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Effect of Nitrogen Fertilization and Seed Contamination on Epiphytic Populations of *Xanthomonas axonopodis* pv. *allii* and Development of Xanthomonas Leaf Blight of Onion

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Abstract

Xanthomonas leaf blight of onion, caused by *Xanthomonas axonopodis* pv. *allii*, is a yield-limiting disease in Colorado, for which few effective management strategies are available. The effects of rates of nitrogen fertilization and levels of seed contamination by *X. axonopodis* pv. *allii* on epiphytic populations of the pathogen and subsequent disease development were determined in field studies over two years. Epiphytic populations of a rifampicin-resistant mutant were quantified from bulked, asymptomatic leaf tissue using a leaf wash assay. There was no significant relationship between nitrogen fertilization treatment and epiphytic population development, but excessive nitrogen (> 200 kg/ha) fertilization increased disease severity 27 to 50% compared with non-fertilized and low (112 kg/ha) nitrogen fertilizer treatments in 2004. Seed inoculated with various levels of *X. axonopodis* pv. *allii* did not result in persistent epiphytic populations of the bacterium or development of symptoms, even at 10^7 CFU/g of seed. The results of these studies suggest moderate rates of nitrogen fertilization may reduce severity of Xanthomonas leaf blight compared to higher rates, and that seed contamination by *X. axonopodis* pv. *allii* may be of limited importance for disease development under the semi-arid conditions of furrow-irrigated onion production in Colorado.

Introduction

Fig. 1. Xanthomonas leaf blight symptoms on the flattened sides of onion leaves with characteristic water-soaked lesions from infection by *Xanthomonas axonopodis* pv. *allii*.

Xanthomonas leaf blight of onion (*Allium cepa*), caused by the bacterium *Xanthomonas axonopodis* pv. *allii*, can be a yield limiting disease of sweet Spanish type onion cultivars in Colorado during warm, rainy weather (21,22). Yield losses of 20% have been reported during epidemics in southern Colorado (21), but complete crop losses may occur in some tropical regions of onion production (13). The disease first appears as small, lenticular-shaped, chlorotic lesions that are most prominent on the flattened sides of leaves. These lesions quickly enlarge and become water-soaked in humid or rainy weather (Fig. 1) (1). The disease results in stunted plant growth and reduced bulb size (12,21,23). A bulb rot is not known to occur in association with this disease. The natural host range of *X. axonopodis* pv. *allii* appears limited to a few *Allium* species (1,3,6,8,17).

Few management strategies are available for management of Xanthomonas leaf blight, but crop rotation (13), host resistance (13), copper or zinc application (14,21), and biological control (15) have been proposed. Most of these management strategies have not been evaluated in Colorado and, consequently, growers have relied largely upon copper-based bactericides for disease suppression (21). The importance of cultural practices, such as rate of nitrogen fertilization, for management of Xanthomonas leaf blight is largely unknown. In other plant diseases caused by xanthomonads, nitrogen fertilization may increase or decrease disease severity (2,5,10,11). However, observations from experimental field plots and natural epidemics of Xanthomonas leaf blight in Colorado suggest nitrogen deficiency may reduce disease severity (Gent and Schwartz, *unpublished data*). No studies, to our knowledge, have examined the effect of rate of nitrogen fertilization on epiphytic *X. axonopodis* pv. *allii* or development of Xanthomonas leaf blight of onion.

The planting of pathogen-free seed also has been recommended for disease management (16), as contaminated seed has been associated with epidemics of Xanthomonas leaf blight when moisture is abundant (18). However, the importance of seed-borne *X. axonopodis* pv. *allii* as a primary inoculum source for Xanthomonas leaf blight in semi-arid environments with furrow-irrigated onion production is unknown.

Development of management strategies for Xanthomonas leaf blight requires an understanding of the effects of cultural practices such as rates of nitrogen fertilization and seed contamination with *X. axonopodis* pv. *allii* on development of Xanthomonas leaf blight. Therefore, these studies were initiated to determine: (i) the influence of rates of nitrogen fertilization on development of epiphytic populations of *X. axonopodis* pv. *allii*; and (ii) the seed contamination threshold with this pathogen for development of Xanthomonas leaf blight under standard grower production practices for Colorado.

Nitrogen Fertility Studies

A rifampicin-resistant mutant of *X. axonopodis* pv. *allii* strain O177 (ATCC 508) was generated as described by Weller and Saettler (25), and is referred to as R-O177 in this article. Strain R-O177 was resistant to rifampicin concentrations > 200 µg/ml, but selection for the pathogen was routinely performed on nutrient agar amended with 50 µg/ml of rifampicin. To prepare inoculum of strain R-O177, bacteria were streaked onto rifampicin-amended nutrient agar medium and plates incubated at 29°C for 72 h in the dark. Cells were harvested from plates by flooding with deionized water and gently scraping the plates with a small, flame-sterilized spatula. The cell suspension was adjusted to 10^8 CFU/ml (OD_{600} = 0.12) before adjusting with 0.01 M magnesium phosphate buffer to the desired concentration for an experiment. Strain R-O177 was preserved in nutrient broth with 15% glycerol at -80°C.

Field studies were established at the Colorado State University Agricultural Research, Development, and Education Center (ARDEC) near Fort Collins, CO to determine the effect of rates of nitrogen fertilization on epiphytic populations of *X. axonopodis* pv. *allii* and subsequent development of Xanthomonas leaf blight. The susceptible onion cultivar Vantage was planted on 7 April 2003 and 2 April 2004 into beds, each with two rows of seed spaced 0.15 m apart and spaced 0.1 m apart within the row. Plots consisted of four contiguous 7.6-m-wide beds 7.6 m in length, separated by 0.1 m, and replicated four times in a randomized complete block design. The field was furrow-irrigated once or twice weekly and was not fertilized. A different area of the same field was used for field plots in 2003 and 2004.

Major and micro-nutrients, soil pH, and soluble salts were determined each year by a private company (MDS Harris, Inc., Lincoln, NE) from bulked soil samples consisting of six randomly collected soil cores (2.5 cm in diameter) taken to a depth of 15 cm from the area of the field where the experiment was conducted. Soil nitrate nitrogen content was used to adjust nitrogen fertilization rates to achieve the desired nitrogen level for each treatment. The field contained 17 and 70 ppm nitrate nitrogen in 2003 and 2004, respectively. Nitrogen content of water used for irrigation contained negligible amounts of nitrogen (< 1 ppm were detected). Nitrogen fertilizer (46-0-0, urea) was applied to each plot by gently loosening the soil between the onion rows on the center two beds per plot using a hoe and banding the fertilizer by hand onto the soil surface. The fertilizer was mechanically incorporated approximately 2.5 cm deep by shallow hoeing. Treatments consisted of nontreated (residual soil nitrogen only), and available nitrogen at 112, 224, or 448 kg/ha per plot. Total nitrogen applied was split in two equal applications at the four-leaf stage and at bulb initiation, respectively. The outer beds of the plot did not receive fertilizer to minimize nitrogen movement among plots, which might confound treatment effects.

The center two beds of each plot were inoculated to runoff with an aqueous suspension of strain R-O177 at 10^6 CFU/ml amended with 0.1% v/v of an organosilicone surfactant (Silwet L-77; Loveland Industries, Greeley, CO) using a CO_2-pressurized backpack sprayer, at 106 and 107 days after planting (approximately at bulb initiation) in 2003 and 2004, respectively. Inoculum was prepared as described above.

Approximately 20 to 40 g of asymptomatic leaf material were collected randomly from each treatment plot (4 plots per treatment; 16 plots in total) weekly after inoculation, placed into resealable plastic bags, and transported to the laboratory on ice for determination of epiphytic populations. Leaves with symptoms of Xanthomonas leaf blight were purposely avoided during sampling to minimize recovery of endophytic populations of *X. axonopodis* pv. *allii*, which may have been released if diseased tissue was included in the leaf wash assay. Five to 10 g of this bulked leaf sample was placed into a sterile 250 ml flask containing 100 ml magnesium sulfate-potassium phosphate buffer (0.01 M magnesium sulfate and 0.01 M potassium phosphate, pH 7.2), and shaken at 250 rpm for 60 min at room temperature (approximately 22°C). Serial dilutions (10-fold) were prepared with sterile magnesium phosphate buffer and spiral plated (Autoplate 4000; Spiral Biotech, Inc., Norwood, MA) onto nutrient agar amended with rifampicin and cycloheximide at 50 µg/ml. All dilutions were plated twice. *X. axonopodis* pv. *allii* colonies were enumerated after 72 h of incubation at 29°C, and a subset of these rifampicin-resistant colonies were confirmed as *X. axonopodis* pv. *allii* by standard physiological and biochemical tests (20), including negative Gram stain reaction, yellow pigmentation on yeast dextrose carbonate medium, lack of fluorescence on King's medium B, negative indole test, lack of growth on 0.1% tetrazolium chloride, absence of oxidase, starch hydrolysis, oxidative utilization of glucose, presence of catalase, production of H_2S from cysteine, lack of arginine dihydrolase, and hydrolysis of casein.

Disease severity was estimated weekly for each plot with a modified Horsfall-Barrett (7) scale after symptoms of Xanthomonas leaf blight developed. At maturity, 20 bulbs or a 3-m section from the center of one row within each plot was topped mechanically, harvested, graded, and weighed to estimate yields.

Statistical analyses were performed using the PROC MIXED function of SAS version 9.1 (SAS Institute, Cary, NC). Bacterial population data were log-transformed to achieve independently and normally distributed experimental errors with a common variance. The area under the bacterial growth curve (AUBGC), calculated from log-transformed values of the epiphytic populations recovered in leaf wash assays, and relative area under the disease progress curve (RAUDPC) were considered response variables. Replications of experimental units were considered random factors.

Epiphytic populations of *X. axonopodis* pv. *allii*, as measured by the AUBGC, differed between years (P = 0.0187), but did not differ among nitrogen fertilization treatments in 2003 or 2004 (P = 0.2914 or 0.2998, respectively). In 2003, epiphytic populations increased almost 5 logarithmic units per gram of fresh leaf tissue by 27 days after inoculation, but decreased one to two logarithmic units in the following 7 days (Fig. 2a). In 2004, epiphytic populations increased 3 to 4 logarithmic units per gram of fresh leaf tissue within 11 to 18 days of inoculation, but populations decreased 1 to 2 logarithmic units over the subsequent 30 to 38 days (Fig. 2b). All bacterial colonies evaluated for physiological and chemical reactions had reactions consistent with those of *X. axonopodis* pv. *allii* and were resistant to rifampicin.

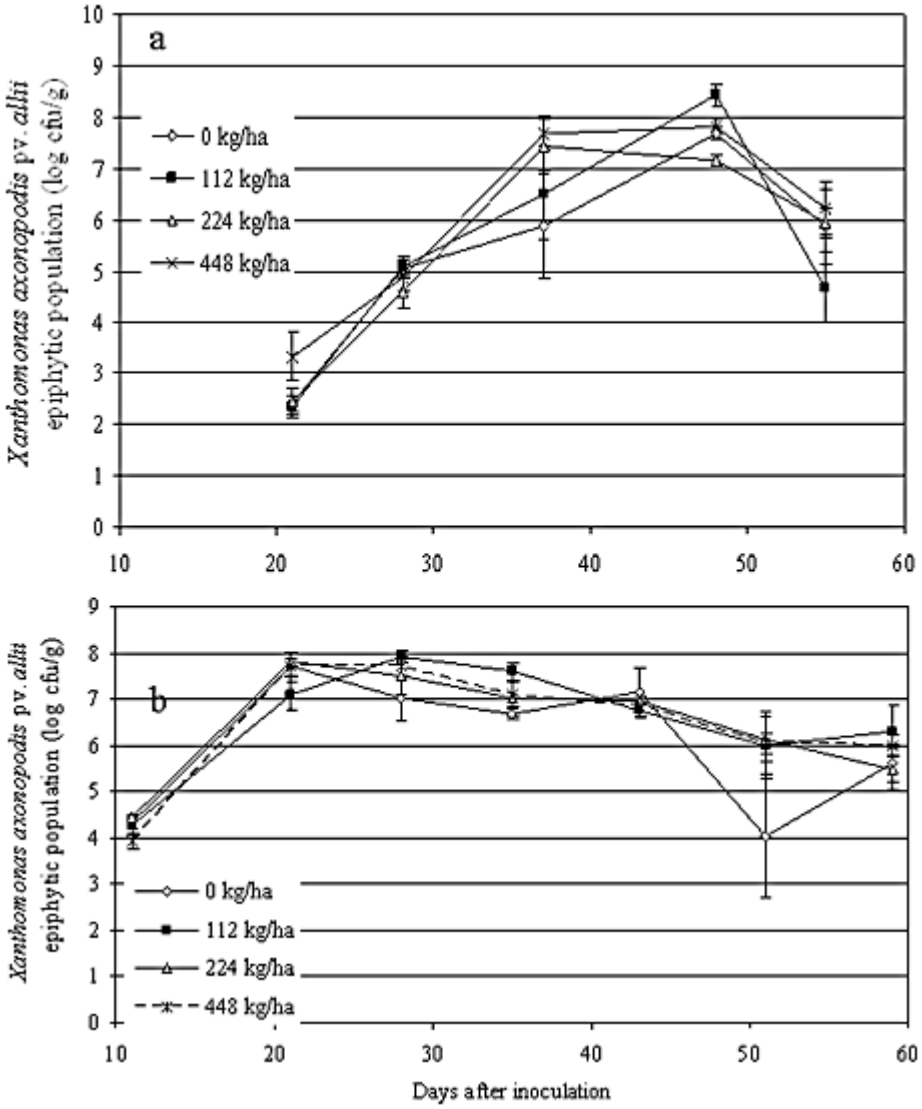

Fig. 2. Epiphytic populations of *Xanthomonas axonopodis* pv. *allii* strain R-O177 on onion in relation to nitrogen fertilization rates in 2003 (a) and 2004 (b). Nitrogen was available to plants at approximately 38 and 155 kg/ha in the 0-kg/ha treatment in 2003 and 2004, respectively, from residual soil nitrates.

The RAUDPC did not differ significantly among nitrogen fertilization treatments in 2003, but in 2004 the RAUDPC increased 27 and 50% with increasing rates of fertilization, and was greater for the 224- and 448-kg/ha treatments compared with the nontreated plots, respectively (Table 1). Fertilization with N at 112 kg/ha did not significantly increase the RAUDPC as compared to the nontreated or 224-kg/ha treatments. Bulb yields (total and by size) were similar among rates of nitrogen fertilization in 2003 and 2004.

Table 1. Severity of Xanthomonas leaf blight and yield of onion in relation to nitrogen fertilization.

Treatment (kg/ha)[x]	Year, disease severity, and yield					
	2003		2004			
				Yield[z]		
	RAUDPC[y]	Yield[z]	RAUDPC[y]	Medium	Jumbo	Total
0	0.18a	13.8a	0.22a	29.0a	3.2a	47.3a
112	0.18a	14.6a	0.24ab	32.2a	10.5a	54.2a
224	0.19a	12.4a	0.28b	34.4a	6.1a	53.0a
448	0.25a	13.3a	0.33c	30.8a	2.2a	46.1a

[x] Total nitrogen fertilizer (46-0-0) was applied in two equal applications by mechanical incorporation between plants on the bed at the four-leaf growth stage and at bulb initiation. Nitrogen was available to plants at approximately 38 and 155 kg/ha in the 0-kg/ha treatment in 2003 and 2004, respectively, from residual soil nitrates.

[y] RAUDPC = relative area under the disease progress curve. RAUDPC was calculated as:

$$\{\Sigma^{n}_{i=1}\,[(x_{i+1} + x_i)/2][t_{i+1} - t_i]\} \;/\; t_n - t_i$$

where x_i is the severity of disease at time (t) i, and n = the number of disease evaluations. Treatments within a column followed by the same letter are not significantly different based on pairwise differences calculated using SAS PROC MIXED. Data are means of four replications.

[z] The Xanthomonas leaf blight susceptible cultivar Vantage was used in 2003 and 2004. Total yield in 2003 was weight (kg) of 20 bulbs harvested randomly from each plot, and in 2004 as tons/ha.

Seed Transmission Studies

Experiments were conducted to determine the seed contamination threshold for development of Xanthomonas leaf blight under standard onion production practices in Colorado. Commercial onion seed lots (n = 107) were screened for *X. axonopodis* pv. *allii* contamination as described previously by Roumagnac et al. (16) using a modified MXP medium (4) containing kasugamycin at 50 µg/ml, cephalexin at 50 µg/ml, and cycloheximide at 50 µg/ml to reduce growth of other bacteria and fungi. This modified medium was used because preliminary studies found several strains of *X. axonopodis* pv. *allii* (including strain R-O177) had low plating efficiencies (less than 25% compared to nutrient agar) on the semi-selective medium NCTM1, and this medium allowed for the growth of copious amounts of secondary bacterial species associated with some onion seedlots (Gent and Schwartz, *data not presented*). The modified MXP medium used in this study was deemed superior to NCTM1 because it had higher plating efficiencies (similar to nutrient agar) for *X. axonopodis* pv. *allii* strains from Colorado, allowed for rapid differentiation of saprophytic bacterial species incapable of hydrolyzing starch, and contained antibiotics readily available from commercial sources. Using this medium, *X. axonopodis* pv. *allii* was not detected in the seedlots of any long-day onion cultivars screened. In the absence of naturally-infested seed, seed was inoculated with a range of concentrations (0, 10^3, 10^5 or 10^7 CFU/g of seed) of *X. axonopodis* pv. *allii*. Seed of the yellow onion cultivar Vantage that tested free of *X. axonopodis* pv. *allii* was rinsed in

running tap water for 30 min to minimize pesticide residues from the commercial seed treatment thiram. Seed was dried overnight on paper towels and then inoculated with the pathogen by vacuum infiltration. Approximately 20 g of seed was submersed in the appropriate concentration of *X. axonopodis* pv. *allii* strain R-O177 and placed under a vacuum (15 mm Hg) for 3 min. After the vacuum was removed, the seed was soaked in the bacterial suspension for an additional 4 h at room temperature (~22°C) and then air-dried overnight. Seed was assayed using a seed wash assay (16) and plating onto rifampicin-amended nutrient agar to determine the level of inoculum delivered to each seed treatment just prior to planting. Populations of *X. axonopodis* pv. *allii* recovered from the inoculated seedlots were within 0.5 log CFU/g of the desired concentration for the treatments in both 2003 and 2004 (*data not presented*). A different seed lot was inoculated and planted in 2003 and 2004, but the same strain of *X. axonopodis* pv. *allii* was used in both years to minimize possible differences in pathogenic variability among strains of *X. axonopodis* pv. *allii*.

Inoculated seed was planted into plots at ARDEC as described for the nitrogen fertility studies. The center two beds of each four-bed wide plot were planted with inoculated seed, and the outer two beds were planted with seed that tested free of *X. axonopodis* pv. *allii*. Plots were monitored weekly for disease development, and epiphytic populations of *X. axonopodis* pv. *allii* strain R-O177 were determined and enumerated at approximately 3-week intervals as described above. Bulb yields were not determined.

Irrespective of the level of *X. axonopodis* pv. *allii* inoculated onto seed, persistent epiphytic populations of *X. axonopodis* pv. *allii* were not detected, nor was development of Xanthomonas leaf blight observed in 2003 or 2004. In 2003, epiphytic *X. axonopodis* pv. *allii* was recovered from plants at the two-leaf stage grown from seed inoculated with 10^3, 10^5, or 10^7 CFU/ml (Fig. 3a). In this case, populations were $< 10^2$ CFU/g of fresh leaf tissue for plants grown from seed inoculated with 10^3 or 10^5 CFU/ml, whereas populations of 10^7 CFU/g were recovered from plants grown from seed inoculated with 10^7 CFU/ml. By the four-leaf stage, epiphytic populations of *X. axonopodis* pv. *allii* were recovered only from the 10^7-CFU/ml seed treatment, and by the six-leaf stage epiphytic *X. axonopodis* pv. *allii* was not detected from any plants in any of the seed treatments.

In 2004, epiphytic populations $< 10^2$ CFU/g of fresh leaf tissue were recovered from plants that grew from any of the inoculated seed (Fig. 3b). Epiphytic *X. axonopodis* pv. *allii* was not detected after the eight-leaf stage, irrespective of seed contamination level. Xanthomonas leaf blight symptoms were not observed on plants from any of the seed treatments.

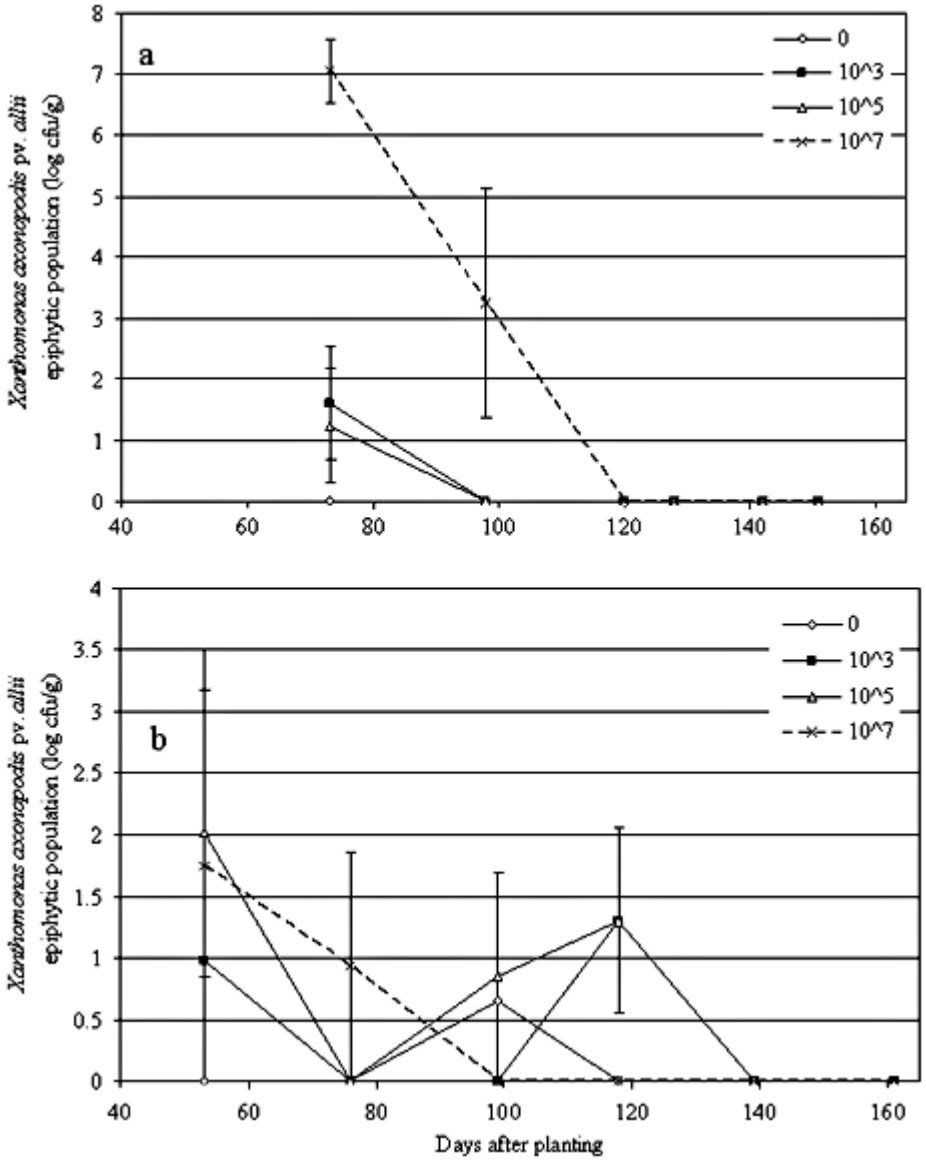

Fig. 3. Epiphytic populations of *Xanthomonas axonopodis* pv. *allii* strain R-O177 on onion in relation to seed contamination levels planted in 2003 (a) and 2004 (b).

Conclusions and Recommendations

Understanding the effects of cultural practices, such as rates of nitrogen fertilization and planting of infested seed, on development of *X. axonopodis* pv. *allii* are essential for the development of ecologically-based and sustainable management programs for this pathogen. In this study, the effect of nitrogen fertilization and levels of seed contamination with *X. axonopodis* pv. *allii* populations on epiphytic populations of the pathogen on onion leaves and subsequent development of Xanthomonas leaf blight of onion were evaluated. Excessive nitrogen fertilization (available nitrogen at 224 or 448 kg/ha) increased the severity of Xanthomonas leaf blight in 2004, but not in 2003, and did not influence epiphytic populations or bulb yields. The level of seed contamination with *X. axonopodis* pv. *allii* at planting did not affect subsequent epiphytic populations or development of symptoms of Xanthomonas leaf blight under the standard onion production practices used in this study.

Little is known about how rates of nitrogen fertilization influence epiphytic bacterial populations of xanthomonads. On tomato (*Lycopersicon esculentum*), populations of *X. axonopodis* pv. *vesicatoria* and defoliation from bacterial spot were greatest on plants fertilized lightly with nitrogen compared to heavily fertilized plants, but other nutrients also may influence epiphytic bacterial

populations and disease severity (11). Abundant nitrogen fertilization may exacerbate bacterial blight of rice (*Oryza sativa*), caused by *X. oryzae* pv. *oryzae*, and reduce grain yields (2,5), but high levels of nitrogen impeded invasion of cabbage (*Brassica oleracea*) leaves by X. *campestris* pv. *campestris* (10). In the study presented in this paper, epiphytic populations of *X. axonopodis* pv. *allii* did not differ significantly among treatments in 2003 or 2004. Significant differences in Xanthomonas leaf blight severity occurred among plants receiving low and high rates of nitrogen in 2004, but not in 2003. The reasons for the observed difference are not clear, but may be related to weather conditions and disease severity in 2003 and 2004. A Colorado Agricultural Meteorological Network CR10X (Campbell Scientific, Inc., Logan, UT) located less than 1 km from the plots recorded mean monthly temperatures of 5 to 6°C higher and 7.5 cm less rainfall during and after bulb initiation in 2003 compared to 2004. These weather conditions are associated with less severe epidemics of Xanthomonas leaf blight in Colorado (22), and, correspondingly, disease severity was less in 2003 than 2004 (Table 1). High rates of nitrogen fertilization may not exacerbate Xanthomonas leaf blight epidemics during years with low disease pressure or when epidemics of Xanthomonas leaf blight are short or disease pressure is low, but may increase disease severity during severe epidemics of the disease. Although residual soil nitrates were higher in 2004 (70 ppm) compared to 2003 (17 ppm), this likely had little affect on disease severity because rates of nitrogen fertilization were adjusted according to soil test results. However, since the first application of nitrogen fertilizer was made at the four-leaf stage in this study, we cannot discount those higher levels of soil nitrogen early in the season may have influenced the effect of later nitrogen fertilization and severity of Xanthomonas leaf blight.

Neither bulb yield nor size was affected by rate of nitrogen fertilization. Based upon soil tests conducted before planting in 2003 and 2004, nitrogen was available to plants at 38 (17 ppm) and 155 (70 ppm) kg/ha in the 2003 and 2004 trials, respectively. Residual soil nitrogen, combined with mineralization of organic nitrogen sources, may have supplied the entire crop demand for nitrogen in this study, and levels of nitrogen above 224 kg/ha increased disease severity in 2004 without increasing yields.

Contaminated seed is known to be an important inoculum source for many diseases caused by xanthomonads (9,19,24,26), including Xanthomonas leaf blight of onion (18). However, under the conditions of this study we were unable to incite an outbreak of Xanthomonas leaf blight, even with high levels (~10^7 CFU/g) of seed-borne inoculum. Seed contamination thresholds for development of carrot (*Daucus carota*) bacterial blight caused by *Xanthomonas campestris* pv. *carotae* were 10^4 to 10^5 CFU/g of seed for the semi-arid region of central California in a carrot crop grown using sprinkler irrigation (24). Similarly, Roumagnac et al. (18) reported contamination of onion seed by 5.1×10^1 to 2.0×10^6 CFU/g (median population of 5.1×10^2 CFU/g) *X. axonopodis* pv. *allii* likely sufficient to incite an epidemic of Xanthomonas leaf blight in a sub-tropical environment, but this was with 30 min of supplemental irrigation each morning and evening to ensure disease development. In our studies without overhead irrigation, epiphytic populations of *X. axonopodis* pv. *allii* did not persist throughout the season. Thus, the results of this study suggest that contaminated seed may not be an important inoculum source for Xanthomonas leaf blight epidemics in Colorado, and perhaps other semi-arid production regions where onion is grown without sprinkler irrigation.

In this study, artificially-inoculated seed was used and it was not determined if the inoculum was external contamination or internal infestation. If the inoculation procedure used in this study deposited the pathogen predominately on the seed surface, it may under-represent the importance of naturally-infested seed. Thus, further research is needed to develop specific management recommendations regarding seed contamination thresholds for Xanthomonas leaf blight in onion crops in Colorado and other regions of production. Nonetheless, moderate rates of nitrogen fertilization and the planting of

pathogen-free seed and transplants should reduce losses from Xanthomonas leaf blight in Colorado and similar regions of onion production.

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