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Bacteria Within Ovules and Seeds

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Surface-sterilized ovules and seeds of 27 species of plants were cultured in the water of syneresis of a nutrient medium low in agar content. Bacteria were obtained from 30% of the ovules, 15% of the seeds of herbaceous plants, 16% of the seeds of woody plants, 5.4% of the overwintered noncereal seeds, and 13.5% of overwintered cereal seeds. In no instance did every ovule or seed of a plant species contain bacteria. No bacteria were obtained from the hard, waxy seeds of mimosa or yellowwood. They were not obtained from ovules with unbroken coats or from seeds with coats that were not ruptured during the swelling of the seed. Only one species of bacteria was recovered in 93% of the instances in which bacteria were obtained. Bacteria were obtained from seeds that were embedded in the acidic parenchyma of the lemon or surrounded by the thickened flesh of the cucurbits. The bacteria were distributed among 19 genera and 46 species. The species isolated in greatest numbers were *Bacillus megaterium, B. cereus, Erwinia herbicola, Flavobacterium devorans,* and *Pseudomonas fluorescens.* Bacteria recovered less frequently were in the genera *Achromobacter, Acinetobacter, Alcaligenes, Brevibacterium, Corynebacterium, Cytophaga, Leuconostoc, Micrococcus, Nocardia, Proteus, Streptococcus, Streptomyces,* and *Xanthomonas.* Members of 11 genera and 15 species of bacteria were isolated once.

Pasteur observed that the juice of the unbroken grape berry was sterile although yeasts populated the surface. Thereby he may have influenced Fernbach (9) to conclude that inner plant tissues are sterile, although he recovered bacteria from tomato, carrot, turnip, and sugar beet tissues. Fernbach attributed the growth of bacteria during his experiments to contamination. It now appears possible that the bacteria were actually present in the tissues (12, 15, 21, 23; J. C. Meneley and M. E. Stanhellini, Prog. Abstr. Annu. Meet. Am. Phytopathol. Soc., 66th, 1974). Schanderl (cf. Burcik, reference 5) later subscribed to Fernbach's belief by attributing the recovery of corynebacteria from plant tissues to spontaneous origin, and Fischer (10) attributed outgrowth of bacteria during the culture of surface-sterilized plant tissues to the extreme resistance of spores and to contamination.

Since Fernbach's paper appeared in 1888, saprophytic, often unidentified bacteria have been isolated from 5% of potato seeds (12), 30 to 40% of corn and pea seeds (20), seeds of *Triticum* spp. (8), seeds of *Vicia faba* or the broad bean (5), and from crushed and also intact cultivated bean seeds (19). Burcik did not obtain bacteria from the seeds of cabbage, sugar beets, or tomatoes. He did demonstrate the presence of *Bacillus mycoides* and staphylococci, but not of *Pseudomonas fluorescens*, in the fruits of tomatoes developing from stigmata inoculated with the bacteria. *Bacillus vulgatus* has been isolated from the seeds of the muskmelon (15) and streptococci have been cultured from the ovules of peas and corn (11, 16).

Burcik (5) obtained bacteria from 9, 11, 0, 45, and 5% of the seeds of the broad bean cultured over a period of 5 successive years, and if bacteria were present in seeds produced on the test plot of ground, they were present the same year in seeds grown on other plots. He isolated staphylococci from 72% or more of tomato seeds cultured with the flesh, but not from seeds cultured without the flesh.

Bacteria may enter the seeds through the vascular system (22), the germ tube of the pollen grain, the hilum of ripened seeds, and cracks and openings in the seed coat (1); they may invade through the dorsal suture of the seed pod to migrate to the funiculus, through the raphe, and from there into the seed coat (24). It has been suggested that they enter the plant at the point of emergence of the secondary root or by passage directly into the meristemic tissue (12), by the return of water of guttation, containing bacteria, into the leaf (2, 6), and colonization of stomatal exudates. The rapid swelling after the inhibition of water by the seed, with the formation of natural rifts that separate the epidermal cells from the cotyledons, has been suggested as a means of sys-

temic invasion (24). Once within the tissues the bacteria either are free, or they may be in membrane-bound vesicles in the cytoplasm, but not in the central vacuoles (16; J. C. Meneley and M. E. Stanhellini, Prog. Abstr. Annu. Meet. Am. Phytopathol. Soc., 66th, 1974).

This study was prompted by inquiries for information on the presence and identity of bacteria in seeds. The number of plant species from which seeds have been obtained for study has been extended, and also the list of identified bacteria obtained from ovules and seeds has been expanded.

MATERIALS AND METHODS

Sources of ovules and seeds. Ovules and seeds were collected locally from gardens, lawns, and roadsides, and obtained from university fields. Seeds to be cultured immediately were kept in glass containers. Over-wintered seeds were stored in paper or glass at −15°C.

Sterilization. Ovules, seeds, and ovule-bearing pods from which the ovules were obtained were surface sterilized in sodium hypochlorite solutions containing Tween 20 (3), to assure maximal contact of the chlorine with the seed, adjusted to pH 6.0, and warmed to 50°C to increase the activity of the hypochlorous acid (13). The concentration varied between 100 and 330 μl/liter, and the duration of surface sterilization varied from 5 to 13 min, as determined by preliminary studies with each type of ovule or seed.

Controls. Burcik's procedure (5) was followed to determine sterilizing concentrations of hypochlorite and time. Seeds were sterilized at 121°C for 15 min, soaked in water for 1 to 2 min to regain moisture, placed for 1 min in saline suspensions containing (per ml) 5×10^6 cells of equal numbers of *Serratia marcescens* and a well-sporulated *Bacillus megaterium*, and then placed on sterile paper towels to dry. The minimum concentrations of hypochlorite and time required to destroy the inoculated cells on specific seeds were used in the surface sterilization of seeds. Seeds of maple, watermelon, and pumpkin could not be sterilized. The test bacteria apparently penetrated between the cotyledons and the seed coat to a depth not achieved by the sterilizing solution. The pumpkin has two large, readily seen openings in the hilum which are connected by a peripheral canal between the seed coat and the cotyledons.

Culture of ovules and seeds. Surface-sterilized ovules and seeds were placed in the water of syneresis which formed at the base of the slant of freshly prepared agar medium. The medium was composed of nutrient broth plus 0.3% yeast extract, 0.5% glucose, 0.5% agar, and 0.20% cycloheximide. The limited amount of agar kept the heavy seeds at the surface, yet enabled the water of syneresis to be extruded to provide both moisture and oxygen to the germinating seed and the bacteria. Tubes were incubated in the dark at 22°C and observed daily for 21 days for broken seed coats and outgrowths of bacteria. The coats of ovules and of seeds with very hard seed coats were punctured with toothed forceps to enable the entry of water into the seed coat. Ovules and seeds with intact coats after incubation were not included in the data.

Ground seeds. Ten-gram samples of surface-sterilized, air-dried seeds of small grain cultivars were blended in 95 ml of half-strength nutrient glucose broth and then maintained in suspension with minimal stirring for 3 h on a magnetic stirrer. Then 1.0-, 0.5-, and 0.1-g samples of the sediment were introduced to each of five tubes of nutrient glucose medium.

Identification of bacteria. Bacteria were identified according to *Bergey's Manual* (4).

RESULTS

Bacteria in ovules. Bacteria were obtained from 95 (30%) of 315 ovules representing 10 genera of plants (Table 1). They were obtained from more than 25% of the ovules of seven plant species, but in no instance was every ovule of a plant species free of bacteria. The green bean ovules were cultured shortly after formation and again at a later stage of maturity when the seed pod had become thin and flaccid (shelly bean stage).

Bacteria in young, mature seeds. Bacteria were obtained from 77 (15%) of 525 seeds of herbaceous plants (Table 2) and from 41 (16%) of 261 seeds of woody plants (Table 3). They were recovered from some seeds of every herba-

TABLE 1. *Presence of bacteria in plant ovules*

Plant ovule	No. cultured	No. positive	% Positive
Phaseolus sp. (very young)	15	1	7
Phaseolus sp. (shelly stage)	15	6	40
Zea mays (corn)	20	11	55
Cucumis sativis (cucumber)	30	11	37
Hibiscus esculentus (okra)	15	4	26
Pisum sp. (English pea)	27	4	15
Raphanus sativus (radish)	25	9	36
Secale cereale (rye)	25	10	40
Glycine max (soybean)	100	37	37
Capsium frutescens (sweet pepper)	18	1	6
Triticum aestivum var. *arthur* (wheat)	25	1	4

TABLE 2. *Numbers of herbaceous seeds containing bacteria*

Plant	No. cultured	No. positive	% Positive
Medicago sativa (alfalfa)	100	13	13
Zea mays (corn)	100	9	9
Cucumis melo (cantaloupe)	30	16	53
Brassica sp. (mustard)	20	2	20
Hibiscus esculentus (okra)	100	6	6
Glycine max (soybean)	100	15	15
Cucurbita sp. (squash, acorn)	20	2	10
Cucurbita sp. (squash, potato)	20	13	65
Vicia sp. (vetch)	35	1	3

TABLE 3. *Numbers of woody plant seeds containing bacteria*

Plant	No. seeds cultured	No. positive	% Positive
Malus sp. (apple)	26	1	4
Malus sp. (crab)	25	1	4
Robinia sp. (locust)	25	1	4
Albizia julibirssin (mimosa)	26	0	0
Koelreuteria sp. (Oriental rain tree)	45	8	18
Poncirus trifoliata (ornamental lemon tree)	25	2	8
Asimina triloba (pawpaw)	19	12	63
Firmiana simplex (parasol tree)	18	15	83
Diospyros virginiana (persimmon)	27	1	4
Cladrastis lutea (yellowwood)	25	0	0

ceous plant examined. The greatest percentage of recovery occurred with the cucurbits, muskmelon and potato squash, whose fruit lie on the ground; however, the percentage of recovery from the seeds of another cucurbit, acorn squash, was low.

Bacteria were isolated from 18% of the seeds of the rain tree, 63% of the seeds of the pawpaw, and 83% of the seeds of the parasol tree. No bacteria were recovered from the mimosa or the yellowwood tree seeds. The seeds of the parasol tree are born openly and without protection on a five-parted, modified carpel that is in the shape of a parasol. The seeds of the pawpaw, mimosa, and yellowwood are hard, but the seeds of the pawpaw have a porous hilum through which bacteria could enter after maturation of the seed.

Overwintered seeds. Bacteria were obtained from 5.4% of the overwintered noncereal seeds (Table 4) and from 13.8% of the cereal seeds. All overwintered seeds of the cucumber, persimmon, soybean, and one variety of squash were sterile, and a decrease occurred in the percentage of seeds of 10 plant species with bacteria. Less than 25% of the bacteria obtained from the noncereal seeds were bacilli, but 48% of the bacteria isolated from the overwintered cereal seeds were *Bacillus* spp.

Estimated numbers of bacteria in cereal seeds. The estimated numbers of bacteria in seeds ranged from less than 2/g in five trials of barley, oats, and wheat, to a maximum of 10/g in the seeds of two varieties of rye and one of wheat.

Identity and frequency in occurrence of bacteria. The identity of 395 cultures of bacteria isolated from ovules and seeds, the frequency in occurrence, and the numbers of individual ovules and seeds from which the bacterial species were isolated are recorded in Table 5. Nineteen genera and 46 species of bacteria were obtained. *Bacillus cereus, B. megaterium, Enterobacter aerogenes, Erwinia herbicola, Flavobacterium devorans,* and *Pseudomonas fluorescens,* in decreasing order of frequency, occurred most frequently. Members of 11 genera and 15 species of bacteria were isolated once.

The gram-negative rods were distributed nearly equally among the ovules and seeds. Nonsporulating gram-positive rods were isolated chiefly from ovules and from the seeds of woody plants. *Bacillus* spp. were obtained largely from the ovules and the seeds of herbaceous plants and the overwintered cereal seeds. *Streptococcus faecium* was isolated only from the seeds of the parasol tree, and *Leuconostoc mesenteroides* was isolated from the seeds of the muskmelon and pawpaw.

DISCUSSION

The presence of bacteria in ovules and seeds appears to be due to chance, and to be determined among different plant species by the structure, physiology, and variety of the plant (19). Elevation above the ground, suggested by Samish et al. (20), appears not to be a factor. The bacteria were obtained from many seeds of several varieties of trees, but from fewer seeds of several herbaceous species of plants in which the seeds are borne closer to the ground. Bacteria were present in many seeds of two varieties of cucurbits, but in few seeds of a third variety. Varietal differences may account for the differences in numbers of seeds of overwintered rye and wheat.

TABLE 4. *Numbers of overwintered seeds containing bacteria*

Plant	No. seeds cultured	No. positive	% Positive
Medicago sativa (alfalfa)	25	1	4
Cucumis sativis (cucumber)	25	0	0
Hibiscus esculentis (okra)	25	2	12
Asimina triloba (pawpaw)	26	5	19
Raphanus sativus (radish)	25	7	28
Diospyros virginiana (persimmon)	25	0	0
Glycine max (soybean)	25	0	0
Cucurbita sp. (squash, acorn)	25	0	0
Cucurbita sp. (squash, potato)	27	5	19
Zea mays (corn)	100	1	1
Hordeum vulgare var. *barsoy* (barley)	100	5	5
H. vulgar var. *volbar* (barley)	150	5	3
Arvena sativa (oats)	100	26	26
Secale cereale var. *balbo* (rye)	100	7	7
S. cereale var. *hiwassee*	150	35	23
Triticum aestivum var. *arthur* (wheat)	100	33	33
T. aestivum var. *blueboy* (wheat)	100	2	2

TABLE 5. *Bacteria isolated from ovules and seeds and frequency in occurrence*

Bacterium	Frequency of occurrence[a]	No. of isolates
Achromobacter sp.[b]	1	1
Acinetobacter calcoaceticus	4	12
Alcaligenes faecalis	1	1
Bacillus brevis	3	4
B. cereus	13	36
B. circulans	5	12
B. megaterium	13	44
B. pumilis	3	19
B. subtilis	7	17
Brevibacterium linens[b]	1	1
Corynebacterium flaccumfaciens	4	14
C. hypertrophicans	2	12
C. michiganense	1	1
C. poinsettiae	3	7
Corynebacterium sp.	1	1
Cytophaga hutchinsonii	1	5
C. rubra	4	5
Enterobacter aerogenes	8	40
Erwinia amylovora	5	13
E. carotovora	6	15
E. herbicola	8	23
Flavobacterium capsulatum	3	7
F. devorans	7	23
F. lutescens	2	3
F. rigense	2	8
Flavobacterium spp.	3	3
Leuconostoc mesenteroides	1	7
Micrococcus luteus	1	1
Micrococcus spp.	3	6
Nocardia salmonicolor	1	1
Proteus vulgaris	1	1
Pseudomonas acidovorans	3	3
P. alcaligenes	1	2
P. caryophilli	1	1
P. facilis	1	1
P. fluorescens	3	22
P. marginata	1	1
P. palleroni	1	3
P. putida	1	1
P. stutzeri	2	3
P. syringae	3	3
P. vesicularis	1	1
Serratia marcescens	2	8
Streptococcus faecium	2	8
Streptomyces albolongus	1	1
Xanthomonas sp.	1	1

[a] Frequency of occurrence among 143 lots of ovules and seeds cultured.

[b] Species *incertae sedis*.

More than 93% of the bacteria were isolated as single species rather than as mixtures from ovules and seeds. The percentage of recovery of bacteria was greatest from ovules, and it decreased in progression to young mature and then to overwintered seeds.

Although not conclusive, the observations suggest that in many, if not the majority, of instances of occurrence of bacteria within seeds, the individual bacteria penetrate the mechanical barriers of the living plant, withstand the vital protective mechanisms (7) that normally function to prevent bacterial parasitism, and are carried to and deposited within the ovule. No selective mechanism exists, since bacteria indigenous to both the plant and to the soil were isolated. The greater frequency in the isolation of the plant-resident bacteria may be explained by the larger numbers of these bacteria on plant surfaces.

The phytopathogens *Corynebacterium, Erwinia, Pseudomonas syringae,* and *Xanthomonas* accounted for 22.5% of the identified bacteria. They possibly represent plant-resident bacteria (14) existing in a nonparasitic state. The gram-negative rods accounted for 42%, and the sporeforming bacteria accounted for 33%, of all bacteria isolated. Bacteria not ordinarily associated with plant residence accounted for less than 10% of the isolates.

The need to rupture the coat of the ovule and some of the hard seed coats suggests that when present, bacteria are found between the seed coat and the cotyledon. This is in contrast with the study by Schnathorst (21), who isolated bacteria from 21.5% of whole bean seeds cultured, but did not recover bacteria from separately cultured seed coats, epicotyls, hypocotyls, or radicles of 18 seeds.

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