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FULL RESEARCH PAPER

Contamination of bean seeds by *Xanthomonas axonopodis* pv. *phaseoli* associated with low bacterial densities in the phyllosphere under field and greenhouse conditions

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Abstract *Xanthomonas axonopodis* pv. *phaseoli* and its variant *fuscans* are the causal agents of common bacterial blight of bean. Production of seeds is recommended in arid climates with the use of pathogen-free seeds. However, contamination of seeds still occurs in these seed production areas. To verify if low contamination levels of sown seeds could explain these field contaminations, we used seeds that were naturally contaminated with CFBP4834-R, a rifamycin-resistant *X. axonopodis* pv. *phaseoli* fuscous strain, to contaminate field plots at different rates. We also inoculated seeds to verify some parameters of plant colonization and seed transmission. In growth chambers, seedling contamination was always successful from seeds contaminated with CFBP4834-R having population sizes greater than 1×10^3 CFU seed^{-1} and were not successful below 1×10^2 CFU seed^{-1}. In the greenhouse, the efficiency of contamination of seeds was not significantly different between contaminated plants that had a low or a high CFBP4834-R population size and reached between 40% and 52% whatever the origin of the inoculum (aerial or seed-borne). In field experiments, under low relative humidity, plots with 0.1–0.003% contamination rates or plots sown with seeds that were inoculated with low CFBP4834-R population sizes (1×10^2 and 1×10^4 CFU seed^{-1}) led to an asymptomatic colonization of bean during the entire growing season with low CFBP4834-R population sizes. Seeds were contaminated both in primary and secondary foci. The contamination of seeds without symptom expression during the growing season represents a risk for eventual disease outbreaks.

Keywords Common bacterial blight · Ecology · Epidemiology · Epiphyte · Plant pathogenic bacteria

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Introduction

Infested seeds represent the most important means of survival for many plant pathogenic bacteria. To limit this major inoculum source, specific seed production areas were created in several countries (Webster et al. 1983). These zones are located in areas where climate is considered to be non-conducive to diseases and/or where seed producers follow strict rules concerning the sanitary quality of stock seeds and cultural conditions—long rotations and isolated location of fields—to limit the introduction and multiplication of inoculum (Schaad 1988; Laurent 1990). Despite these

measures that considerably reduce disease incidence (Schaad 1988; Laurent 1990), epidemics sometimes occur. Such outbreaks could originate from contamination of stock seeds that was not detected due either to low population sizes per seed or to low rates of seed contamination that were below the detection threshold. The few available data on the inoculum threshold of seedborne bacteria sufficient for epidemic development indicate that these thresholds are dependent on plant-pathogen-environment interactions (Schaad 1988). For the halo blight pathogen *Pseudomonas savastanoi* pv. *phaseolicola*, Taylor estimated that analysis of 10,000 seeds was necessary for sanitary control of the disease in the UK (Taylor 1970). Trigalet and Bidaud (1978) found that one seed out of 20,000 was the tolerable level of contamination for which no epidemic of halo blight develops even under the most favourable environmental conditions in France.

Different means of seed contamination have been reported for diseases caused by bacteria (Maude 1996). Seed contamination can be external as a consequence of contact with bacterial populations on symptomatic tissue, or a high inoculum load on the pods, or during threshing with residues carrying large bacterial populations (Weller and Saettler 1980b). Seeds may also become infested by invasion of floral structures as demonstrated for bacterial blight of soybean (Kaufman and Leben 1974) and more recently for bacterial fruit blotch of watermelon (Walcott et al. 2003). Seeds can be internally contaminated by systemic movement of the pathogen from infected mother plants. For example, common bacterial blight of bean can occur after pedicel infiltration (Aggour et al. 1989). The relative impact of these different means of seed contamination in the field remains unknown.

Xanthomonas axonopodis pv. *phaseoli* (Vauterin et al. 1995, 2000) (*Xap*) and its variant *fuscans* (*Xapf*) are causal agents of common bacterial blight of bean (*Phaseolus vulgaris*). Both externally and internally contaminated seeds can transmit common bacterial blight (Weller and Saettler 1980b). From a contaminated seed, bacteria first colonize seedlings asymptomatically and disperse in the field (Ishimaru et al. 1991; Jacques et al. 2005; Weller and Saettler 1980a). *Xapf* strains are well adapted to colonize foliar surfaces as they are organized in biofilms that protect them from environmental stresses (Jacques et al. 2005). A threshold of population size (1×10^5–1×10^6 CFU g^{-1} of fresh leaf tissue) is necessary to induce symptoms (Jacques et al. 2005; Rudolph 1993; Weller and Saettler 1980a).

Symptoms of common bacterial blight of bean develop on aerial parts of plants. When grown from infected seeds, seedlings will have injured growing tips. Small angular water-soaked lesions develop on the undersurface of leaves. Lesions later evolve into necroses usually surrounded by a bright yellow halo. Spots can coalesce with adjacent lesions and, for severe infections, symptoms can cause leaf death and defoliation. Small lesions can also be observed on stems sometimes causing girdling. On pods, water-soaked areas enlarge with time, become brick red and slightly sunken; bacterial exudates can be seen covering the lesion. Brown spots can be observed in the hilum or on the coat of white seeds but are not apparent on dark-coloured seeds. Contaminated seeds can also be symptomless (Gilbertson and Maxwell 1992; Vidaver 1993).

Prophylactic measures for controlling common bacterial blight include the use of bacterial-free seeds. In France these are produced according to strict rules in two registered areas (Laurent 1990). Sanitary quality of stock seeds must conform to current quarantine regulations in Europe imposing the absence of common blight bacteria in lots of 30,000 seeds (Laurent 1990). In these registered areas despite all measures to limit inoculum introduction and multiplication, contamination of seeds sometimes occurs. Such outbreaks could originate from seed contamination that was not detected. Indeed, the real biological significance of such low levels of contamination in temperate climates has not been determined for this disease, in contrast to the well-established biological significance of the contamination thresholds for halo blight disease of bean (Taylor 1970; Trigalet and Bidaud 1978).

The objective of this research was to determine if low levels of contamination of bean seed lots by *Xapf* could result in field contamination of beans and finally in the transmission of the pathogen to the seeds. To test this hypothesis, we established bean plots using seed lots at known contamination rates in areas isolated from other bean fields and eliminated other potential inoculum sources such as contaminated debris, volunteer beans and other plants likely

to harbour epiphytic populations of *Xap* such as onion (Gent et al. 2005).

Materials and methods

Bacteria

All experiments were performed with a rifamycin-resistant fuscous strain of *X. axonopodis* pv. *phaseoli*, CFBP4834-R (Jacques et al. 2005). This strain is highly aggressive on cv. Flavert bean. CFBP4834-R was stored at −80°C in 40% (w/v) glycerol and routinely grown at 28°C on selective medium 10% TSA-R (1.7 g l^{-1} tryptone, 0.3 g l^{-1} soybean peptone, 0.25 g l^{-1} glucose, 0.5 g l^{-1} NaCl, 0.5 g l^{-1} K_2HPO_4 and 15 g l^{-1} agar supplemented with 50 mg l^{-1} rifamycin, 50 mg l^{-1} cycloheximide and 10 mg l^{-1} propiconazole). Cycloheximide and propiconazole were added to inhibit fungal growth and both these fungicides had no inhibitory effect against CFBP4834-R. To prepare inoculum, CFBP4834-R was grown for 48 h on 10% TSA-R. Bacterial cells were suspended in sterile distilled water (SDW). Suspensions were calibrated to 1×10^8 CFU ml^{-1} with a turbidimeter (Hach Company, Colo. US) and adjusted to the desired final concentrations with SDW.

Monitoring of CFBP4834-R population sizes

Seeds were soaked in 2 ml of SDW per g of seeds (5.56 seeds g^{-1}) or per seed for individual analysis overnight at 4°C. Then samples were vortexed for 30 s at maximal speed three times. Individual leaves and bulks of flowers or pods per plant were ground (Stomacher 80; Seward, London, UK) for 2 min at maximum power in 5–10 ml of SDW. Aliquots of 100 µl (5 × 100 µl for samples of 1,000 seeds) and serial dilutions were plated on 10%TSA-R medium to quantify CFBP4834-R population sizes.

Production of the typical fuscous pigment on TSA (17 g l^{-1} tryptone, 3 g l^{-1} soybean peptone, 2.5 g l^{-1} glucose, 5 g l^{-1} NaCl, 5 g l^{-1} K_2HPO_4, 15 g l^{-1} agar), rifamycin resistance and typical yellow mucoid colony morphology were criteria used to confirm that isolates were CFBP4834-R. When colony morphology of these fuscous colonies was not typical, identification was confirmed using a specific PCR test. The X4e and X4c primers of Audy et al. (1996) were used with 35 two-step cycles as recommended by the authors in a Genamp PCR system 9700 thermocycler (Applied biosystems, Courtaboeuf, France): 94°C for 1 min and 72°C for 2 min. The reaction mix (25 µl) contained 200 µM dNTP, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 0.05 U $µl^{-1}$ Taq polymerase (Goldstar Red, Eurogentec, Angers, France) and 5 µl of a boiled bacterial suspension (10^7 CFU ml^{-1}). Agarose electrophoresis (1.2%) revealed a 730 bp DNA fragment for a *Xapf* isolate. Negative and positive controls, of the PCR reaction, were systematically run on SDW and a suspension of CFPB4834-R at 10^6 CFU ml^{-1}, respectively. The pathogenicity of rifamycin-resistant *Xapf* isolates recovered from samples was also verified. *Xapf* isolates were inoculated by immersing the second trifoliate leaf of 3 week-old beans in suspensions of 10^7 CFU ml^{-1} for 30 s. Beans were maintained under high humidity for 4 days then were kept at standard greenhouse conditions during the remaining 9 days. Three beans were inoculated per isolate. Symptoms were recorded daily for 11 days.

The contamination rate of a seed lot (p) was calculated from the analysis of a sample divided in N groups according to the formula $p = 1-(Y/N)^{1/n}$ (Maury et al. 1985), where n is the number of seeds in each group and Y the number of healthy groups.

Plant material

Experiments were performed with the flageolet bean (*Phaseolus vulgaris*) cv. Flavert (Vilmorin, La Ménitré, France). This cultivar is susceptible to common bacterial blight. Three different lots of healthy stock seeds (harvested in 2000, 2001 and 2002) were provided by Vilmorin and were considered to be free of *Xap* and *Xapf* following the analysis of about 100,000 seeds per lot with standard tests (International Seed Testing Association 2007). Three different lots of stock seeds were produced by spray-inoculating field-grown beans at the first trifoliate stage (Michael 1994) with suspensions of CFBP4834-R at 1×10^8 and 1.5×10^7 CFU ml^{-1} in 2001 and 2002, respectively. Seeds produced by these contaminated plants were hand-harvested and are referred to as lots of naturally contaminated stock seeds (lots A and B harvested in 2001 and lot C harvested in 2002) in contrast to seeds that were inoculated by soaking. Contamination rates of these lots and the quantity of

CFBP4834-R per contaminated seed were determined (Table 1) in order to determine the quantity of seeds to be used to establish disease foci in field experiments.

Table 1 Contamination rates of seed lots B and C naturally contaminated with *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* CFBP4834-R

Selected groups	CFBP4834-R population sizes (CFU seed^{-1})	Contamination rate (corrected contamination rate)[a]
Contaminated groups from the analysis of 18 groups of six seeds (lot B)		
1	35	
2	3.6×10^4	
3	1.8×10^2	
4	18	$N = 18$, $n = 6$, $Y = 5$
5	2	$p = 19{,}2\%$
6	5	($N = 18$, $n = 6$, $Y = 14$
7	5.7×10^5	cp = 4%)
8	37	
10	2	
12	2.8×10^2	
13	5	
14	3	
15	77	
Contaminated groups from the analysis of 30 groups of one seed (lot C)		
2	4	
8	3.3×10^5	$N = 30$, $n = 1$, $Y = 31$
10	12	$p = 30\%$
11	8	($N = 30$, $n = 1$, $Y = 28$
12	4	cp = 6%)
19	4	
22	1	
23	4	
28	5×10^6	

To determine the contamination rates of the two seed lots, shown to be highly contaminated by previous analysis, 18 groups of six seeds were analyzed for lot B and 30 groups of 1 seed for lot C

[a] p is the contamination rate of seed lots. It was calculated according to the formula $p = 1-(Y/N)^{1/n}$, where N is the number of analyzed groups, n the number of seeds in a group and Y the number of healthy groups (Maury et al. 1985). The corrected contamination rate (cp) takes into account only seeds for which CFBP4834-R is present at population sizes >10^2 CFU seed^{-1}

Contamination of seeds under greenhouse conditions

Experiments were conducted in the greenhouse to determine the effect of the inoculum source on the dynamics of *Xapf* CFBP4834-R populations on bean plants inoculated via the seeds or leaves and on the rates of subsequent contamination of the seeds from these plants. Eighty healthy bean seeds were vacuum infiltrated by soaking seeds for 30 min at room temperature in a suspension of CFBP4834-R at 2.3×10^8 CFU ml^{-1} (2 ml seed^{-1}). The vacuum was applied for 3 min. Seeds were then dried for 2 h in a sterile environment. CFBP4834-R population sizes were determined on six inoculated seeds at the time of inoculation. The remaining inoculated seeds were sown at the same time as 160 healthy seeds each in $10 \times 10 \times 18$ cm pots (1 seed per pot) containing soil substrate (Neuhaus humin substrat S NF 11-44-551, Proveg, La Rochelle, France). Plants were watered daily and supplemented with 0.3 g l^{-1} NPK (18/14/18) once a week. Five weeks after sowing, when plants reached the flower bud stage (Michael 1994), 80 plants from healthy stock seeds were spray-inoculated until run-off with a suspension of CFBP4834-R at 2.5×10^6 CFU ml^{-1} and 80 other plants with a suspension at 1.9×10^4 CFU ml^{-1}. Population sizes of CFBP4834-R were determined just after spray-inoculation and at 1, 6, 13, 20, 27, 34 and 41 days after spray-inoculation on one young trifoliate leaf per plant for six plants per treatment and per sampling date. CFBP4834-R population sizes were determined on flowers and pods 34 and 41 days after inoculation. Flowers and pods were bulked per plant. Six weeks after spray-inoculation, seeds of 21–25 plants per inoculation condition were analyzed for presence of CFPB4834-R. Seeds were bulked per plant.

Effect of seedborne bacterial population sizes on seedling contamination

Four replicates of 23 seeds of lot A naturally contaminated with CFBP4834-R and one additional healthy seed per replicate as a negative control were analyzed for the presence of CFBP4834-R by washing and dilution plating as described above. Four other replicates of 23 seeds of lot A and one healthy seed per repeat as a negative control were incubated

in sterile germination boxes (17.5 × 11.5 × 15.5 cm) on moistened sterile paper at 28°C. Three days later, seedlings were analyzed for the presence of CFBP4834-R.

Seeds that were artificially contaminated with CFBP4834-R were also tested. Forty-two healthy seeds per inoculum dose were vacuum-infiltrated. Three inoculum doses (1.5×10^3 CFU ml^{-1}, 1.5×10^5 CFU ml^{-1} and 1.5×10^7 CFU ml^{-1}) were used. After inoculation, 21 seeds per inoculum dose and one healthy seed as a negative control were analyzed for the presence of CFBP4834-R. The remaining inoculated seeds were incubated for germination and, three days later, 21 seedlings per inoculum dose and one healthy seedling as a negative control were analyzed for CFBP4834-R. In both experiments, every seed or seedling was analyzed individually.

Impact of low contamination rates of bean seed lots on the establishment of *Xapf* on bean plants

Two field experiments were conducted to evaluate epidemiological risks conferred by different contamination rates of seed lots (0.003%, 0.03% and 0.1%) by *Xapf*. Working with low contamination rates requires large plots that could be difficult to survey precisely. To avoid this problem, plots were seeded first with healthy seeds. On the same day, seeds naturally contaminated with CFBP4834-R were sown by hand in the same field and their position was labelled with a post to ensure a reliable survey of primary foci. This strategy avoided further sowing by machine and subsequent contamination of heavy farm equipment that would be difficult to disinfect. To prevent contamination of these experimental fields by exogenous inoculum sources, experimental plots had no history of bean cultivation, were located among orchards, and were in a different site each year.

Seeds were sown in experimental fields (INRA experimental station, Maine-et-Loire, France) in 2002 on May 27 and in 2003 on May 23 at a density of 16 seeds linear m^{-1} with 0.6 m between rows. Beans were cultivated under traditional agricultural methods for bean seed production in the Loire valley. Three plots were sown per contamination rate and for the negative control (Fig. 1). Contaminated seeds (from lots B and C) were sown among healthy seeds in a regular pattern to favour a homogeneous inoculum distribution (Fig. 1). The rates of natural contamination for seed lots B (used in 2002 field experiment) and C (used in 2003 field experiment) were first determined to be 19.2% and 30% (Table 1), respectively. However, many seeds harboured very low CFBP4834-R population sizes (below 1×10^2 CFU $seed^{-1}$) that likely failed to transmit bacteria to the seedling (Weller and Saettler 1980b, this paper). Hence, corrected contamination rates were calculated by taking into account only contaminated seeds with bacterial densities higher than 1×10^2 CFU $seed^{-1}$. Corrected contamination rates were determined to be 4% and 6% for lots B and C, respectively (Table 1). Germination rates of lot B and C were 100% and 89%, respectively. Consequently, 83 and 62 seeds from contaminated seed lots B and C, respectively, were sown on a regular grid in each plot of the 0.003% treatment to establish 3 foci per plot and per year. For the 0.03% and 0.1% treatments, 125 and 94 seeds from contaminated seed lots B and C, respectively were sown in order to establish five foci per plot each year. Every seed from naturally contaminated seed lots was considered as a potential focus as we had no means to precisely determine which specific seeds were contaminated in the lot. In order to limit and to detect cross-contaminations, two windbreaks were sown between each contamination rate. They consisted of one row of maize, two rows of healthy bean trap plants and one row of maize. Each windbreak was separated from the next one by a 1.2 m of raw soil.

If rain did not occur naturally, plots were watered to mimic naturally occurring rainfalls thereby yielding around 10 mm of simulated rain per week. In addition, plots were watered twice with 50 mm (07/01/2002 and 07/15/2002) to mimic rainstorm conditions. In 2003, as rainstorms naturally occurred, no additional watering was applied. Temperature and relative humidity (RH) were monitored during the two growing seasons.

Presence of common blight symptoms (all those described in the introduction section) was monitored every week in the potential contaminated foci as these were potential primary foci. Their surrounding plants were also surveyed. Control plots were surveyed by passing in one every five rows. Population sizes of CFBP4834-R were monitored on one leaflet of a trifoliate leaf per plant in every focus of

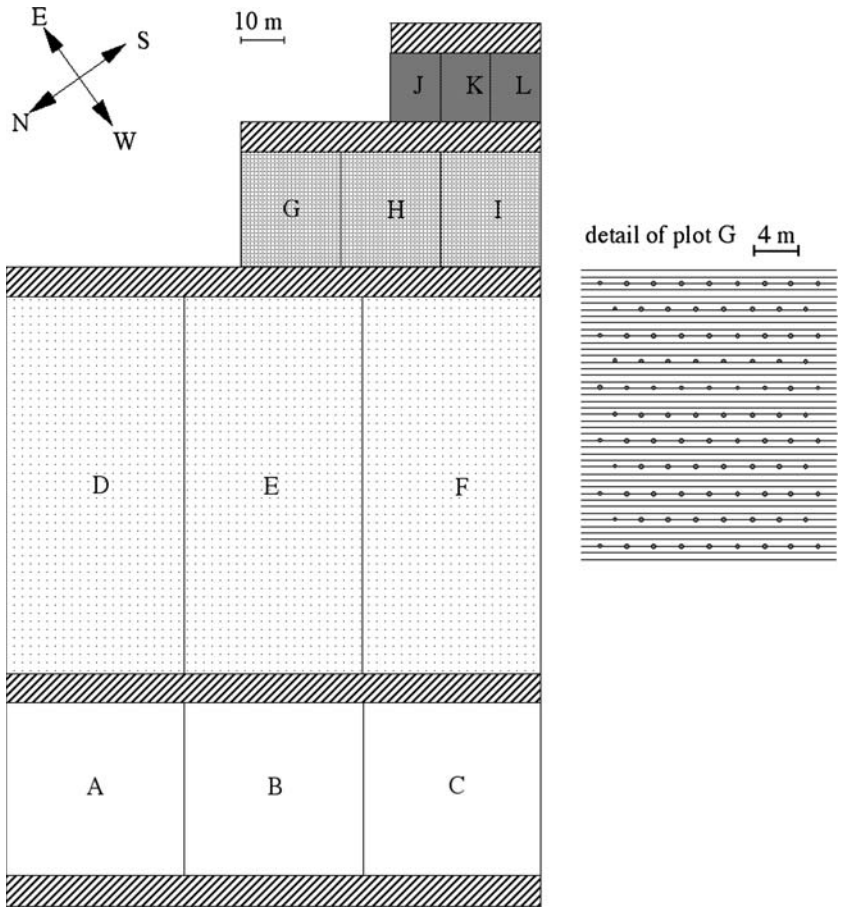

Fig. 1 Design of experimental fields aimed at evaluating epidemiological risks of different contamination rates of bean seed lots by *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* CFBP4834-R. Decreasing dot densities correspond to 0.1% (plots J, K and L), 0.03% (plots G, H and I) and 0.003% (plots D, E and F) contamination rates and control plots (plots A, B and C). Hatching corresponds to windbreaks. Continuous lines indicate limits of plots. As an example, the details of plot G illustrate potential foci (circles) distribution in rows (continuous lines) of healthy seeds

plot G (Fig. 1) at least twice per experiment. Leaf samples were always analyzed individually.

For each plot, one area of nine rows (corresponding to the width of the planter) for a 10 m length was hand-harvested 18 weeks after sowing. Harvested plants were dried a few days in the greenhouse and threshed manually. Harvested lots were treated individually and harvesting equipment was disinfected with sodium hypochlorite and 70% ethanol between each lot. For each plot, 30 samples of 1,000 seeds were analyzed for the presence of CFBP4834-R in harvests. One sample of 1,000 healthy seeds was added as a negative control. Pathogenicity of at least 25 isolates from each of two harvested seed lots per year was confirmed.

In order to study contamination of seeds for secondary foci, 72 bean trap plants included in windbreak plantations (nine bean plants per each of eight windbreaks) were harvested individually. Seeds were bulked per plant and analyzed for the presence of CFBP4834-R.

Effect of seed-borne populations of CFBP4834-R on contamination of plants under field conditions

A field experiment was performed to study the effect of seed-borne bacterial population sizes on the contamination rates of plants and seeds. Thirty seeds per inoculum dose were vacuum-inoculated with CFBP4834-R suspensions at inoculum doses of 10^4 and 10^6 CFU ml^{-1}. Seeds were sown (06/21/2002) in alternation with healthy seeds, in a row and between rows. Seed density and agricultural practices were similar to those used for other field experiments. Presence of symptoms was monitored every week on each plant. Population sizes of CFBP4834-R were monitored as described above twice during the growing season on one leaflet for ten plants per

treatment. Every plant was harvested individually (09/16/2002). Seeds were bulked per plant and analyzed for the presence of CFBP4834-R.

Statistical analyses

Statistical analyses were performed using Statbox Pro software (Grimmer Logiciels, Optima France). Properties of the data were verified (homogeneity of variances with Bartlett's test, normality of $\log_{10}$ transformed population size distribution with Pearson coefficients). Analyses of variance were conducted when data were log-normally distributed and variances were homogenous; means were compared with the Newman-Keuls test (Sokal and Rohlf 1969). The Kruskal–Wallis and Mann–Whitney tests were used when there was significant deviation of the data from a log-normal distribution and/or when variances were not homogenous. Comparisons of proportions of contaminated seeds were based on a Pearson's χ^2 test.

Results

Effect of mode of contamination of plants on seed contamination under greenhouse conditions

Seeds inoculated with a suspension of CFBP4834-R at 2.3×10^8 CFU ml^{-1} were contaminated at a mean population size of 4.7×10^6 CFU seed^{-1}. Under greenhouse conditions, these seeds gave rise to plants on which the population dynamics of CFBP4834-R on young trifoliate leaves, flowers and pods were not different ($P > 0.05$) from those on plants that were spray-inoculated at flower bud stage with a suspension at 1.9×10^4 CFU ml^{-1} (Fig. 2). During the bean growing season, CFBP4834-R population sizes on leaves remained as low as 1×10^2 CFU organ^{-1} (Fig. 2). Population sizes on pods were also generally similar for plants inoculated by these two different methods and means did not exceed 1.2×10^4 CFU organ^{-1}. No symptoms were observed throughout bean development on any aerial parts of these plants. In contrast, plants that were spray-inoculated with more bacteria (2.5×10^6 CFU ml^{-1}) (Fig. 2) displayed significantly higher population sizes on trifoliate leaves (up to 1×10^6 CFU organ^{-1}) and pods (9.7×10^8 CFU organ^{-1}). Symptoms were observed on pods of plants inoculated with suspensions at 2.5×10^6 CFU ml^{-1}. Despite these differences in population sizes and presence or absence of symptoms, no significant differences (calculated $\chi^2 = 0.93$; $\chi^2_{.05[2]} = 5.991$) were recorded in the frequencies at which the seeds were contaminated. For plants grown from contaminated seeds (4.7×10^6 CFU seed^{-1}) seeds from ten plants out of 25 (40%) were contaminated, for plants spray-inoculated with an inoculum of 1.9×10^4 CFU ml^{-1}, seeds from eight plants out of 19 (42%) were contaminated, and for 2.5×10^6 CFU ml^{-1}, seeds from 11 plants out of 21 (52%) were contaminated.

Effect of seedborne bacterial population sizes on seedling contamination

For naturally contaminated seeds a population of at least 1×10^3 CFU seed^{-1} was required for an effective seedling contamination. As the analysis of CFBP4834-R population sizes on seeds and seedlings showed no significant effect of replicates ($P > 0.05$), data from the four replicates were pooled. The number of contaminated individuals was significantly ($P < 0.05$) higher for seeds (42.4%) than for seedlings (7.6%). The distribution of contaminated individuals in classes of population sizes was also different between seeds and seedlings (Table 2) (calculated $\chi^2 = 29.88$; $\chi^2_{.005[4]} = 14.86$). The number of seeds harbouring more than 1×10^3 CFBP4834-R CFU seed^{-1} was not significantly different (calculated $\chi^2 = 0.073$ and $\chi^2_{.01[1]} = 6.635$) from the number of contaminated seedlings. Hence, seeds harbouring population sizes lower than 1×10^3 CFU seed^{-1} did not give rise to contaminated seedlings.

The *Xapf* population size necessary for seedling contamination was also determined by artificial inoculation of seeds. In this case, we observed that a CFBP4834-R population of a least 1×10^2 CFU seed^{-1} was required for seedling contamination. With a high inoculum dose (1.5×10^7 CFU ml^{-1}), all seeds were contaminated with at least 1×10^4 CFU seed^{-1} and all seedlings were contaminated (Fig. 3). For the lower inoculum concentrations, the number of seeds harbouring fewer than 1×10^2 CFU seed^{-1} was not significantly different from the number of uncontaminated seedlings (calculated $\chi^2 = 0.7$ and 0.14 for inoculum doses of 1.5×10^3 and 1.5×10^5, respectively with a critical value of $\chi^2_{.05[1]} = 3.841$) showing the inability of seeds contaminated with a

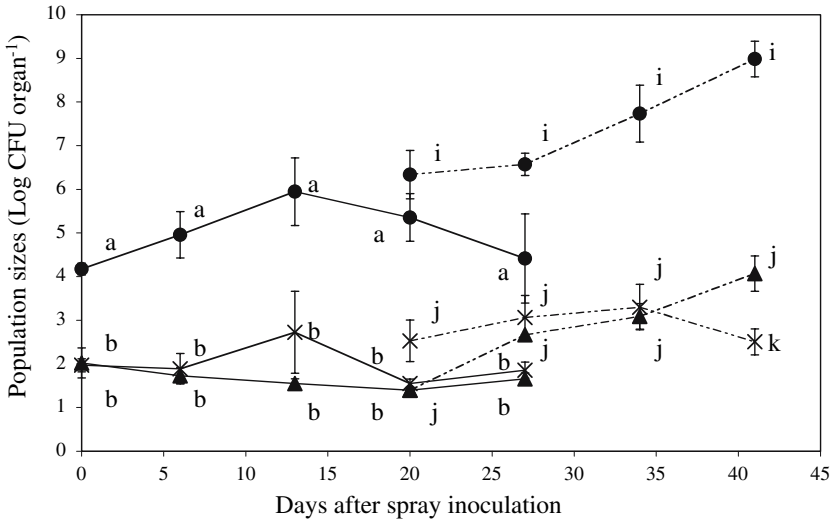

Fig. 2 *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* CFBP4834-R population size dynamics on leaves and pods on plants inoculated in the greenhouse. Inoculum came from contaminated seeds or plants were spray-inoculated. Population sizes were determined on young trifoliate leaves (continuous line) and pods (dotted line) of plants inoculated at the flower bud stage with CFBP4834-R at 2.5×10^6 CFU ml^{-1} (circle) and 1.9×10^4 CFU ml^{-1} (cross) and from plants of the same age grown from contaminated seeds (4.7×10^6 CFU $seed^{-1}$) (triangle). Means of six values are presented with bars representing the standard error. At each sampling date and for a type of organ, means followed by different letters are significantly different ($P < 0.05$) on the basis of Mann–Whitney tests. The detection threshold was 25 CFU $organ^{-1}$

Table 2 Distribution of *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* CFBP4834-R population sizes in naturally contaminated seed lot A evaluated using individual stock seeds or seedlings after 3 days germination

	% samples per contamination class[a]					% of contaminated samples[c]
	<DT[b]	$2.5 \leq n < 10^2$	$10^2 \leq n < 10^3$	$10^3 \leq n < 10^4$	$n \geq 10^4$	
Stock seeds	57.6	18.5	15.2	6.5	2.2	42.4 a
Seedlings	92.4	3.3	2.2	1.1	1.1	7.6 b

[a] Expressed in CFBP4834-R CFU per sample

[b] Below detection threshold of 2.5 CFU per sample

[c] % contaminated samples calculated on stock seeds (92 samples) and on seedlings (92 samples). Letters indicate significantly different values according to a χ^2 test

low population size to give rise to contaminated seedlings.

Impact of low contamination rates of bean seed lots on the epidemiological cycle of *Xapf*

Throughout the entire growing season, population sizes of CFBP4834-R in the field remained low, they did not exceed 6.9×10^3 CFU $organ^{-1}$ in 2002 and 2.5×10^5 CFU $organ^{-1}$ in 2003. In the 2002 experiment, the number of contaminated foci fluctuated during the growing season (Table 3). In the 2003 experiment, the number of contaminated foci increased during the growing season and exceeded the expected value at the last sampling date (Table 3). For both years, no symptoms of common bacterial blight were observed either in contaminated foci and their surrounding plants or in the control treatment.

Asymptomatic transmission of CFBP4834-R to seeds was detected for every level of seed lot contamination in both field experiments (Table 4). Contamination rates of seeds for these experiments ranged from 0.003% to 0.022%. For plots sown at an initial contamination rate of 0.003%, each harvested area contained at least between one and four potential foci, for the 0.03% treatment they contained between

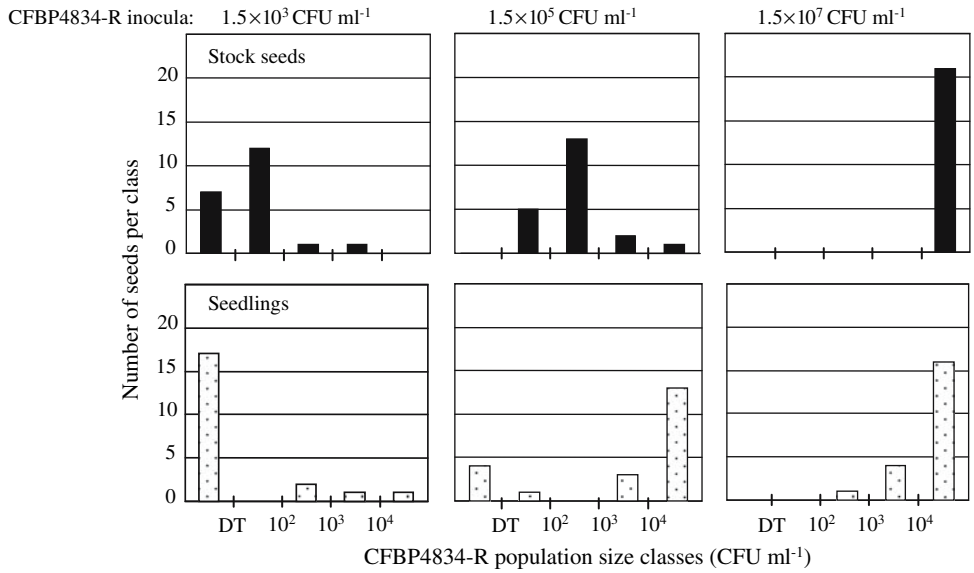

Fig. 3 *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* CFBP4834-R population sizes on seeds and seedlings. Bean seeds were inoculated with 1.5×10^3, 1.5×10^5 and 1.5×10^7 CFU ml^{-1} suspensions of CFBP4834-R. A sample of 21 seeds was analyzed directly after inoculation (stock seeds: black area) and another sample was analyzed after 3 days of germination (21 seedlings: dotted area) for each inoculum dose. Population sizes (CFU sample^{-1}) are distributed into five classes, each of one $\log_{10}$ unit, extending from under the detection threshold (DT <10 CFU sample^{-1}) to $>1 \times 10^4$ CFU sample^{-1}

Table 3 Transmission ratios (TR) of *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* CFBP4834-R from seed to plant calculated at different sampling dates in plot G planted with contaminated seeds at a rate of 0.03%

Year	No. contaminated seeds (>10^2 CFU seed^{-1})[a]	1st sampling date[b]		2nd sampling date		3rd sampling date	
		No. detected foci (population sizes CFU leaflet^{-1})	TR[c]	No. detected foci (population sizes CFU leaflet^{-1})	TR	No. detected foci (population sizes CFU leaflet^{-1})	TR
2002	24[d]	nd[e]	nd	8	3:1	2	12:1
	(5)	nd	nd	(1×10^2–6.9×10^3)	(0.6:1)	(50–1.5×10^2)	(2.5:1)
2003	25[f]	1	25:1	2	12.5:1	10	2.5:1
	(5)	(50)	(5:1)	(50)	(2.5:1)	(1.5×10^3–2.5×10^5)	(0.5:1)

[a] No. contaminated seeds and (no. contaminated seeds with a bacterial population size >100 CFU seed^{-1}) sown per plot

[b] Sampling dates correspond to 07/16/02 and 07/31/02 for 2002 experiments and to 06/18/03, 07/21/03 and 07/31/03 for 2003 experiment

[c] No. contaminated seeds sown: no. contaminated plants detected in the plot (no. seeds sown carrying a bacterial load >10^2 CFU seed^{-1}: no. contaminated plants detected in the plot)

[d] 24 = no. seeds corresponding to % contaminated seeds (19.2%) present among the 125 seeds sown of lot B

[e] Not determined

[f] 25 = no. seeds corresponding to % contaminated seeds (30%): present among the 94 seeds sown of lot C that will germinate at only 89%

11 and 18 potential foci and for the 0.1% treatment there were between 35 and 41 potential foci per area. As a general tendency, contamination rates of harvests were below initial contamination rates. CFBP4834-R population sizes were also generally lower in harvests than in sown seeds. However, increases in contamination rates (plots E and F in the 2003 experiment, Table 4) and in population sizes of

Table 4 Detection of *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* CFBP4834-R in harvests of 2002 and 2003 field experimental plots

Initial contamination rate of plots		2002 experiment		2003 experiment	
		Contamination rate of harvests[a] (%)	CFBP4834-R population sizes (CFU sample^{-1})	Contamination rate of harvests (%)	CFBP4834-R population sizes (CFU sample^{-1})
Control	A	–[b]	–	0.010	2×10^3
	B	–	–	–	–
	C	–	–	–	–
0.003%	D	0.003	7×10^2	–	–
	E	–	–	0.022	2×10^3
	F	–	–	0.018	2×10^4
0.03%	G	0.003	2×10^3	0.003	6×10^2
	H	0.007	4×10^3	0.003	2×10^3
	I	0.007	2×10^3	–	–
0.1%	J	0.003	1×10^3	0.003	2×10^3
	K	0.018	3×10^5	–	–
	L	0.010	1×10^6	0.003	6×10^2

[a] Contamination rate of seed lots calculated according to Maury et al. (1985) using 30 samples of 1,000 seeds each

[b] – indicates negative result for isolation

CFBP4834-R (plot L in the 2002 experiment, Table 4) were observed in harvested seeds.

In the 2003 test, harvested seeds from the negative control were contaminated by the strain CFBP4834-R confirming dispersion events in the field (Table 4). Furthermore, in windbreak plantations, among the 72 harvested bean trap plants, nine plants produced seeds contaminated with CFBP4834-R but in which bacterial population sizes did not exceed 3×10^3 CFU per sample.

Tests of pathogenicity on beans revealed that recovered *Xapf* isolates in harvested seeds were highly aggressive on bean. The type of symptom (water-soaked spots followed by necrosis on inoculated leaves), abundance of symptoms (totality of the surface of inoculated leaves) and length of latent period from inoculation to symptom appearance (8 days) did not deviate from that caused by CFBP4834-R.

Environmental conditions during 2002 field experiments were quite cool when compared with the past 10 years, and in 2003 temperatures were particularly warm with a mean temperature 2°C higher than the mean for the past 10 years. However, during these two field experiments, RH was below average with a deficit in hours during which RH was >80%. A daily mean of 8.3 h and 8.7 h with a RH >80% was measured from May 20th to August 31th in 2002 and 2003, respectively compared to 11 h during the same period in 1992–2001. In 2002, an increase of hours during which RH was <40% was recorded (4.0 h in 2002 and 1.6 in 1992–2001, from May 20th to August 31th). Winds were primarily westerly.

Effect of CFBP4834-R population sizes per seed on contamination of plants under field conditions

During the field growing season, no symptoms typical of common bacterial blight were observed on plants grown from inoculated seeds. Seeds inoculated with suspensions at 10^4 and 10^6 CFU ml^{-1} had mean population sizes of 4.6×10^2 and 4.2×10^4 CFU seed^{-1}, respectively. Among the 30 plants from seeds contaminated at a density of 4.6×10^2 CFU per seed, only one plant yielded seeds on which CFBP4834-R was detected. This sample harboured 16 CFU. Among the other 30 plants from seeds contaminated at a density of 4.2×10^4 CFU per seed, four plants yielded contaminated seeds, on which the maximum CFBP4834-R population size was 1.3×10^4 CFU. This result showed that even low initial bacterial population sizes per seed could lead to successful contamination of the plant and seeds under field conditions.

Discussion

In this work, we have show that *Xapf* is able to contaminate bean seeds without any symptom expression on its host. The disease cycle of common bacterial blight has been well described (Gilbertson and Maxwell 1992). However, this is the first report of seed contamination in association with low bacterial population sizes in the phyllosphere and in the absence of symptoms on a susceptible host during the entire growing cycle under field conditions. Infestation of seeds of resistant or susceptible cultivars of bean has been associated with symptoms on pods or sometimes with high *Xap* and *Xapf* population sizes on asymptomatic pods (Cafati and Saettler 1980; Weller and Saettler 1980a). The asymptomatic contamination of bean seeds by *Xapf* reported here was observed both in field conditions and in greenhouse experiments. It was associated with low *Xapf* population sizes on seeds and in the phyllosphere. Indeed, when bean plants were spray-inoculated with high inoculum concentrations under the same environmental conditions, symptoms developed on plants. Contamination of seeds has been reported in asymptomatic watermelons by *Acidovorax avenae* subsp. *citrulli* (Walcott et al. 2003). However, these results were obtained following blossom inoculations. Their natural occurrence is not known.

Absence of any symptoms of common bacterial blight in a bean field is difficult to prove. However, the position of potential primary foci was precisely labelled in the fields thereby facilitating symptom surveys. It is unlikely that symptoms would first have developed in secondary foci. Weller and Saettler (1980 a) showed that initiation of secondary contaminations occurred before symptom development but that symptoms appeared first in primary foci. Our fields had no history of bean cultivation and therefore no inoculum on residues. Fields were located among orchards far away from any bean cultivation area and no known alternate hosts of *Xap* and *Xapf* were found in these fields. Furthermore, we used a strain with resistance to rifamycin. Such resistance occurs in *Xap* and *Xapf* populations at a frequency of less than 10^{-7}. Therefore, we are confident that the rifamycin-resistant isolates recovered from seeds originated only from the inoculations with CFBP4834-R that we conducted for these experiments and the subsequent colonization of plant tissues by this strain.

We conducted our experiments in a temperate climate where the main inoculum source is the contaminated seed and obviously where climate does not always favour bacterial multiplication. The asymptomatic transmission could be less important in tropical and subtropical areas where environmental conditions are frequently favourable to bacterial multiplication and symptom development and where other inoculum sources could predominate such as infested debris, contaminated volunteer plants or weeds (Fininsa and Tefera 2001; Stall et al. 1993).

The asymptomatic colonization of bean seeds by *Xapf* could explain re-emergent epidemics, despite the rather efficient prophylaxis obtained by using seed lots with non-detected levels of contamination. *Xapf* strains isolated from harvested seeds were highly aggressive when inoculated on beans under favourable environmental conditions. Results showed that low population densities of *Xapf* per seed, as low as 4.6×10^2 CFU seed^{-1}, could lead to the asymptomatic transmission of the bacterium to the next generation with a low (30:1) seed:seed bacterial transmission efficiency. Indeed, the current routine protocols for *Xapf* detection in seed lots (International Seed Testing Association 2007; Laurent 1990) will not allow detection of contaminations as low as one seed with 1×10^2 CFU in a lot of 30,000 seeds. However, such contamination could lead to subsequent contamination of the seeds in a field of about 3 ha. Results established that the transmission frequency from seed to seedling is 30:1 under conditions that do not favour disease development. Furthermore, bean seed is planted at a density of about 30 seeds m^{-2}. Hence, 3 ha would represent enough seeds to assure the presence of at least 30 seeds contaminated with 1×10^2 CFU, a frequency that is not detectable with the currently used protocols. One way to overcome this problem will be to improve sampling and detection tools.

Absence of symptoms and low *Xapf* population sizes can be explained by environmental factors. Low RH is unfavourable to bacterial multiplication and was below normal levels in the field experiments. In previous work (Jacques et al. 2005) we showed that low RH leads to a decrease of solitary *Xapf* population sizes but has no effect on the aggregated or biofilm part of the population. Field populations of *Xapf* were likely to have been mainly aggregated and hence not available for infection if the behaviour of

Xapf is similar to that of *X. campestris* pv. *campestris* where non-aggregated cells are the invasive form responsible for symptom development (Dow et al. 2003). However, low RH can have opposing effects as Fininsa and Yuen (2002) observed in Ethiopia where an increase of bacterial common blight in beans was observed in the field at low RH.

Can we extrapolate the existence of an asymptomatic contamination of bean seeds in *Xap* from our observations based on *Xapf*? Indeed, *Xapf* is genetically different from *Xap* to some extent (Schaad et al. 2005; Mahuku et al. 2006). We experimented with a *Xapf* strain as this was isolated initially from a field in Anjou and because *Xapf* strains are more frequently isolated in Europe than *Xap* strains. Arguments in favour of extrapolating are (i) that both *Xap* and *Xapf* can invade the xylem of their host (Vidaver 1993), (ii) no epidemiological differences have been reported for these two bacteria (Vidaver 1993) and (iii) the *Xapf* strain used in our study is highly aggressive on bean (data not shown). It has been shown that *Xapf* strains are more aggressive than *Xap* strains (Chan and Goodwin 1999). We suggest that it is reasonable to assume that if a certain trait is linked to the absence of virulence for an otherwise highly aggressive strain (such as for *Xapf*), then it also has the same relationship to virulence in a less aggressive strain (*Xap*).

The asymptomatic contamination of seeds combined with an asymptomatic survival on rotational crops could complicate the effectiveness of disease management strategies. Here, we avoided other potential inoculum sources such as epiphytic populations on other crops or weeds. Under real cropping conditions, however, such sources might not be non-negligible as was shown in Colorado, USA with the asymptomatic survival of *Xap* on onion (Gent et al. 2005).

In primary foci, asymptomatic contamination of seeds could originate from systemic infection of plants contaminated at low levels. Systemic infections of bean by *Xap* were previously reported (Weller and Saettler 1980a; Zaumeyer 1930) but their importance in epidemiological cycles remains unclear. Population densities remained low on leaves of plants grown from contaminated seeds throughout the growing season both in field and greenhouse experiments but vascular populations could have been higher in stems.

Airborne contamination of secondary foci results also in asymptomatic contamination of the seeds. Secondary asymptomatic contamination of seeds was observed in beans in windbreak plantations and in spray-inoculated beans in the greenhouse. How did this aerial contamination of plants result in contamination of seeds? External contamination of seeds generally results from contact with populations on symptomatic pods in the field or during threshing when seeds contact high bacterial populations on plant residues (Weller and Saettler 1980b). However in our experiments only low bacterial population sizes were detected in aerial parts of plants. Are these low population sizes able to externally contaminate seeds during threshing? It is possible that even at low population densities bacteria enter the plant through flowers and hence internally contaminate the seeds. A better knowledge of the mode of seed contamination is necessary to answer these questions and understand the asymptomatic colonization of host plants by pathogenic bacteria.

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