Microb Ecol (1987) 14:29–37

MICROBIAL ECOLOGY

Identity and Properties of Bacteria Inhabiting Seeds of Selected Broadleaf Weed Species

Robert J. Kremer

U.S. Department of Agriculture, Agricultural Research Service and Department of Agronomy, University of Missouri, Columbia, Missouri 65211, USA

Abstract. Seeds of five weed species were examined for the presence of seedborne bacteria. A total of 459 isolates were obtained from 1,740 seeds. The bacteria were identified and examined for distribution among seed viability classes, antifungal activity, and potential phytopathogenicity. Weed seeds varied for the prevalence of bacteria and in the types of bacteria associated with each plant species. Antifungal activity exhibited by 80% of the bacteria may limit seed deterioration by potential fungal seed pathogens. Some of the seedborne bacteria (15%) were potentially phytopathogenic. It is suggested that the complex nature of the weed seed-bacteria associations may be an obstacle to the development of biotic agents for manipulating weed seed activity in soil.

Introduction

The presence of bacteria within seeds of various plant species is documented [7, 14, 16, 18, 21, 22], but little information is available regarding the function of the bacteria in the seed environment. Seed-inhabiting bacteria may be detrimental because they cause seed decay under certain conditions, inhibiting seedling emergence or producing diseased seedlings. Alternatively, the bacteria may be beneficial because they protect the seeds against fungal invasion, producing germination stimulants or inhibiting detrimental microorganisms to enhance seed longevity. *Bacillus subtilis* has been reported to occur in *Arachis hypogeae* kernels [16] and in *Glycine max* seeds [21] and is capable of decreasing germination at temperatures $\geq 40°C$ and of producing antifungal substances. Unidentified bacteria associated with seeds of various cereal crops were shown to produce antibiotics against seedborne fungal pathogens [3, 14].

Previous studies in this laboratory have focused on microorganisms associated with mature seeds of the weed *Abutilon theophrasti* to establish criteria for investigations of weed seed deterioration as a potential weed control method [11]. Various genera of bacteria were isolated from surface-sterilized, newly harvested, and apparently normal *A. theophrasti* seeds. Preliminary assays also revealed that several of the bacterial isolates were antagonistic to their seedborne fungal counterparts [11].

These observations, along with the consistent occurrence of specific bacteria within *A. theophrasti* seeds, prompted a detailed study of the identity and

behavior of these bacteria in the seed environment and the possible relationship to weed seed vigor. The study was expanded to examine bacteria associated with seeds of other weed species and *Glycine max.*

Materials and Methods

Seeds

Seeds of *A. theophrasti* were harvested in the field during 1982, 1983, and 1984 at two sites in central Missouri (Boone and Osage counties). Seeds of *Datura stramonium* were collected during 1983 in Osage County. Seeds of *Ipomoea hederacea, Polygonum pensylvanicum, Xanthium strumarium,* and *G. max* (soybean) were harvested during 1983 from a soybean production field in Boone County. All seed lots were stored at −10°C and were assayed for viability and for the presence of microorganisms within 10 days after harvest. Seeds of *X. strumarium* were aseptically removed from the burs prior to the assays.

Sterilization

Seeds were surface-sterilized by immersion in 1.25% (w/v) sodium hypochlorite, rinsing in sterile distilled water, immersion in 70% (v/v) ethanol, rinsing 5 times in a total of 1 liter sterile distilled water, and blotting on autocleaved paper towels. The duration of surface-sterilization in each solution varied from 4–15 min as determined by surface-sterilization effectiveness tests with each type of seed. Effectiveness was determined by streaking intact seeds after each time interval of surface-sterilization on nutrient agar (Scott Laboratories, Fiskeville, Rhode Island). After incubation (25°C for 4 days), the absence of bacterial growth indicated the minimum time of surface-sterilization required for removal of external microorganisms.

To test the possibility that bacteria might withstand the surface-sterilization procedures, autoclaved seeds of each plant species were inoculated with 10^6 cells/ml nutrient broth culture of *Bacillus subtilis* (isolate 2211) and subjected to surface-sterilization. When effectiveness tests were performed on these surface-sterilized seeds, bacterial growth was not observed.

Culture of Seeds

Blotted surface-sterilized seeds were placed on the surface of nutrient agar at five seeds/plate. Plates were incubated in the dark at 27°C and examined every 24 hours for up to 5 days. Bacterial isolates were randomly selected from outgrowths on and around seeds and streaked on nutrient agar and glucose peptone agar [6] until pure cultures were obtained for characterization and identification. For detection of anaerobic bacteria, surface-sterilized seeds were plated on anaerobic agar (BBL, Cockeysville, Maryland), placed in anaerobic chambers (GasPak; BBL), and incubated at 27°C.

Identification of Bacteria

Isolates were identified according to *Bergey's Manual of Determinative Bacteriology* [1]. Bacteria were first examined for Gram's stain, oxidase reaction, motility, and morphology. Both oxidase positive and oxidase negative, gram-negative bacteria were characterized by using a battery of biochemical tests that were developed based on existing identification systems [8, 15]. Gram-positive bacteria were identified only by Gram's reaction and colony and microscopic morphology, except for *Bacillus* spp. which were characterized based on biochemical tests suggested by Gibson and Gordon [6].

Fungal Antagonism

Bacteria were tested for antagonism against seed- and soilborne fungi obtained previously [11]. The fungi used to study the antagonistic activity by bacteria were *Alternaria alternata, Cladosporium cladosporioides, Epicoccum purpurascens, Fusarium* sp., *Gliocladium roseum,* and *Penicillium diversum.* Fungal isolates were cultured on Czapek-Dox agar [9] for 10 days at 27°C. Spore suspensions were obtained by flooding the fungal growth on the plates with sterile 0.05% (v/v) Tween 40. Spore suspensions (0.5 ml) were spread-plated on potato dextrose agar [9] in triplicate and allowed to dry for 2–3 hours. Agar cores (5 mm diam) from a nutrient agar plate containing a 3–5 day lawn of test bacteria were aseptically removed and transferred to plates seeded with the fungal spores. The core was placed with the bacterial growth in contact with the fungal spores. Each plate accommodated 12 cores. The plates were incubated at 27°C for 10 days. The areas of the zones of fungal growth inhibition around each core were calculated and recorded. The experiment was repeated three times to verify the consistency of the results.

Antimetabolite Production

Production of antimetabolite toxins by bacteria was assayed using an indicator technique described by Gasson [5]. Freshly grown cultures of bacterial isolates were stabbed in minimal agar medium [5] containing ca. 10^8 cells of *Eschericha coli* strain B/ml. A clear zone of inhibition of *E. coli* growth after 48 hours at 27°C indicated antimetabolite production. The reactions of all isolates tested were compared to that of a known culture of the phytopathogen *Pseudomonas syringae* pv. *pisi.*

Germination of Seeds

Four replications of 50 seeds of each plant species were germinated on filter paper substrata in the dark at 27°C. Seeds with radicles of normal appearance >2 mm were considered as germinated at the end of a 4 day germination period. The remaining seeds were classed and recorded as either hard (nonimbibed, viable) or nonviable (imbibed, nongerminating).

Results

Bacteria were cultured from 459 of 1,740 (26.4%) seeds obtained from five weed species and soybean (Table 1). Seeds of *A. theophrasti* and *D. stramonium* had the highest incidence of bacteria while those of *I. hederacea, X. strumarium,* and *G. max* possessed bacteria to a lesser extent. Only two of 262 seeds of *P. pensylvanicum* contained bacteria. Over 95% of the bacteria were isolated as single species rather than as mixtures from seeds. The presence of bacteria within the seed is supported with more direct evidence obtained by light and electron microscopy (unpublished) which indicates that bacteria in infected seeds of *A. theophrasti* exist within the subpalisade cell layer of the seedcoat. The majority of seedborne bacteria was isolated from either germinating or imbibed seeds (Table 2). However, over 50% of the seedborne bacteria from *A. theophrasti* was isolated from the hard-seeded component. Generally, a direct relationship did not exist between the percentage of seed classes with bacteria and the proportion of the seed viability classes determined for a particular species.

Table 1. Prevalence of bacteria within seeds of weed species and *G. max*

Plant seed	No. of seeds cultured	% containing bacteria
A. theophrasti	578	42.9
D. stramonium	300	44.0
I. hederacea	200	13.0
P. pensylvanicum	262	0.8
X. strumarium	200	14.0
G. max	200	11.5

Table 2. Seed viability and occurrence of bacteria within classes of surface-sterilized seeds of weed species and soybean

	Seed viability (%)			% of seed classes with bacteria		
Plant species	Germination	Hard-seeded	Imbibed	Germinated	Hard-seeded	Imbibed
A. theophrasti	35.0	60.0	5.0	31.2	51.0	30.0
D. stramonium	47.5	0	52.5	42.1	0	46.0
I. hederacea	21.2	74.0	4.8	30.0	6.8	66.6
P. pensylvanicum	0	100.0	0	0	2.0	0
X. strumarium[a]	33.0	0	67.0	18.2	0	9.0
G. max	64.0	3.0	33.0	4.6	0	9.1

[a] Seeds removed from burs prior to testing

Of the 10 genera of bacteria identified in the examination of all 459 isolates from the plant species, *Bacillus, Erwinia, Pseudomonas,* and *Flavobacterium* were most predominant, in that order (Table 3). Seeds of *A. theophrasti* possessed the greatest variety of bacterial species (21 species) followed by *D. stramonium* (13 species). *Bacillus* spp. were isolated from seeds of all plant species except *P. pensylvanicum.* The majority of *Erwinia* spp. was isolated from *D. stramonium* while the majority of *Pseudomonas, Flavobacterium,* and *Alcaligenes* spp. was isolated from *A. theophrasti.* Of the seeds assayed for anaerobic bacteria, only 1% of *A. theophrasti* seeds yielded cultures tentatively identified as *Lactobacillus* spp.

Antifungal activity was a characteristic of over 80% of all seedborne bacteria. For example, representative bacterial isolates from *A. theophrasti* seeds displayed a wide spectrum of activity against their companion seedborne fungi and two soilborne fungi (Table 4). *Bacillus subtilis, B. megaterium,* and *Flavobacterium* sp. isolates were highly antagonistic to all test fungi. *Alternaria alternata* and *G. roseum* were susceptible to the highest number of seedborne bacteria.

Approximately 28% of the gram-negative bacteria produced antimetabolites which inhibited the growth of *E. coli* on minimal media. This was only 15% of the total number of bacteria isolated. Most of the isolates producing antimetabolites were *Pseudomonas, Flavobacterium,* and *Erwinia* species (Table

Table 3. Frequency of isolation of various genera and species of bacteria derived from seeds of weed species and soybean[a]

Bacterium	*A. theophrasti*		*D. stramonium*		*I. hederacea*		*G. max*	
	No. identified[b]	% of total	No. identified	% of total	No. identified	% of total	No. identified	% of total
Acinetobacter spp.	2	0.8	1	0.8	0	0	0	0
Alcaligenes spp.	33	13.3	5	3.8	0	0	0	0
Bacillus cereus	0	0	3	2.2	0	0	0	0
B. licheniformis	0	0	0	0	1	3.8	0	0
B. megaterium	30	12.1	16	12.1	5	19.2	3	13.0
B. pumilus	9	3.6	6	4.5	0	0	0	0
B. subtilis	35	14.1	36	27.2	2	7.7	13	56.5
Citrobacter freundii	1	0.4	0	0	0	0	0	0
Enterobacter spp.	3	1.2	9	6.8	0	0	0	0
Erwinia amylovora	5	2.0	5	3.8	0	0	0	0
E. herbicola	10	4.0	40	30.3	16	61.5	1	4.3
Erwinia sp.	4	1.6	0	0	0	0	0	0
Flavobacterium spp.	33	13.3	7	5.3	0	0	4	17.4
Moraxella sp.	2	0.8	2	1.5	1	3.8	0	0
Pseudomonas acidovarans	6	2.4	0	0	0	0	0	0
P. alcaligenes	8	3.2	0	0	0	0	0	0
P. cepacia	14	5.6	0	0	0	0	0	0
P. fluorescens	5	2.0	0	0	0	0	2	8.7
P. putida	7	2.8	1	0.8	0	0	0	0
P. stutzeri	17	6.9	0	0	1	3.8	0	0
P. syringae	6	2.4	0	0	0	0	0	0
Pseudomonas spp.	9	3.6	0	0	0	0	0	0
Xanthomonas spp.	9	3.6	1	0.8	0	0	0	0
Total	248	99.7	132	99.9	26	99.8	23	99.9

[a] All bacteria isolated from *P. pensylvanicum* and *X. strumarium* were identified as *Erwinia* and *Bacillus* spp., respectively

[b] Numbers of bacterial species identified within the collection of isolates obtained from seeds examined for each weed species

5). Many of the isolates appeared to produce greater amounts of an antimetabolite-inhibiting *E. coli* than did a standard strain of *P. syringae* pv. *pisi.*

Discussion

The characterization of the bacteria recovered from various weed seeds provides a significant basis for selecting and evaluating potential biotic agents for control of weed seeds in cultivated soils. The diversity of the bacteria isolated from the weed species illustrates the complex nature of microorganism-seed associations that must be considered in the development of biotic agents directed at weed seeds. The presence and frequency of bacteria in the seeds appear to be determined among the different plant species by structural and/or physiological characteristics of the seeds.

Table 4. Effect of selected bacteria on the growth of velvetleaf seedborne and two soilborne fungi

Bacterium	Inhibition zone (mm^2)						
	Seedborne fungi				Soilborne fungi		
	A. alternata	*C. cladosporioides*	*E. purpurascens*	*Fusarium* sp.	*G. roseum*	*P. diversum*	LSD (0.05)[a,b]
Alcaligenes faecalis (29)[c]	224	113	343	120	182	70	48
Bacillus megaterium (58)	341	244	473	167	198	208	92
B. subtilis (25)	258	266	146	121	266	178	77
Enterobacter sp. (12)	235	59	186	54	120	72	40
Erwinia amylovora (16)	0	0	204	0	82	0	42
Flavobacterium sp. (10)	190	113	502	163	266	159	70
Pseudomonas cepacia (6)	128	786	0	0	132	0	199
P. fluorescens (94)	90	0	450	668	454	0	168
P. stutzeri (21)	54	896	0	163	277	75	164
Pseudomonas sp. (7)	134	62	90	186	1,370	44	110
LSD (0.05)[a,d]	62	132	53	116	124	140	

[a] LSD (0.05) = least significant difference between paired means at the 5% level of probability
[b] LSD (0.05) for comparisons of means within a row
[c] Figures in parentheses denote the accession number of each bacterial isolate
[d] LSD (0.05) for comparisons of means within a column

Table 5. Inhibition of *E. coli* growth on minimal medium by seedborne bacteria from several weed species

Bacterial genus	Number of isolates		
	Total tested	Inhibitory	% of total
Alcaligenes	38	4	10.5
Enterobacter	12	0	0
Erwinia	80	16	20.0
Flavobacterium	40	16	40.0
Pseudomonas	74	34	45.9
Xanthomonas	10	1	10.0

Seeds of *X. strumarium* and *G. max* are borne within closed fruiting structures which may limit potential seed infection by bacteria. The higher incidence of bacteria in seeds of *A. theophrasti* and *D. stramonium* may result from exposure of the maturing seeds to the environment during the dehiscence of the seed capsules on these plant species. However, the seeds of *P. polygonum* are borne openly and unprotected, yet this species had the lowest incidence of seedborne bacteria.

The variability in seedcoat structures among the plant species may contribute to the observed differences in bacterial frequency. For example, a majority of seeds of *A. theophrasti, I. hederacea,* and *P. polygonum* possess a hard-seeded trait generally characterized by a densely packed layer of palisade cells within the seedcoat [11, 23], which could be a mechanical barrier to bacterial pene-

tration. Although *A. theophrasti* seeds have this structure, they also possess a natural opening in the palisade layer at the chalazal region [23] which very likely provides entry for bacteria. In the present study, about 90% of the bacteria isolated were observed to initiate growth at this area of the seed. Bacteria cultured from the hard seeds of the other weed species may have evolved from natural fissures or fractures within the seedcoat. The germination process possibly allowed the release of some bacteria which were detected in this study. Bacteria may produce various metabolic by-products which either stimulate or inhibit seed germination [3, 12]. Therefore, certain bacteria isolated from either germinating or imbibed and nongerminating seeds may have been exerting these effects. Other physiological or physical (or both) factors contributing to the variable susceptibility of the plant species to attack by seedborne bacteria may exist. However, since less than 30% of all seeds examined in this study possessed bacteria, normal protective mechanisms may also exist to exclude bacteria.

Certain groups of bacteria appeared to be associated with different seeds which suggests a certain specificity for each seed-bacteria association. An earlier study examining the bacteria associated with crop seeds found a similar specificity [22]. However, Mundt and Hinkle [13], studying bacteria isolated from seeds and ovules of 27 plant species, concluded that infection was largely nonspecific. In the present study, each plant species appeared to have a specific bacterial association. Seeds of *G. max,* included in this study as a control species, possessed *B. subtilis* as the predominant species. This bacterium had previously been reported as frequently occurring in *G. max* seeds [21]. The largest number of bacterial species occurred in *A. theophrasti* seeds indicating that these seeds presented a suitable nutrient source for a variety of bacteria. The presence of only a few bacterial species in other weed seeds may indicate preferential associations with these particular hosts. This is illustrated by the predominance of *Erwinia* spp. associated with *I. hederacea* and *D. stramonium* seeds compared with those of other plant species. Although *Erwinia* spp. are ubiquitous epiphytes on most plant species [3], the seed may provide a more selective environment for certain bacterial types than that provided by other plant surfaces.

The test for antifungal activity by bacteria on artificial media provided presumptive evidence for the occurrence of antagonism in the seed environment. A variety of bacterial species exhibited a range of antagonism toward seedborne and selected soil fungi. Bacterial antagonism toward seedborne fungal pathogens of crops has been previously reported for *Bacillus* spp. [2, 14, 21], *Erwinia* spp. [3], *Flavobacterium* spp. [3], and *Pseudomonas* spp. [3]. Antagonistic activity might be a survival mechanism for these bacteria in the seed environment. Consequently, the ability of antagonistic bacteria to displace potential seed-colonizing fungi may partially allow weed seeds to resist fungal attack and persist in a nondecayed state on the plant and in the soil environment. A previous study examining the ecology of *A. theophrasti* seeds in contact with soil showed that microorganisms associated with the seed surface greatly hindered establishment of soil microorganisms on the seeds [10]. These results also support the likely existence of an antagonistic defense mechanism against potential weed seed pathogens.

A minority of the bacteria exhibited potential phytopathogenic activity based on the *E. coli* indicator assay developed by Gasson [5]. Although direct proof for phytopathogenic activity is not presented, the results suggest that germination and seedling growth of weeds might be detrimentally affected by specific seedborne bacteria. Other studies have shown that seedborne bacteria capable of producing phytotoxins can reduce seed viability and seedling vigor in various plants [3, 4, 20].

The need for critical assessment of the impact of seedborne microorganisms on the persistence and deterioration resistance of weed seeds has been emphasized [11, 17, 19]. The complex nature of seedborne bacteria associated with various weed seeds was illustrated in the present study. Each weed species appeared to possess a distinct bacterial association which could reduce efficacy of biotic agents targeted at the manipulation of weed seed activity. Additionally, antifungal activity by a majority of the seedborne bacteria could perplex weed seed control based on the use of fungal agents. Yet the existence of seedborne bacteria exhibiting potential phytopathogenicity indicates that these bacteria might be exploited as possible biotic agents in the form of inocula deleterious to weed seedling growth.

Acknowledgments. Contribution from the Missouri Agricultural Experiment Station. Journal Series No. 10,002. Approved by the director. Appreciation is expressed to A. P. Harrison, Jr. and D. Keppler for supplying cultures of *E. coli* strain B and *P. syringae* pv. *pisi*, respectively.

References

1. Buchanan RE, Gibbons NE (1974) Bergey's manual of determinative bacteriology, 8th ed. Williams & Wilkins Co, Baltimore
2. Cubeta MA, Hartman GL, Sinclair JB (1985) Interaction between *Bacillus subtilis* and fungi associated with soybean seeds. Plant Dis 69:506–509
3. Durbin RD (1982) Toxins and pathogenesis. In: Mount MS, Lacy GH (eds) Phytopathogenic prokaryotes. Vol. 1. Academic Press, New York, pp 423–441
4. Frederickson JK, Elliott LF (1985) Effects on winter wheat seedling growth by toxin-producing rhizobacteria. Plant Soil 83:399–409
5. Gasson MJ (1980) Indicator technique for antimetabolite toxin production by pathogenic species of *Pseudomonas*. Appl Env Microbiol 39:25–29
6. Gibson T, Gordon RE (1974) Genus I. *Bacillus*. In: Buchanan RE, Gibbons NE (eds) Bergey's manual of determinative bacteriology, 8th ed. Williams & Wilkins Co, Baltimore, pp 529–551
7. Graham DC, Hodgkiss W (1967) Identity of gram-negative, yellow pigmented, fermentative bacteria isolated from plants and animals. J Appl Bacteriol 30:175–189
8. Isenberg HD, Sampson-Scherer J (1977) Clinical laboratory evaluation of a system approach to the recognition of nonfermentative or oxidase-producing gram-negative, rod-shaped bacteria. J Clin Microbiol 5:336–340
9. Johnson LF, Curl EA (1972) Methods for research on the ecology of soilborne plant pathogens. Burgess Publishing Co, Minneapolis
10. Kremer RJ (1986) Microorganisms associated with velvetleaf (*Abutilon theophrasti*) seeds on the soil surface. Weed Sci 34:233–236
11. Kremer RJ, Hughes LB Jr, Aldrich RJ (1984) Examination of microorganisms and deterioration resistance mechanisms associated with velvetleaf seed. Agronomy J 76:745–749
12. Mayer AM, Poljakoff-Mayber A (1982) The germination of seeds. Pergamon Press, Oxford

13. Mundt JO, Hinkle NF (1976) Bacteria within ovules and seeds. Appl Env Microbiol 32: 694–698
14. Neergaard P (1977) Seed pathology. Vol. 1. John Wiley & Sons, New York
15. Oberhofer TR, Rowen JW, Cunningham GS (1977) Characterization and identification of gram-negative, nonfermentative bacteria. J Clin Microbiol 5:208–220
16. Pettit RE, Taber RA, Foster BG (1968) Occurrence of *Bacillus subtilis* in peanut kernels. Phytopathology 58:254–255
17. Putnam AR, Duke WB (1978) Allelopathy in agro-ecosystems. Ann Rev Phytopathol 16: 431–451
18. Simpson, ME, Marsh PB, Merola GV, Ferretti RJ, Filsinger EC (1973) Fungi that infect cottonseeds before harvest. Appl Microbiol 26:608–613
19. Stoller EW, Wax LM (1974) Dormancy changes and fate of some annual weed seeds in the soil. Weed Sci 22:151–155
20. Suslow TV, Schroth MN (1982) Role of deleterious rhizobacteria as minor pathogens in reducing crop growth. Phytopathology 72:111–115
21. Tenne FD, Foor SR, Sinclair JB (1977) Association of *Bacillus subtilis* with soybean seeds. Seed Sci Technol 5:763–769
22. Wallace RH, Lochhead AG (1951) Bacteria associated with seeds of various crop plants. Soil Sci 71:157–166
23. Winter DM (1960) The development of the seed of *Abutilon theophrasti*. I. Seed coat. Am J Bot 47:157–162