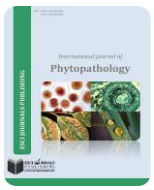

Available Online at ESci Journals
International Journal of Phytopathology
ISSN: 2305-106X (Online), 2306-1650 (Print)
http://www.escijournals.net/phytopathology

CHARACTERIZATION OF WHEAT GENOTYPES AS SOURCES OF ICE NUCLEATION ACTIVE BACTERIA FOR BIOPRECIPITATION AEROSOLS

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A B S T R A C T

As water resources become more and more scarce, production of crops under dry land conditions brings agriculture into potential conflict with other uses of water. There is an emerging awareness that the orientation of the goals of plant breeding can be shifted to create crops that can offset the negative impacts of agriculture on the environment in order to make agriculture more sustainable. Here we have explored the possibility to select lines of wheat, the crop that occupies more land than any individual crop, that contribute to the bioprecipitation cycle. In this cycle the ice nucleation (IN) active component of the microflora on leaves contributes to the ice nuclei in the atmosphere that activate processes in clouds necessary for rainfall. In line with this long term goal, we have deter-mined the capacity of breeding lines of wheat, adapted to dry land conditions, to harbor IN active bacteria. In particular, we focused on *Pseudomonas syringae*, the most ubiquitous of the IN active bacteria on plants. Because strains of this bacterium can be a plant pathogen, we evaluated the abundance of non-pathogenic IN active strains of *P. syringae* on a range of wheat genotypes from the research program of the International Center for Agricultural Research in the Dry Areas. Of the 25 genotypes of bread wheat examined, leaves of 12 genotypes naturally harbored *P. syringae* in the field. Eight of these genotypes harbored populations of IN active *P. syringae* with an impaired Type 3 Secretion System (involved in pathogenicity) as high as 4 x 105 105 CFU g-1 of leaf tissue. Three of these 8 wheat genotypes harbored IN active *P. syringae* that were not virulent on either bread or durum wheat and for 1 of these wheat genotypes the strains of IN active P. syringae were virulent on only 1 of the 13 plant species on which pathogenicity was tested. For the wheat geno-types that had *P. syringae* on leaves in the field, bacteria were naturally transmitted to seed but dur-ing seed storage the bacterium could be detected on only half of the genotypes after 3 months of storage. To explore the possibility of enhancing the IN active microflora on leaves, we assessed the capacity of bacteria inoculated on seed to be transmitted to seed. The effectiveness of the trans-mission depended on an interaction of wheat genotype and bacterial strain. Overall, this work points to the possibility of selecting plants with the goal of changing their microflora for purposes other than resistance to plant disease and in this case for the purpose of contributing to processes that could favor rainfall.

Keywords: *Pseudomonas syringae*, epiphytic bacteria, seed microbiology, bioprecipitation, Syria.

INTRODUCTION

Dry lands constitute about 20% of the Earth's land surface and are found on every continent where there is agriculture (Millenium Ecosystem Assessment, 2005). When dry land is cultivated, numerous agronomic practices are put into place to assure sufficient yield in face of plant disease and of water stress, in particular. Improvement of these agronomic practices, including the creation of disease and drought-tolerant cultivars, depends heavily on the international research network of public institutes and private foundations. Since the advent of international wheat breeding programs in 1944 destined to improve wheat for the developing world, 80% of the resources dedicated to this effort have been consecrated to improvement of yield and disease

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resistance and 20% to development of resistance to abiotic stresses (Rajaram 1995). In Eastern and Southern Africa, for example, up to 80% of the acreage for wheat constitutes improved varieties whereas only 20% are land races (Heisey and Lantican, 2000) thereby illustrating the impact of this research effort on the traits of the wheat varieties that cover dry land landscapes.

Cultivation of wheat in dry land contexts is highly dependent on and limited by rainfall (Qin *et al.,* 2013). Marked increases in yield can be obtained by supplementing water input to attain a total (rain + irrigation) water input of about 500 to 800 mm. For example, in the loess plains of northern China where annual precipitation ranges from 400 to 650 mm, the addition of 200 to 300 mm of irrigation increased grain yield by 60 to 100% (Qin *et al.,* 2013). Likewise in Syria, supplementing rainfall with irrigation to assure a seasonal input of 500 mm of water enhanced the effect of nitrogen fertilization on yield (Zhang *et al.,* 1998). But as water resources become more and more scarce, irrigation is increasingly challenging. Production of crops under dry land conditions brings agriculture into potential conflict with other uses of water. The important ecosystem service rendered by agriculture (food production) is offset by a marked negative impact on the environment in terms of water consumption.

There is an emerging awareness that the goals of plant breeding should be expanded beyond selecting traits directly linked to food production to encompass various environmental and ecosystem services valuable for sustainable agriculture (Brummer *et al.,* 2011). Cultivated and otherwise-managed land now represents 50% of the vegetated surface of Earth, having significantly displaced wild and semi-wild vegetated areas (Ellis *et al.,* 2010). Wheat occupies more land than any other individual crop. In Syria, for example, wheat is the most important winter crop, grown on about 1.8 million hectares or on about 32% of the total cultivated area of that country. Hence, cropped plants in general, and wheat in particular, are in ripe contexts to play roles in plant-mediated ecosystem services not only because of the vast surfaces they occupy but also because they benefit from concerted health management and genetic improvement schemes as well as means to facilitate world-wide distribution of useful genetic resources and of knowledge on agronomic practices.

Plants play an important role in the water cycle, not only as consumers of water but also as sources of atmospheric water vapor and aerosols involved in the physical processes in clouds required to initiate rainfall (Morris *et al.,* 2014). More specifically, the water vapor and microorganisms emitted from their leaves are transported up into clouds where they can have considerable impacts on atmospheric processes that define regional climates. In this light, cultivated plants could be deployed to strategically influence rainfall. Here we have investigated the potential for wheat to be deployed with the intent of contributing to atmospheric aerosols that are favorable for rainfall. In particular, we have assessed the variability of dry land wheat to harbor the ice nucleation (IN) active bacterium *Pseudomonas syringae*. This bacterium is one of the most efficient naturally-occurring IN active aerosols in the environment. Numerous research reports suggest that *P. syringae*, as well as other IN active microorganisms, can initiate the physical processes in clouds needed for rainfall when they are sufficiently abundant (Morris *et al.,* 2014). Variability of wheat in terms of their leaf surface populations of *P. syringae* would open the possibility to optimize emissions of this bacterium into the atmosphere. We have also assessed the variability of wheat genotypes to deliver the bacterium to leaves from infested seeds. Optimizing this transmission would foster the development of technology for inoculation of seeds with specific strains of *P. syringae* best adapted as colonizers and ice nucleators while respecting plant health considerations.

Agriculture in many regions is very dependent on natural cycles of rainfall. A case in point, Syria, depends on a wide base of varied natural resources extending over five agro-ecological zones differing in total precipitation, soil structure, and water resources such as rivers, springs, dams, and groundwater which supply water for about 851,000 ha (61% of the total irrigated areas). However, precipitation is considered the main source of water needed to maintain the widespread rain fed system of agriculture, which occupies 70% of the cultivated area in Syria (Karrou *et al.,* 2011). The production of this crop in the Aleppo region represents around 20% of the total production of the country (Rajaram, 1995). Since the possibility of affecting the climate in a region is our long term objective, by optimizing the release of aerosols of ice nucleating bacteria from a crop, we isolated and characterized a wide range of IN active strains of *P. syringae* from numerous advanced genotypes of bread wheat cultivars grown at Tel Hadya near Aleppo, Syria.

The population density of IN active bacteria on leaves can vary 1000 to 10000 fold among different plant species (Lindow *et al.,* 1978) and cultivars of the same species (Georgakopoulos and Sands, 1992). For *P. syringae* in particular, cultivars of snap beans and barley vary 1000-fold in the population densities of this bacterium that they harbor on leaves (Georgakopoulos and Sands, 1992; Daub and Hagedorn, 1981). For snap beans, these differences when assessed as the number of leaf associated IN active bacteria are associated with quantitative trait loci (Navarro et al, 2007). Studies of the interaction of *P. syringae* with plants have usually focused on damage to crops due to the plant pathogenicity of this group of bacteria. However, about 20% of the strains of this bacterium naturally occurring with plants and in other environments have little pathogenic potential (Demba-Diallo *et al.,* 2012; Morris *et al.,* 2013) because they possess defects in or variants of the Type 3 Secretion System, generally responsible for injecting virulence factors (called effectors) into hosts thereby conferring pathogenicity, and are therefore or inefficient for this function (Bartoli *et al.,* 2014). Many of these non- or weakly pathogenic strains are IN active nevertheless (Bartoli *et al.,* 2014; Berge *et al.,* 2014). Hence, we were particularly interested to identify genotypes of wheat that could support populations of IN active *P. syringae* with little pathogenic potential.

At present, production of field crops such as wheat and barley are driven by yield considerations. There is growing interest in how crops influence the environment and how the so-called ecological services they render could be improved through breeding (Brummer *et al.,* 2011), but attention has not yet been given to how crops might be selected or bred as sources of ice nucleation-active particles that could influence weather. This study took place in Syria where rainfall is a key determinant of cereal crop production and at a location where hundreds of cultivars of wheat and barley are evaluated annually for agronomic fitness. The objective of our work has been to determine which combinations of bacteria and cereal grain cultivars might result in production of aerosols of ice nucleating bacteria without adversely effecting crop health. Given the portent of climate change and its effect on crop production, this kind of approach may have important ramifications.

MATERIALS AND METHODS

Plant Materials as Sources of *P. syringae*: To characterize wheat genotypes for their capacity to harbor *P. syringae* and for their disease sensitivity, 100 bread wheat genotypes were obtained from the bread wheat breeding program at ICARDA in Aleppo, Syria. The seeds from the each genotype were tested for the presence of *P. syringae* using the KBC selective agar medium (Mohan and Schaad, 1987), a test recommended by the International Seed Testing Association (ISTA, 2008), before planting in the field in December 2010.

The wheat genotypes were planted in the midst of wheat and barley fields at the Tel-Hadya ICARDA field station using an alpha design with three replicates. Each plot was planted in 5 rows (25 cm apart) 150 cm long. In May 2011, each wheat genotype was scored visually in the field for the presence of bacterial-like symptoms on leaves collected in the field. Random samples of leaves were taken from the leaves of the 25 genotypes that showed bacterial like symptoms on their leaves (Table 1), put in sterilized paper bags and stored at 4 °C until testing.

To isolate bacteria, the leaf samples were transferred to zip-lock plastic bags and macerated manually in 0.1M phosphate buffer (8.75 g K_2HPO_4 and 6.75 g KH_2PO_4 per liter, pH 6.8). The volume of buffer used was proportional to the mass of tissue processed. Afterwards the bags were subjected to one minute of manual squashing to release the microbes from the surface of leaves. Serial dilutions from each sample were plated onto KBC medium to determine the population size of *P. syringae*.

Plates were incubated for up to 3 days at 22–25 ^{o}C. Production of fluorescent pigment on KBC was checked at 2 days after incubation with a long wavelength ultraviolet lamp (356 nm) and definitive counts of fluorescent and total colonies were realized after 5 days of incubation. Fluorescent colonies were transferred to KBC medium and the absence of cytochrome C oxidase was verified by streaking single colonies of a 48 h culture with a sterile toothpick onto filter paper imbibed with a fresh solution of 1% N,N,N,N, tetramethyl-p-phenylenediamine dihydrochloride (Sigma Chemical Company, Munich, Germany). The presence of arginine dihydrolase was tested as previously described by (Lelliot *et al.,* 1966).

Out of the 25 genotypes of bread wheat leaves, 20 genotypes showed bacterial colonization but only 12 of the strains isolated proved to be *P. syringae* (Table 1 and 2).

Table 1. Population density of P. syringae like bacteria on bread wheat leaves.

Code	Genotype name	Population size (*P. syringae*)	Code	Genotype name	Population size (*P. syringae*)
22/1	Gonglase-1	ND[2]	234/1	Massira/Jadida-2	4.38
22/2	Gonglase-1	5.32	234/2	Massira/Jadida-2	5.38
23/1	Weaver\Jacana	ND	292/1	Faris-16	4.92
115/1	Pearl-10	3.33	306/1	Leith-4	3.43
122/2	Zad-1	5.36	338/1	Baasha-29	ND
122/3	Zad-1	5.48	349/3	Ghali-2	5.91
128/1	Alshoroq-1	2.98	364/2	Zafir-5	5.62
128/3	Alshoroq-1	5.62	369/1	N-Azraq-7	ND
133/2	Samira-20	4.77	369/3	N-Azraq-7	ND
185/1	Sabah-1	4.41	472/1	Alshoroq-1	4.70
306/3	Leith-4	5.18	472/2	Alshoroq-1	4.11
219/1	Anwar-1	3.02	499/1	Itir-2	3.67
219/2	Anwar-1	4.73			

Evaluation of Host Range and Factors Related to Pathogenicity: *P. syringae* like strains isolated from seed were characterized for their host range, for traits related to pathogenicity, and for IN activity. Strains were considered to be *P. syringae* like if they produced fluorescent pigment on KB medium and gave negative responses in the tests for arginine dihydrolase and cytochrome C oxidase. Strains were purified and stored in 0.1 M phosphate buffer at 4 °C and in 40% glycerol at -80 °C before further testing.

The capacity of strains to induce a hypersensitive response was determined in tobacco by infiltrating, with a syringe, leaves of *Nicotiana tabacum* L. cv. Burley at the 10 leaf stage (bacterial suspensions of 48 h cultures, at approximately $1x10^8$ CFU ml^{-1}). Pathogenicity of each strain was determined on a range of field crops and vegetable crops representing a wide range of plant families and commonly grown in the dry land regions in the Middle East (Table 2): Bread Wheat (*Triticum aestivum* cv. Cham 4); Durum Wheat (*Triticum durum* cv. Cham 1); Barley (*Hordeum vulgare* cv. Arabi Aswad); Oat (*Avena sativa* cv. Local = not identified); Faba bean (*Vicia faba* cv. Romi); Lentil (*Lens exculenta* cv. Idleb 3); Chickpea (*Cicer arietinum* cv. Ghab 3); Pea (*Pisum sativum* cv. Local); Grass pea (*Lathyrus sativus* cv. Local) Coriander (*Coriandrum sativum* cv. Local); Cumin (*Cuminum cyminum* cv. Local); Black Seed (*Nigella sativa* cv. Local); and Tomato (*Solanum lycopersicum* cv. Local).

Fifteen plants of each crop (three replicates of 5 plants) were inoculated with each strain (14 total strains) and were incubated under controlled conditions. Tomato is of particular interest for this study because, in Syria, it is subject to recurrent and serious blight caused by *P. syringae*. All treatments were inoculated at the two-leaf stage by abrading the leaves with a bacterial inoculum loaded toothbrush and then covering the leaves with plastic bags in light for 5-7 days at 22-25 °C. The inoculum consisted of aqueous suspensions of 48-h bacterial cultures from KB plates adjusted to $3X10^8$ CFU ml^{-1}.

A well characterized non-pathogenic strain of *P. syringae* (R1) (resistant to rifampicin at 50 µg ml^{-1}) was used as a negative control and sterilized distilled water was used as an additional negative control. Symptoms were recorded on a daily basis, and the reaction of the plant was scored at 7 days after inoculation, using the following previously described scale (Morris *et al.,* 2000): 0 (no obvious symptoms); 1 (hypersensitive-like reaction restricted to the area of inoculation); 2 (slight expansion of a necrotic zone of tissue away from the area of inoculation and/or necrosis and breaking of the petiole); and 3 (expansion of a necrotic zone of tissue away from the area of inoculation, leading to wilting or death of the entire leaf or the entire plant). Bacteria were re-isolated from selected plants to confirm their presence by taking samples from the infected tissues, macerating in distilled sterilized water and spread-plating on KBC medium.

Production of syringomycin was evaluated on Nutrient Agar medium (Gross, 1985), according to the bioassay based on the sensitivity of *Geotrichum candidum* (Gross and DeVay, 1977). The distance between the border of the bacterial colony and the edge of the fungal lawn (zone of inhibition) was measured after 3 and 6 days of incubation. Ice-nucleation activity at -2 to -8 °C was assessed with 30

µL drops of aqueous bacterial suspensions containing a total of 10^7 cells as described before (Morris *et al.,* 2008).

Efficiency of Transmission of INA Strains of *P. syringae* from Artificially Inoculated Seeds to Cotyledons: The ability of *P. syringae* to be transmitted from seeds to seedlings was determined with two seed treatment methods using four lines of *P. syringae* and six bread wheat genotypes obtained from the bread wheat breeding program at ICARDA (Giza-168, Daira-14, Reyna-8, Seri.1B, Karawan-1/Mellal-1, and Massira/Jassira/Jadida-2). To monitor the bacteria after inoculation into seeds, lines of parental strains were selected that were resistant to rifampicin (50 µg ml^{-1}). Three rifampicin-resistant lines from strain CC1504 (referred to as R1, R2 and R3) and one resistant line from strain TA0043 (referred to as R043) were isolated. The parental lines are ice nucleation active and incapable of inducing hypersensitivity and were isolated from environmental sources as described previously (Berge et al, 2014).

The strains were grown on KBC medium and incubated at 22-25 °C for 48-50 hours. The bacterial cells were washed off using a 0.1 M phosphate buffer solution with carboxymethyl cellulose sodium salt (0.5 %) (Sigma-Aldrich) and transferred to sterilized test tubes; a mixture of the four lines of rifampicin-resistant bacteria was prepared as an additional treatment, giving a total of 6 treatments including the control treatment. The final concentration for each strain was determined by dilution plating on KBC medium.

The seeds were treated with the bacterial suspension using two methods: The first method consisted of coating seeds with a mixture of bacterial suspension and talcum powder. For this method, 1 ml of bacterial suspension (final concentration 1X 10^7 CFU ml^{-1})from each strain was added to 15 seeds (3 replicates) in a sterile flask (in average 6.5X10^5 CFU/seed), then the talcum powder was added gradually (about 1 g) with shaking. The flasks were wrapped in aluminum sheets and transferred immediately to the greenhouse for planting. For the control treatment, seeds were treated with a 1 ml solution of sterile 0.1 molar phosphate buffer with talcum powder. The second method consisted of soaking the seeds in a bacterial suspension: the seeds were treated with the same bacterial suspension in the same way as for the first method but they were then incubated at 22-25 °C overnight. On the next day talcum powder was added gradually. The control seeds were treated with a solution of 1 ml of phosphate buffer and talcum powder incubated at 22- 25 °C overnight and then transferred to the a greenhouse for planting.

For each inoculation method, three replicates of 10 seeds were treated. Seeds were sown in 10 cm pots containing soil, sand and organic matter at 2.7:1.3:1 (v:v:v). This mixture was disinfected in an autoclave at 121 °C for 20 min prior to sowing. Sown seeds were incubated at 22-25 °C until germination, after which each pot was covered with a nylon bag to prevent the transmission of bacteria between treatments. Pots were irrigated with low water influx on the soil surface.

At 15 days after germination, pots were transferred to the laboratory to determine the presence of *P. syringae* on leaves either by dilution plating to measure the population density of the bacteria or by evaluating the IN activity of the leaf tissue and 0.4 g from the upper half of leaves of all plants in the same pot were sampled, macerated in test tubes containing phosphate buffer 0.1 M (10ml), agitated for 1 min and stored in the refrigerator at 4 °C. To determine the abundance of *P. syringae* on leaves, three tenfold dilutions were prepared and plated on KBC medium and incubated for up to 5 days at 22–25 °C. The definitive counts of fluorescent and total colonies were realized after 5 days of incubation of plates. Ice nucleation activity was revealed by determining the freezing temperature of the suspensions, from -2 to -9 °C, by immersing the test tubes containing the leaf pieces in a cooling bath (3 replicates per strain and per genotype). The negative control was the suspension of control treatment leaves.

Verification of Bacterial Transmission from Leaves to Seed: The plots which showed bacterial colonization by *Pseudomonas* syringae on leaves were harvested and tested to study the transmission of the bacterium from leaves to seeds. This step was performed to evaluate the sustainability of the cycle of transmission of *P. syringae* from seed to leaves to seeds.

Two methods to isolate bacteria from seeds were applied to validate transmission, 1) seed soaking and 2) direct plating. For the seed soaking method, 1000 seeds from each genotype that showed *P. syringae* colonization of leaves (12 genotypes) were soaked in cold 0.85% NaCl (saline) containing 0.02% v/v Tween 20 (Sigma) and incubated at 4 °C overnight (16 h). After shaking vigorously for about 1 h, the sample was allowed to settle for 1 min and then tenfold dilutions in cold sterile saline were plated on KBC medium. The direct seed plating method was implemented also by placing 250 seeds (25 seeds per plate). Before plating,

seeds were treated with running tap water (non-chlorinated) and air dried. Colonies that produced a diffusible fluorescent pigment after 3–4 days incubation at 22- 25 °C were collected, purified and stored in 0.1 M phosphate buffer at 4 °C and in 40% glycerol at -80 °C, and later confirmed by physiological tests (absence of arginine dihydrolase and cytochrome C oxidase as described above) to be strains of *P. syringae.* The percent of the population that was recovered was estimated for the plating method and the population density on seeds (CFU/g) was estimated for the soaking method.

RESULTS

Natural Populations of *P. syringe* on Leaves of Bread Wheat Breeding Lines in Syria: Natural populations of *P. syringae* on wheat leaves were highly variable depending on the wheat genotype (Table 1) with some genotypes (128/3, 349/3, 364/2) supporting high populations of *P. syringae*, while on other genotypes the populations were much lower (genotypes 128/1 and 219/1).

Out of the 25 bacteria strains obtained from leaf samples, 12 were shown to be *P. syringae*. Interestingly, the majority of the strains isolated (8 / 12) could not induce hypersensitivity on tobacco (Table 2.). Nearly all (7/8) of these strains came from lines of wheat that harbored population densities of *P. syringae* on their leaves of at least 10^4 CFU g^{-1} and as high as 4 x 10^5 CFU g^{-1} (line 128/3).

Table 2. Traits of *P. syringae* like bacteria isolated from bread wheat leaves.

Physiological traits						Host Range[a]												
Strain Code[b]	Levan production	Potato Rot	Tobacco Hypersensitivity	Ice Nucleation [c]	Toxin Production[d]	bread wheat	duram wheat	Barley	Oat	Fava bean	Lentil	Chickpea	Grass pea	Pea	Coriander	Cumin	Black seed	Tomato
22/2	+	-	+	-6 °C	5													
115/1	-	+	-	-5 °C	13													
122/2	-	+	-	-6 °C	0													
122/3	-	+	+	-5 °C	17													
128/3	-	+	-	-5 °C	0													
133/2	+	-	-	-5 °C	8													
234/1	+	-	-	-4 °C	23													
234/2	+	-	+	-5 °C	13													
292/1	+	-	-	-4 °C	18													
306/3	+	+	-	-4 °C	0													
472/2	+	+	+	-5 °C	0													
499/1	+	-	-	-4 °C	13													

a. Pathogenicity was considered positive if more than half of the inoculated plants gave compatible reactions of score 3 (black squares) or score 2 (medium grey squares). Some strains caused compatible reactions on some plants, but fewer than half of the plants tested (light grey squares) and some strains never caused compatible reactions (white squares).

b. The origin of strains is indicated by the sample code. These codes are indicated in Table 1.

c. Ice nucleation activity (INA). For each strain, 3 droplets of 30 µL of an aqueous suspension containing 107 cells per droplet were tested at temperatures of 1°C intervals from -2 C to -8 C. Freezing was considered positive when at least 2 of the 3 droplets froze.

d. The width (mm) of the zone of inhibition against *Geotrichum candidum* is indicated.

The incapacity to induce hypersensitivity in tobacco was not necessarily an indication of the absence of pathogenicity in *P. syringae* as has been observed elsewhere (Bartoli *et al.,* 2014; Demba Diallo *et al.,* 2012). Host range of the strains was highly variable, spanning from one host (strain 234/1) to all of the plant species and cultivars tested (strains 133/2 and 272/1). Although the strains that could not induce hypersensitivity in tobacco included those with the most restricted host range (234/1 and 128/3), they also included the strains with the broadest host range.

The Inoculation of Wheat Seed with INA Strains of *P. syringae* Assures the Transfer of Bacteria to Seedlings: For these trials, the movement of strains from inoculated seeds to leaves of seedlings was verified by using spontaneous variants of strains that were resistant to the antibiotic rifampicin. Selection and characterization of these bacterial variants was time-consuming. Therefore, the strains of *P. syringae* used for this part of the study came from previous work as described above. The capacity of *P. syringae* to move from the seed to the seedling depended on the strain of the bacterium and on the genotype of the wheat (Table 3). Strain R1 was able to move from the seed to seedlings of all wheat genotypes, attaining population densities up to 10^6 CFU g^{-1}. Strains R2 and R3 were more inconsistent in their capacity to move from seeds to seedlings and R043 was not detected on any seedlings by dilution plating. When detected, bacterial population densities varied up to 100 fold among the different plant genotypes with the Seri.1B genotype harboring the highest population densities on leaves. In ice nucleation assays, none of the leaf tissue emerging from seeds treated with sterile buffer froze in the range of temperatures tested. In contrast, nearly all of the leaf tissue collected from plants from seeds inoculated with *P. syringae* froze at -9 °C or warmer suggesting that IN active cells of the bacterium were present even though they could not be detected with dilution plating. The warmest temperatures of freezing were not necessarily associated with the largest population densities of *P. syringae* on leaf tissue suggesting that there was an interaction of plant genotype and bacterial strain on IN rate of *P. syringae*.

Table 3. Populations of *P. syringae* on leaves and the concomitant IN activity of leaves from plants emerging from inoculated seed.

Log_{10} mean population density of *P. syringae* (CFU/g)/Warmest freezing temperature of 0.4 g leaf tissue pieces (°C)										
Strain	R1		R2		R3		R043		Mixture	
Treatment	Coating	Soaking	Coating	Soaking	Coating	Soaking	Coating	Soaking	Coating	Soaking
Giza-168	3.7 / -8	ND[1] / -8	ND /-8	ND /-9	4.1 / -8	ND / -7.5	ND / -8	ND / ND	4.5 / -7	3.8 / -9
Daira-14	4.4 / -8.5	ND / -9	2.8 / -9	ND / -8	ND / ND	ND / -7	ND / ND	ND / -7	5.0 / -8	ND / -8
Renya-8	4.4 / -9	ND / -8.5	ND / -8	ND / -9	ND / -7	ND / -8	ND / -9	ND / ND	4.7 / -7	4.6 / -9
Seri.1B	6.1 / -9	5.6 / ND	4.6 / -8	ND / -9	2.3 / -9	3.9 / -8	ND / -9	ND / -9.5	6.8 / -7	4.8 / -7
Karawan-1	5.3 / -8	ND / -8	ND / -9	ND / -9	ND / -7	ND / -8	ND / ND	ND / -9	4.9 / -8	5.4 / -8
Massira	5.6 / -9	1.0 / -9	1.9 / -8	ND / -8	ND / -9	ND / ND	ND / -9	ND / ND	4.4 / -8	5.8 / ND

[1]Not detected. For populations on leaves the detection threshold was 7.5X 103 CFU/g. For the IN test, freezing was reported as not detected if only 1 or no tubes froze in the range of temperatures tested (-2 ° to -9 °C).

Transmission of INA Bacteria from Wheat Leaves to Seeds under Natural Conditions: Among the 25 wheat genotypes tested in the field for epiphytic populations, only 12 carried *P. syringae*. The transmission of *P. syringae* to seeds harvested from these 12 genotypes was then determined. Seed-borne populations were assessed at two storage periods: immediately after seed harvest and after 3 months of storage.

Immediately after harvest *P. syringae* was detected on the seeds of all 12 genotypes, depending on the method of detection (Table 4). Interestingly, there was no apparent correlation of the size of the population on leaves in the field with the percent of seeds carrying *P. syringae* or with the size of the populations on the seed. After 3 months of storage, *P. syringae* was not detected on the seeds of any of the genotypes via the seed soaking method whereas it was detected on a small percent of the seeds for 6 of the genotypes via the direct plating method. In spite of the fewer seeds that were used in the direct plating method compared to the seed soaking method, the direct plating method was more efficient in detecting *P. syringae* most likely due to enhancement of bacterial growth by the presence of the seed on the plating medium.

DISCUSSION

Our results demonstrate that wheat breeding lines and cultivars adapted to dry land agriculture vary considerably in the number and diversity of *P. syringae* that populate their leaf surfaces both in terms of their potential as pathogens and as ice nuclei. These wheat lines also differ in their propensity for epiphytic P. syringae to be transmitted to and survive on the seed and subsequently for the colonization of leaves from seed-borne populations. Furthermore, there is a marked interaction between strains and wheat genotype in these processes. Interestingly, strains of *P. syringae* with little pathogenic potential and with marked IN activity at

relatively warm temperatures (> -10 °C) were readily detected on the wheat phyllosphere and were transmitted from leaves to seeds and vise-versa. These results illustrate that wheat lines could be selected to favor the non-pathogenic IN active component of the indigenous microflora. Likewise they also suggest that the microflora of wheat could be further altered by inoculating seed with the strains of *P. syringae* that are most fit for colonization and with the least potential as pathogens and the most potential as ice nucleators. Overall, this work points to the possibility of selecting plants with the goal of changing their microflora for purposes other than resistance to plant disease. In this case we propose altering the microbiology of plant surfaces for the eventual effect that these microorganisms can have on the atmosphere when they are removed from plants by air turbulence and transported into the troposphere. All microorganisms on plant surfaces are susceptible to being released into the atmosphere, however the traits of plants that confer the intensity of this release are not currently considered in plant breeding. Selecting plants for enhanced microflora that render various ecological services could be extended to other services such as antagonistic biocontrol activity toward pathogens. For example, most of the strains of *P. syringae* characterized in this study produced a toxin that inhibited fungi, a trait that could potentially be useful in limiting certain fungal pathogens.

Table 4. Detection of naturally occurring *P. syringae* on seeds of wheat from field-grown plants.

Plant genotype	Thousand seed weight (g)	*P. syringae* on seeds[1] immediately after harvest		at 3 months after harvest	
		CFU[2]/ g	% infected seed	CFU/ g	% infected seed
22/2	49.5	>900	40	ND[3]	ND
115/1	55	150	10	ND	ND
122/2	53	690	20	ND	ND
122/3	53	160	ND	ND	ND
128/3	56	490	26	ND	ND
133/2	52.5	560	24	ND	2
234/1	57.5	870	64	ND	16
234/2	57.5	470	24	ND	1
292/1	49	530	16	ND	4
306/3	54	710	52	ND	1
472/2	51	300	18	ND	ND
499/1	53.5	330	56	ND	18

[1]The presence of P. syringae on seed was determined either by dilution plating the buffer used to wash 1000 seeds (expressed as CFU/g of seed) or by allowing bacteria to grow from 250 seeds place directly on nutrient medium (expressed as the % of seeds carrying the bacterium).

[2]Colony-forming units

[3]Not Detected

Our results also illustrate that breeding and selection of wheat for traits such as yield or disease resistance could have inadvertently selected against abundance of IN active *P. syringae* or other biological IN active microorganisms. Direct selection against IN active *P. syringae* is likely when the size of populations that this bacterium can attain on leaves is correlated with resistance to disease caused by this bacterium as in the case of snap beans (Daub and Hagedorn, 1981). In regions where drought is a major limitation for crop production, it would be important to develop breeding and production strategies that protect crop health while fostering the contribution of plants to the water budget – in terms of tolerance to water limitation as well as contribution to the bioprecipitation cycle. As cultivated and managed land grew to represent 50% of the vegetated surface of Earth (Ellis *et al.,* 2010), the areas of land planted to genetically homogenous crops has increased and in particular for the major staple crops. In the developing world this homogeneity has resulted mainly from the efforts of the major international agricultural research centers to offer farmers the necessary resources for assuring the best possible yields. Via the networks of these international research centers for distributing plant cultivars and monitoring their performance, the environmental impact of

breeding plants to make beneficial contributions to the water cycle could be significant and could be set in a context where it could be evaluated on a large scale.

CONCLUSIONS

We have demonstrated that wheat breeding lines adapted to dry land agriculture vary in the size of the populations of weakly pathogenic and IN active *P. syringae* that they harbor on their leaves and in their capacity to transmit these bacteria to seeds and subsequently from seeds to leaves. Furthermore, we have also revealed a marked interaction between strains of *P. syringae* and the capacity of wheat breeding lines to foster leaf colonization and transmission from leaf to seed and back to leaves. This variability could be the basis of a breeding program that targets the development of wheat lines that contribute to the bioprecipitation cycle by generating aerosols of IN active bacteria that could favor rainfall under those conditions in clouds that are not favorable for other common atmospheric ice nuclei such dust and mineral particles to induce rain. Seed treatment with specific strains of *P. syringae* enhanced the colonization of leaves of certain wheat genotypes by these bacteria. Hence, the creation of plant cultivars that favor the abundance of IN active *P. syringae* could be coupled to seed inoculation methods in an attempt to develop a new generation of wheat lines adapted to dry land agriculture.

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