higher populations of indigenous endophytes with trituration of plant tissue agrees with the results by Bell et al. (1995) for grapevine and Gardner et al. (1982) for citrus using the vacuum extraction procedure, and we suggest an explanation as follows. Anatomically, the trituration method is more inclusive than the Scholander pressure bomb method, sampling both cortex and vascular systems of the roots. In contrast, the pressure bomb method expresses only vascular sap. Previous work by Quadt-Hallmann and Kloepper (1996) suggested that endophytic bacteria comprise two groups in relation to the habitat colonized within plants: cortical colonists found predominantly in the root cortex but not in the vascular region, and not recovered from stems, and systemic colonists which can be recovered from stems as well as roots. Hence, we consider that the trituration method recovers endophytes which exhibit both cortical and systemic colonization, while the Scholander pressure bomb method recovers predominantly systemic colonists.

In our study, the taxonomic identification of indigenous endophytes recovered using each technique supports the hypothesis that each method samples a different group of endophytes. Gram-positive *Bacillus* spp. were detected primarily with the trituration method, while with both methods, the predominant genera were gram negative, with *Pseudomonas*, *Phyllobacterium*, and *Agrobacterium* being the most common. This is in agreement with previous reports by Bell et al. (1995), Gardner et al. (1982), and McInroy and Kloepper (1995).

An advantage of the Scholander pressure bomb is that it is less labour intensive than trituration. However, limitations of the pressure bomb technique were encountered when juvenile, fleshy tissues of cucumber and tomato were used. These tissues were damaged by pressure, making expression of plant sap unreliable. Jeschke et al. (1995) reported that the amount of extractable plant sap is directly correlated with increased pressure; however, rapid changes in pressure can cause plant cell breakdown. We conclude that, depending on plant species and tissue being sampled, the Scholander pressure bomb is a suitable method for recovery of endophytic bacteria from root tissue. To recover the maximum diversity of endophytes with both root cortical colonization and systemic colonization patterns, it is recommended that both the trituration and Scholander pressure bomb methods be used.

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