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Research Article

Mutual effects of seed-borne bacterial pathogens, *Xanthomonas phaseoli* pv. *phaseoli* and *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in co-infected bean seeds

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Abstract: Most plant pathology research has focused on single-host–single pathogen interactions. Here, are the consequences of co-infection of bean seeds with two important seed-borne pathogens, *Xanthomonas phaseoli* pv. *phaseoli* (*Xpp*) and *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*) were investigated in terms of disease severity and bacterial population dynamics. *Cff* and *Xpp* isolates were collected from infected bean seeds and were identified by PCR with specific primers. Some physiological, pathogenicity, and antagonistic traits of *Cff* and *Xpp* were compared. These pathogens exhibited different characteristics, such as the production of extracellular compounds, including indole acetic acid, biofilm formation, and motility which can potentially affect each other and host plants. The results revealed that simultaneous infection of bean seeds with two pathogens increased the area under disease progress curve (AUDPC) by 1.71 and 2.38 times compared to a single infection of those with *Xpp* and *Cff*, respectively. Pathogen populations in stems and leaves were different under co-infection and single conditions. The data exhibited that the ascending *Xpp* population in the leaves developed from bean seeds co-infected by *Cff* and *Xpp* resulted in a descending *Cff* population. *Xpp* isolate displayed greater motility, colonized the plant earlier than *Cff*, and accelerated disease onset. More biofilm production, confirmed in both pathogens, under co-infection conditions caused earlier plant death via water movement restriction. Our results substantiated that the higher pathogenicity abilities of *Xpp* played a more critical role in the disease progression in plants developed from bean seeds co-infected by *Cff* and *Xpp*. This study provides evidence for the co-occurrence of *Xpp* and *Cff* in nature, highlighting the importance of co-infection in common bacterial blight (CBB) and bacterial wilt (BW) disease dynamics.

Keywords: Bacterial wilt, Common bacterial blight, Co-infection, Disease severity, Bacterial population

Introduction

The different interactions of the microorganisms, including parasitic, competitive, commensalism, and mutualistic, are described (Thebault *et al*., 2010; Coyte *et al*., 2015). The co-infection of plants by different pathogens affects the host plant and could change the potential for disease development and transmission rate (Dutt *et al*., 2022). Multi-infection of several pathogenic species has been reported in different plants using metagenomics and culture-based studies

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(Tollenaere *et al*., 2016). In mixed infection, disease outcomes depend on the interaction type between pathogenic species (Afkhamifar *et al*., 2023; Sadhukhan *et al*., 2024), The pathogenic species may interact directly through mechanical and chemical interaction and indirectly by host defense modifications (Vaumourin *et al*., 2015).

Xanthomonas phaseoli pv. *Phaseoli* (*Xpp*) and *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*) are the most economically significant seed-borne pathogens on beans, widely distributed in many parts of the world, such as Iran, and cause common bacterial blight (CBB) and bacterial wilt (BW) disease, respectively, on beans (; EPPO 2011; Osdaghi *et al*., 2015; Osdaghi and Zademohamad, 2016; Gonçalves *et al*., 2017; Gudero and Terefe 2018). Molecular-based analysis showed that Iranian strains of *Xpp* were relatively genetically homogenous (Zamani *et al*., 2011) but *Cff* strains are polyphyletic and heterogeneous (Osdaghi *et al*., 2018).

The inoculum of the seeds by *Xpp* and *Cff* influences the pathogen survival, development, and dissemination (Fatmi *et al*., 2017; Bastas and Sahin, 2017; Osdaghi *et al*., 2020). Epiphytic populations of *Xpp* (> 10^5 cfu/g) caused disease development and yield losses under favorable environmental conditions (Ishimaru *et al*., 1991; Gilbertson *et al*., 2017). The disease symptoms of CBB appear on the leaves and develop into seeds, causing discoloration of the seed's hilum and wrinkling of the seed coat. Seed discoloration and necrosis on the leaves are also a common symptom of bacterial wilt disease (Huang *et al*., 2009). BW and CBB caused similar lesions, but *Cff* infection is often accompanied by plant wilting and death (Osdaghi *et al*., 2020).

Cff and *Xpp* survive on seed coats and, during seed germination, enter the vascular tissue and systematically develop there at the seedling stage (Bianchini *et al*., 2005; EPPO 2011; He and Munkvold, 2013; Soares *et al*., 2018). Colonization of vascular tissues by *Cff* caused wilting symptoms, and a high amount of obstruction of primary xylem vessels was seen in susceptible bean cultivars (Maringoni *et al*., 2015).

Cff and *Xpp* transmission rates from seed to plant were 5.5-74.2% and 49.9-61%, respectively (Camara *et al*., 2009; He and Munkvold, 2013). Differences in the seed transmission rates have been reported for *Xpp* and *Cff* based on cultivar susceptibility and pathogen aggressiveness (Camara *et al*., 2009; He and Munkvold, 2013).

Bean plants may harbor pathogens in the field that are infected by *Cff* and *Xpp* (Thomas and Graham,1952). Research exhibited that under multi-infection status, individual pathogens' population, transmission rate, aggressivity, disease dynamics, and severity were influenced by other pathogens (Tollenaere *et al*., 2016; Dutt *et al*., 2022). The interactions between the pathogens may be synergistic or antagonistic, and one pathogen can facilitate or inhibit subsequent infections by other pathogens. Therefore, understanding the interaction type between the pathogens within the multi-pathogen-host system can lead to the development of novel disease management strategies to prevent more widespread disease outbreaks. The objectives of this study were to (a) compare the pathogenic abilities and population dynamic of *Xpp* and *Cff* in bean, and (b) investigate the co-infection of bean seeds by *Xpp* and *Cff* in terms of CBB and BW diseases development and severity.

Materials and Methods

Seed samples and bacterial isolation

Bean seeds of Yaghout, Dadfar, Saleh, and Almas cultivars, grown in six fields (Lorestan and Markazi provinces of Iran), were used in this study. Seeds from symptomatic plants displaying necrotic lesions on the leaves surrounded by a yellow halo were sampled according to the International Seed Testing Association (ISTA) instructions in October 2020. Seed samples were rinsed using tap water and suspended in the sterile saline buffer (containing 0.02 % v/v tween 20 and 0.85% NaCl) for four hours. Serial dilutions of each suspension were cultured on a Yeast dextrose carbonate agar (YDC) medium, as described by Grimault *et al*. (2014). Colonies with yellow, orange, and red color on YDC were

selected for general biochemical tests such as gram reaction, oxidase, and catalase (Schaad *et al*., 2001). The isolates obtained from one infected sample with both seed-borne pathogens, *Xpp* and *Cff*, were chosen for further experiments. Two reference isolates of *Xpp* (Xph1) and *Cff* (2En) were received from the microbial culture collection of the University of Valiasr Rafsanjan and Seed and Plant Certification and Registration Institute, respectively.

Hypersensitive response and pathogenicity tests

Hypersensitivity reaction was performed by infiltrating bacterial suspensions ($1*10^8$ CFU/ml) into tobacco leaves. Inoculated leaves were analyzed after 24 hours (Klement *et al*., 1964). Pathogenicity tests of isolates were carried out using the common seed inoculation method on a susceptible bean cultivar (Yaghuot) under greenhouse conditions. Seed inoculation was performed based on the method described by Tegli *et al*., 2017. Hilum-injured seeds were soaked in freshly bacterial suspensions of the strains suspected to *Xpp* and *Cff* separately for one hour. The planted seeds were incubated in a growth chamber at 28 ± 2 °C, 8–16 h day/night cycle.

Molecular detection of *Xpp* and *Cff* using PCR

The specific primers of p7X4c (5′-GGCAACACCCGATCCCTAAACAGG -3′) and p7X4e (5′- CGCCGGAAGCACGATC CTCGAAG -3′) that amplified a fragment of 800bp were used for detection of *Xpp* (Audy *et al*., 1994). The PCR master mix was prepared in a total volume of 25 µl containing Taq 2x Master Mix Red (Ampliqon, Denmark) buffer, 2 µL of template DNA, and 0.2 µM of each primer pair based on the manufacturer′s instruction. The reaction was performed in a thermocycler (Eppendorf, USA). Thermal cycles of the reaction include 3 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 2 min at 72 °C and finally 10 min at 72 °C.

The primer pairs CffFOR2 (5′-GTTATGACTGAACTTCACTCC-3′) and CffREV4 (5′- GATGTTCCCGGTGTTCAG-3′) were used for detection of *Cff* (Tegli *et al*. 2002). PCR was performed in the 25 µl reaction using Taq 2x Master Mix Red (Ampliqon, Denmark) buffer, 0.5 µL template DNA, and 0.1 µM forward and reverse primers. PCR program used for the detection of *Cff* was: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 45 s, and extension at 72°C for 30 s and a final extension at 72 °C for 10 min (Puia *et al*., 2021).

Inhibition zone assay

The inhibitory activity of each strain against the other was evaluated separately by spreading 100 µl of bacterial suspension of each strain on the nutrient agar plates. Following that, the other strain was spot-inoculated on the treated plates. Inhibition zones were measured after 72 h incubation at 28 °C.

Production of extracellular compounds

Cff colonies (48h-old culture) were scrapped from the nutrient agar plate, and the residues were thoroughly washed with sterile distilled water. A chloroform-impregnated sterile cotton was placed into the lid of the Petri dishes and was placed upside down to expose them to chloroform vapor for 30 s. After the chloroform vapor was evaporated entirely, 200 µl *Xpp* suspension (1×10^3 CFU/ml) was spread uniformly on the Petri dishes and incubated for 48 h. The results were evaluated by counting *Xpp* colonies. The same experiment was also performed for *Xpp* against *Cff*.

Biofilm formation

Biofilm forming ability of *Xpp* and *Cff* was analyzed by quantitative crystal violet staining method on 96-well microplates with three replicates. Bacterial isolates were cultured on a nutrient broth medium for 24 h at 28 °C. Bacterial suspension was transferred to each well of ELISA plate. Plates were incubated for 48 h at 28 °C, and then the medium was removed and rinsed with 0.85% NaCl solution. Biofilm was fixed by filling the wells with 200 µl methanol. After 15 minutes, wells were rinsed with water and 1% crystal violet (CV) solution to stain adhered cells. The wells were washed with rinsed water, and the bounded crystal violet

was solubilized by ethanol-acetone solution (4:1, vol/vol) (Nagorska *et al*., 2008). OD_{590} of dissolved crystal violet was recorded by a microplate reader (Elx808, Bio-Tek, Winooski, VT, United States). The data were analyzed as described by Basson *et al*. (2008).

Bacterial motility

Swimming and swarming motility of *Cff* and *Xpp* isolates were examined in nutrient broth medium with 0.3% and 0.5% (w/v) agar, respectively (O'May and Tufenkji, 2011). An individual colony of each isolate was inoculated in the middle of each mentioned medium, and motility diameter was measured after seven days of incubation at 28 °C.

Phosphate solubilizing capability, and indole acetic acid, lipase, proteinase, and cellulase production

Indole acetic acid production was evaluated by the Salkowski reagent (Bent *et al*., 2001). Phosphate solubilizing capability was examined using the method described by Castagno *et al*. (2011). The ability of *Cff* and *Xpp* isolates to produce cellulase was assayed using Czapek mineral salt agar medium (Borkar, 2017). Also, proteinase and lipase production were evaluated as described by Majumdar and Chakraborty (2017) and Tom and Crisan (1975).

Assessments of the interaction between *Xpp* and *Cff* on disease progression

Two infection methods were used to assess the mutual effects of *Xpp* and *Cff* isolates on bean plants (Yaghout cultivar). In the first method, the seeds were sown in infected soil. For single-pathogen inoculation,100 grams of soil (peat moss 25%, perlite 25%, and soil 50%) mixed with 100 mL of bacterial suspension (*Xpp* or *Cff*) at a concentration of 1×10^9 CFU/mL and in co-inoculation with both pathogens, double the soil volume was used with 100 mL of each pathogen suspension at the same concentration.

In the second method, bean seeds were injured by piercing the hilum with a sterilized needle; then the seeds were soaked in *Xpp* and *Cff* suspension (1×10^8 CFU/ml) plus 1% CMC for 1 h (separately and in the combination of two pathogens). Seeds in *Xpp*- and *Cff*- free soil were considered as negative control. The planted seeds were incubated in a growth chamber at 28 ± 2 °C, 8–16 h day/night cycle, and ~90% relative humidity. Experiments were performed with three technical and biological replicates. Each replication consisted of ten seeds in each pot. Symptom incidence was assessed daily from leaflet appearance to 20 days after planting to calculate the disease severity index and area under the disease progress curve (AUDPC).

The disease severity index (DSI) was determined on ten plants in each replication. Scales of 0–6 were used as 0: no symptoms; 1: wilt or necrotic lesions (may be surrounded by yellow halo) that covered 1-10 % of leaves; 2: wilt or expended lesions on 10-20% of leaves; 3: wilt or expended lesions on 20-50% of leaves; 4: wilt or expended lesions on 50-80% of leaves; 5: wilt or expended lesions on 80-100% of leaves and 6: death of plantlets.

$$\text{Disease severity index (\%)} = \frac{\text{sum (class frequency} \times \text{score of rating class)}}{\text{(total number of plants)} \times \text{(maximal disease index)}} \times 100$$

The AUDPC was estimated based on recorded DSI on the 10^{th} and 20^{th} days after culture. Experiments were executed in a completely randomized design. Statistical analysis was performed with SPSS (SPSS Inc., Chicago).

Determination of bacterial population and transmission rate

The population size and transmission rate of *Xpp* and *Cff* were estimated in the stem and leaf of plants at 5, 10, and 15 days after soil inoculation by *Xpp* and *Cff*, individually or in combination with each other (Osdaghi *et al*., 2016). Three subsamples of each treatment in tree replicates were harvested from stems and leaves separately. Samples were macerated in saline buffer (1 gr/10 ml buffer). Serial dilution of extracts was plated onto YDC medium. Bacterial populations were determined five days post-incubation at 28 °C (Toussaint *et al*., 2012). Confirmation of the re-isolated pathogens was performed using specific PCR methods, as mentioned earlier.

The transmission of *Xpp* and *Cff* to daughter plants was confirmed by comparing the morphology of the colonies and specific PCR with isolates used in seed treatment. For each pathogen, three biological and three technical replicates were performed.

Survey of co-infection of *XPP* and *Cff* in the field

Bean seeds (Yaghout cultivar) sampled (28 samples, and 200 seeds from each sample) from infected fields located in three provinces of Iran (Lorestan, Zanjan, and Markazi) from 2021 to 2023 were planted in a field plot in four rows (75 cm row spacing and 25 cm seed spacing in each row) in Alborz province. Natural co-infections of bean plants (leaf) by *Xpp* and *Cff* were assessed after selecting ones displaying severe disease symptoms by culturing on YDC and performing PCR.

Results

Bacterial isolation, selection, and pathogenicity tests

Based on colony morphology on YDC, 16 isolates, including 12 gram-positive orange, red, or yellow colonies, and four gram-negative light yellow mucoidal colonies were obtained from bean samples, as shown in Table 1. Gram stain and hypersensitivity reaction on tobacco leaves and pathogenicity test on bean (Yaghout cultivar) were differentiated pathogenic isolates. Four gram-positive isolates suspected to *Cff* exhibited pathogenicity on beans and negative hypersensitivity reaction on tobacco (Table 1). Also, two isolates suspected to *Xpp* as pathogenic gram-negative bacteria displaying light yellow convex mucoid colonies on YDC showed positive pathogenicity and hypersensitivity reactions (Table 1). All pathogenic isolates suspected to *Xpp* and *Cff* were confirmed by PCR analysis. The specific PCR products of *Xpp* and *Cff* were approximately 800 bp and 306 bp, respectively (Fig. 1 D, E).

The first disease symptoms of common bacterial blight (CBB) and bacterial wilt (BW) on bean seedlings were observed seven and nine days after seed inoculation by *Xpp* (LA7) and *Cff* (X0S), respectively (Fig. 2A). The visual symptoms caused by *Xpp* on the leaves were observed earlier than those caused by *Cff* (Fig. 2A). The first symptoms in the co-infection of *Xpp-cff* appeared around the same time as the *Xpp* infection (Fig. 2A). However, co-infected plants exhibited more significant visual symptoms. The entire plant death occurs much earlier than plants infected by single pathogens (Fig. 1F). In the single-infected and co-infected plants, chlorosis symptoms appeared along the margins of the leaves and expanded into brown lesions, surrounded by yellow halo areas (Fig. 1 A, B).

Cff and *Xpp* isolates exhibited different traits potentially affecting each other and host plants (Table 2). *Cff* isolate (LA7) exhibited cellulase and protease activity and the *Cff*'s ability in biofilm production was estimated three times higher than that of *Xpp* (Table 1). *Xpp (X0S)* showed more abilities, compared to *Cff* (LA7), regarding swimming and swarming traits. The data presented in Table 2 exhibited an inverse relationship between biofilm formation and motility of the pathogens.

Colonization of leaf and stem tissues by pathogenic isolates "LA7" and X0S" was determined using culture on YDC and PCR. In addition to *Cff* (LA7), *Xpp* isolates (X0S) can also enter the vascular system and develop systemically. Population size of both pathogens varied in co-infected leaf and stem tissues. The results showed that *Xpp* is a more successful pathogen occupying the leaf tissue under competition conditions in co-infection.

The population of *Xpp* in leaf tissue tended to increase over time within mix and single infection. Comparing bacterial population in co-infected and single-infected beans indicated that ascending *Xpp* population in the leaf developed from co-infected seeds resulted in a descending *Cff* population. In contrast, this trend is not observed in the stem (Fig. 2B, C). This study found that *Xpp* and *Cff* population higher than 10^6 CFU/g in tissue, created disease symptoms in single and co-infection.

The development of the CBB and BW diseases occurred in both soil and seed inoculation, and disease severity index (DSI) and area under the disease progress curve (AUDPC) are significantly different (P > 0.01) in single infection of *Xpp* and *Cff* with co-infection of the pathogens (Table 3). Results showed that seed inoculation was more affected DSI and AUDPC than soil inoculation. However, seed germination was influenced by hilum injuring and soaking the seeds in the bacterial suspension.

Table 1 Bean seed samples, type of isolated bacteria, colony morphology and pathogenicity tests on bean leaf and pod.

Samples name	Cultivar- location	*Xpp* infection	*Cff* infection	colony color on YDC		Hypersensitivity reaction		Gram stain		Water soaked on bean pod		Pathogenicity on bean	
				Xpp	*Cff*	*Xpp*	*Cff*	*Xpp*	*Cff*	*Xpp*	*Cff*	*Xpp*	*Cff*
MYNB99	Yaghout- Markazi	+ (MY29)	-	yellow	N	+	N	-	N	+	N	+	N
LYDA00	Yaghout-Lorestan	-	+ (LY0)	N	red	N	-	N	+	N	-	N	+
MYMT00	Yaghout- Markazi	-	+ (MY1)	N	orange	N	-	N	+	N	-	N	+
FDa00	Dadfar-Lorestan	-	+ (FD8)	N	orange	N	-	N	+	N	-	N	+
LA00	Almas- Lorestan	+ (X0S)	+ (LA7)	yellow	orange	+	-	-	+	+	-	+	+
KHS00	Saleh- Markazi	-	-	N	N	N	N	N	N	N	N	N	N

Xpp: *Xanthomonas phaseoli* pv. *Phaseoli, Cff*: *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*
+: Positive; -: Negative; N: not found

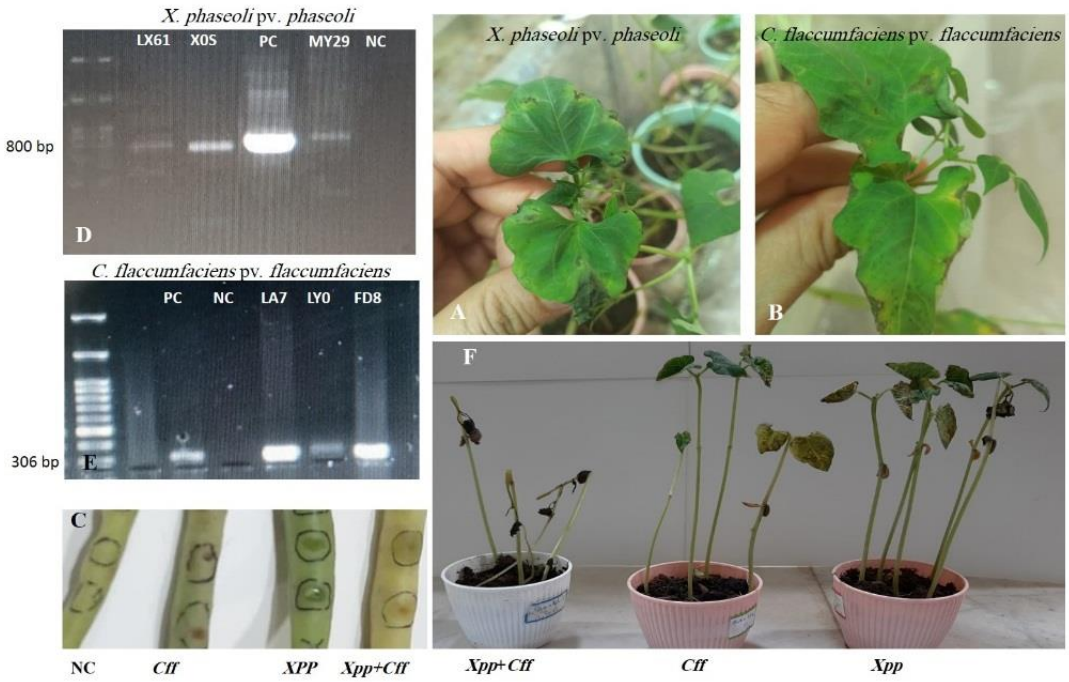

Figure 1 Visible symptoms of inoculated bean plants and pods with *Xpp* and *Cff* and specific detection of the pathogens with PCR analysis. A, B: Similar necrosis symptoms surrounded by yellow halo caused by *Xpp* and *Cff*. C: Water soak lesion on bean pod induces by *Xpp* and *Xpp* + *Cff* and necrosis lesion with *Cff*. D, E: Reproduction of 800-bp and 306-bp DNA fragments (specific to *Xpp* and *Cff*, respectively) with polymerase chain reaction (PCR) using the oligonucleotide primers p7X4c/p7X4e, CffFOR2/CffREV4; F: Increasing disease severity after co-inoculation of bean seed with *Xpp* + *Cff* , 15 Day after sowing inoculated seeds (Yaghut cultivar). *Xpp*: *Xanthomonas phaseoli* pv. *Phaseoli, Cff*: *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*), PC: positive control, NC: negative control.

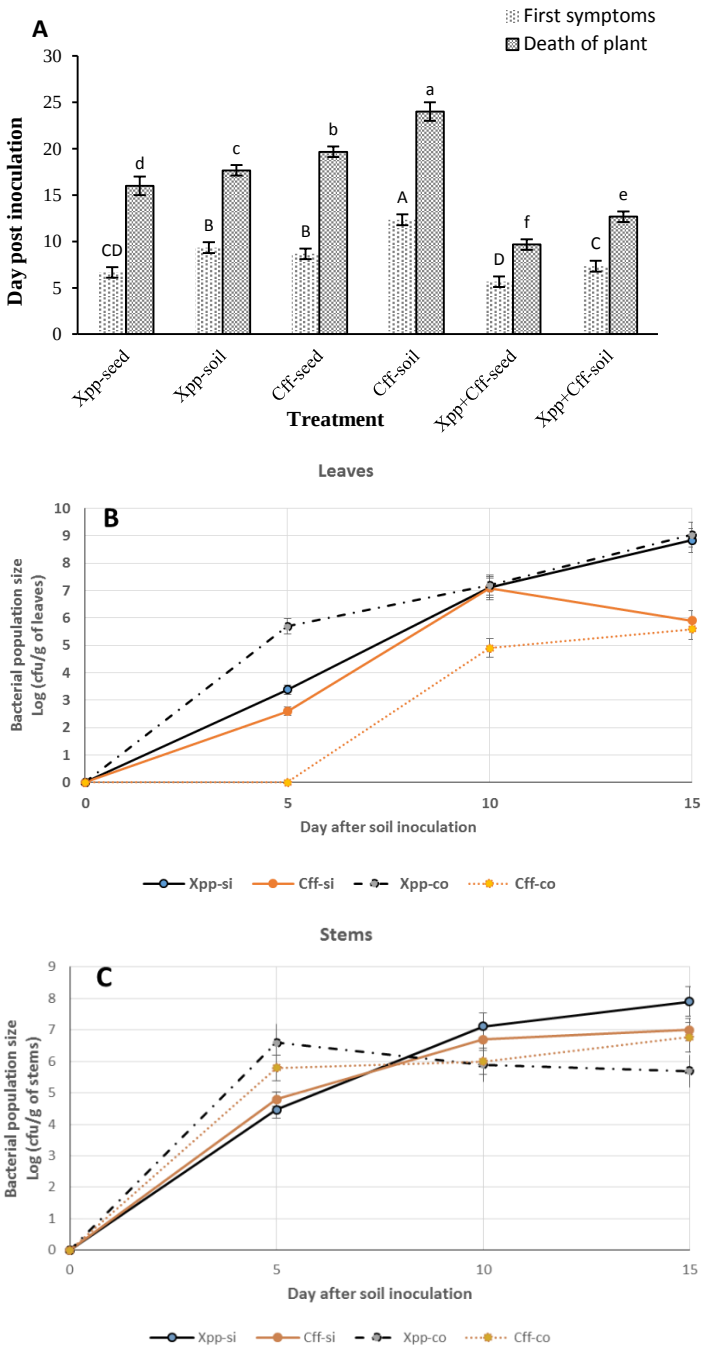

Figure 2 Time of disease symptoms appearances and the bacterial population changes in a single infection of *Xanthomonas phaseoli* pv. *phaseoli* and *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* and a co-infection of pathogens. Statistical comparison of the time for first symptoms appearance and plant death in a single infection and a co-infection of pathogens (A). *Xpp* and *Cff* population dynamics in the bean stem and leaves in a single and a co-infection after soil inoculation (B, C). *Xpp*-si: population of *Xpp* in single infection of *Xpp*., *Cff*-si: population of *Cff* in single infection of *Cff*., *Xpp*-co: population of *Xpp* in co- infection., *Cff*-co: population of *Cff* in co- infection. *Xpp*: *Xanthomonas phaseoli* pv. *Phaseoli*, *Cff*: *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*).

Table 2 Comparison of some physiological, pathogenicity and antagonistic traits of *Cff* and *Xpp*.

Traits	*Cff*	*Xpp*
Swarming	+	+++
Swimming	+	++
Biofilm (OD:590)	+++	+
Extracellular Substances	-	++
Volatile compounds	-	-
HR	-	+
Nitrogen Fixation	++	-
Protease	++	++
Cellulase	++	-
IAA	++	+
Zone of Inhibition	Against *Xpp* (-)	Against *Cff* (++)

Cff: *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*; *Xpp*: *Xanthomonas phaseoli* pv. *phaseoli*
- negative; + weak positive; ++ intermediate; +++ strong positive

The progression of both diseases was slower in the soil inoculation than seed one. A two-day delay in the appearance of CBB and BW symptoms in soil inoculations (Fig. 2A) was accompanied by lower DSI and AUDPC (Table 4). The AUDPC value after co-infection of seeds increased up to 1.7 and 2.3 times more than single infection of seeds by *Xpp* and *Cff*, respectively. At the same time, AUDPC in mixed soil contamination was estimated 1.3 and 5.2 higher than the individual soil contamination by *Xpp* and *Cff*, respectively (Table 4). Our finding exhibited that *Xpp* can establish, occupy the tissues, and initiate symptoms faster than *Cff* (Fig. 2). More abilities of *Xpp* (X0S) in swimming and swarming (Table 2) can help the pathogen to establish faster, as compared *Cff*, on seeds from inoculated soil. The mean comparison of DSI and AUDPC in single and co-infection in seed and soil inoculation indicated that the pathogenicity of *Xpp* was more than that of *Cff* (Table 3).

This study revealed that co-infection of *Xpp* and *Cff* is common in nature and aggravates the disease severity. The field assay exhibited that out of 5600 evaluated bean plants, only 3 plants of susceptible Yaghout cultivar were infected by both pathogens in natural conditions (Table 5).

Table 3 Variance analysis for disease severity index (DSI) and area under the disease progress curve (AUDPC) after seeds and soil inoculation by *Xpp* and *Cff* (separately) and co-infection of *Xpp* and *Cff*.

SOV	df	Mean square				AUDPC (10-20 dpi)	
		DSI 10dpi		DSI 20dpi			
		Soil	Seed	Soil	Seed	Soil	Seed
Treatment	2	**2.071	**5.076	**9.384	17.710**	**487.271	**1042.682
Error	9	0.024	0.27	0.861	0.320	28.192	27.770
%CV		14.86	17.61	19.14	13.25	18.43	14.60

** Significant at 1% probability level; DF: Degree of freedom; MS = Mean square; and CV = Coefficient of variation.

Table 4 Mean comparison of DSI and the AUDPC after seed and soil inoculation with *Xpp*, *Cff* and co-infection of *Xpp* and *Cff*.

Seed treatment	DSI 10dpi		DSI 20 dpi		AUDPC in period of 10–20 DPI	
	Soil	Seed	Soil	Seed	Soil	Seed
Xpp	1.13 [b]	2.41 [b]	4.0 [a]	3.9 [b]	25.6 [a]	31.56 [b]
Cff	0.16 [c]	1.16 [c]	0.93 [b]	3.4 [b]	5.5 [b]	22.68 [c]
Co-infection	1.81 [a]	5.26 [a]	4.1 [a]	5.5 [a]	29.08 [a]	54.01 [a]

Means with the same letter are not significantly different from each other (P > 0.01).

Table 5 The number of natural co-infected plants with *Xanthomonas phaseoli* pv. *phaseoli* and *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in experimental plots.

Year	Number of evaluated plants	*Xpp*	*Cff*	Co-infection of *Xpp* and *Cff*
2021	900	15	12	2
2022	2600	27	9	1
2023	2100	6	11	0

Discussion

The present study showed that *Cff* and *Xpp*, alone and simultaneously, were detected from bean seed samples. Detection of both pathogen in seeds and plant tissues were confirmed by PCR analysis in agreement with the results obtained by Audy *et al*. (1994) and Tegli *et al*. (2002). Thomas and Graham (1952) reported that bean plants can harbor both *Xpp* and *Cff* populations.

Symptoms of both pathogens are similar. They may be confused with each other (Osdaghi *et al*. 2020). Júnior *et al*. (2012) previously reported that necrosis lesion surrounded by a yellow halo at the edges of the leaflets is another symptom of BW disease besides wilting, similar to what happens in CBB disease.

The bacterial pathogens use a multitude of mechanisms and harbor physiological, pathogenicity and antagonistic traits, which enable the pathogens to cause disease or interact with other microorganisms. Both pathogens produced indole-3-acetic acid (IAA), which can play critical roles in crosstalk between plants and microbes. During plant-microbe interaction, IAA biosynthesis by bacteria, physiologically facilitates root colonization (Ahmad *et al*., 2022). The research highlighted bacterial IAA's role in their colonization strategy through phytostimulation and circumvention of basal plant defense mechanisms (Spaepen *et al*., 2007). Bacteria have evolved diverse mechanisms to influence their host plants (Chepsergon and Moleleki, 2023) and to establish negative (competition), positive (cooperation), or neutral interaction with other microbial populations, which can implicate the health status of the plant host (Granato *et al*., 2019; Chepsergon and Moleleki, 2023).

Cff isolate (LA7) exhibited cellulase and protease activity, which can significantly affect disease development. *Cff's* pathogenicity is related to lytic enzyme activity, such as pectate lyase, serine protease, cellulases, and xylanase (Chen *et al*., 2021; Li and Sun, 2021). *Cff* colonizes the xylem system of the host plant and acquires nutrients via disruption of cell walls by enzymes (Thapa *et al*., 2019). Evseev *et al*. (2022) demonstrated that two adjacent genes of cellulases and cellulose-binding proteins and six genes of trypsin-like serine proteases may be related to virulences of *Cff*.

Biofilm is a virulence factor in early colonization and infection of some bacterial pathogens (Flemming and Wingender, 2010; Guttenplan and Kearns, 2013). It has been indicated that *Cff* and *Xpp*, as bean bacterial blight (BB) disease complex agents, produce biofilms during their disease cycles (Harding *et al*., 2019). They can develop systemically in vascular system so, wilting symptoms can result from vascular occupation by the pathogens (Aggour *et al*., 1989). The biofilm substrates occupy and block the vessels that can restrict water movement, resulting in plant wilting or, in more severe cases, the entire plant death (Harding *et al*., 2019).

Our data showed *Cff*'s ability in biofilm production was higher than that of *Xpp* (Table 1) but *Xpp* (X0S) showed more abilities in swimming and swarming traits. Table 2 exhibited that motility and biofilms are oppositely controlled because it seems one of the essential events in biofilm formation is the inhibition of motility (Guttenplan and Kearns, 2013).

On the other hand, bacterial motility promotes its entry into stomata or wounds and facilitates its adhesion and initial attachment (Danhorn and Fuqua, 2007). Swimming motility can vary widely depending on the bacterial host range (Sena-Vélez *et al*., 2015). Bacterial strains with a restricted host range require a more specific niche. So, they need higher swimming ability to find colonization sites (Sena-Vélez *et al*., 2015). Belete and Bastas (2017) demonstrated that *Cff* isolates with a broader host range showed lower motility than *Xpp* with a limited host range (Osdaghi *et al*., 2015).

The disease symptoms of co-infected plants appeared more significant than that of *Xpp* and *Cff* alone. Co-infection outcomes can differ depending on the interaction type of pathogenic species (Sadhukhan *et al*., 2024). However, disease severity increment under

mixed infections condition as compared to single-infection has been frequently reported (Stromberg *et al*., 2000; Kůdela *et al*., 2010; Lamichhane and Venturi, 2015; Moretti *et al*. 2021; Marchetto and Power, 2018; Tambong, 2022).

The bacterial population assay in leaves of co-infected beans showed that ascending *Xpp* population resulted in a descending *Cff* population (against stems tissue). The competition between pathogens with similar nutritional requirements likely happened in leaves, as nutrient niches, more than stems (West *et al*., 2006). Although CBB is mainly considered as a foliar disease, systemic development of *Xpp* via the vascular system in the plant tissue and wilting has also been reported (Belete and Bastas., 2017). On the contrary, *Cff* mostly colonizes xylem vessels in stems and the petiole bases of leaves (Maringoni *et al*., 2015). The population dynamics depend on co-infecting partners and competition resources which can modulate cooperative or competitive interactions (Sadhukhan *et al*., 2024). The population of both pathogens higher than 10^6 CFU/g, need to cause disease in single and co-infection as reported by Belete and Bastas (2017) and Sammer and Reiher (2012).

The diseases progressive are significantly different in single infection of *Xpp* and *Cff* with co-infection of the pathogens. Studies revealed that co-infections can increase disease severity up to the sudden collapse of host plants (Kůdela *et al*., 2010; Marchetto and Power, 2018). On the other hand, releasing cellulase enzymes by *Cff* in co-infection can improve the nutrients acquisition through cell-wall degrading (Rohmer *et al*., 2011) which provide advantages for co-occurring pathogen (*Xpp*) (Abdullah *et al*., 2017).

The inoculation methods influence the establishment and persistence of microorganism populations in the host plant and can alter infection time (Afzal *et al*., 2013; Lopes *et al*., 2021). Results showed that seed inoculation was more affected diseases progressive but seeds germination were decrease due to hilum injuring in this method. The soil inoculation method was appropriate, probably due to simulating natural conditions. However, low mobility and microbial population reduction in the soil, can affect the ability of microbes (Paravar *et al*., 2023). Studies revealed that co-infection timing had an essential effect on population dynamics and disease impact predictions (Marchetto and Power, 2018).

Our study reveals that *Xpp* was more aggressive pathogen than *Cff*. The influential and diverse pathogenic factors of *Xpp* made it superior as compared *Cff* in terms of bacterial competition. *Xanthomonas citri* pv *fuscans* (*Xcf*), as the other causal agents of CBB disease, is the closely related pathogens to *Xpp* which its virulence factors have been determined. *Xpp* acquired pathogenicity genes from *Xcf* via horizontal gene transfer (Chen *et al*., 2018). Darsonval *et al*. (2008) demonstrated that the type III secretion system and *hrp* regulatory genes are involved in systemic colonization and vascular transmission of *Xcf*. Several proteins involved in bacterial virulence are transmitted from the bacterial cell into the plant cell via this secretion system (de Paiva *et al*., 2022).

Other pathogenicity factors are biofilm and xanthan which are secreted by *Xanthomonas* spp. and play a crucial role in the pathogenesis and suppression of calluses as the first line of plant defense (Gaudin *et al*., 2023; Harding *et al*., 2019; Yun *et al*., 2006). Lack of type III secretion system (T3SS) was reported for *Cff*, and it seems the pathogenicity of *Cff* is related to some enzymes, toxins, and biofilm (Osdaghi *et al*., 2020; Chen *et al*., 2021). Synergistic interactions of *Cff* and *Xpp* may be mediated by the production of various virulence compounds, resource availability, and high biofilm output in the host plant (Abdullah *et al*., 2017).

Conclusion

This study showed the co-infection of *Xpp* and *Cff* occurred in nature. Our co-inoculation experiments under controlled conditions confirmed that co-infection by both pathogens could significantly change the disease load, increase disease severity and AUDPC, and

influenced population dynamics. Different virulence factors, such as biofilm, IAA and enzyme production, and also motility abilities, are thought to affect the colonization and the disease severity. However, the role of these factors in the interactions of two pathogens with each other and the host plant requires more research in the future. Our results substantiated that *Xpp* had more achievement in colonization and pathogenicity than *Cff* and imposed higher disease severity. The present study was outlined a deeper understanding of various aspects of microbial co-infections in beans which is a crucial consideration for disease control and can inspire novel management strategies.

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اثرات متقابل باکتری‌های بذرزاد *Xanthomonas phaseoli* pv. *phaseoli* و *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* در آلودگی همزمان در بذر لوبیا

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چکیده: اکثر پژوهش‌ها در بیماری‌شناسی گیاهی، روی برهمکنش‌های تک‌میزبان-تک بیمارگر متمرکز شده‌اند. تحقیق حاضر به بررسی اثرات آلودگی همزمان لوبیا با دو بیمارگر مهم بذرزاد *Xanthomonas phaseoli* pv. *phaseoli* (*Xpp*) و *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*)، بر شدت بیماری و پویایی جمعیت باکتری‌های مورد اشاره پرداخته است. جدایه‌های *Cff* و *Xpp* از بذرهای لوبیا آلوده جمع‌آوری و با استفاده از PCR و پرایمرهای اختصاصی شناسایی شدند. برخی از صفات فیزیولوژیکی، بیماری‌زایی و آنتاگونیستی *Cff* و *Xpp* با یک‌دیگر مقایسه شدند. هر دو بیمارگر صفات مختلفی از جمله تولید آنزیم‌ها، ایندول استیک‌اسید ، تشکیل بیوفیلم و تحرک را از خود نشان دادند که این ویژگی‌ها به‌طور بالقوه می‌توانند باعث اثرات دو بیمارگر بر یک‌دیگر و بر گیاهان میزبان شوند. نتایج ما نشان داد که آلودگی همزمان بذرهای لوبیا با دو عامل بیماری‌زا باعث افزایش سطح زیرمنحنی پیشرفت بیماری (AUDPC) به ترتیب ۱/۷۱ و ۲/۳۸ برابر نسبت به آلودگی انفرادی *Xpp* و *Cff* شد. جمعیت بیمارگرها در ساقه و برگ در اثر آلودگی همزمان تغییر یافت. داده‌های به‌دست آمده نشان داد، زمانی که جمعیت *Xpp* شروع به افزایش در بافت‌های برگ نمود، کاهش قابل‌توجهی در جمعیت *Cff* مشاهده شد. به‌نظر می‌رسد جدایه *Xpp*، با تحرک بیش‌تر، زودتر از *Cff* گیاه را اشغال نمود و شروع بیماری را باعث شد. تولید بیوفیلم بیش‌تر در اثر هم‌افزایی دو عامل بیمارگر در آلودگی همزمان، باعث مرگ زودرس گیاه از طریق محدود نمودن حرکت آب می‌شود. براساس نتایج به‌دست آمده توانایی‌های بیماری‌زایی بالاتر *Xpp*، نقش مهم‌تری در پیشرفت بیماری در آلودگی ترکیبی ایفا می‌نماید. همچنین این مطالعه وقوع همزمان دو عامل بیمارگر را در طبیعت ارائه نموده است که اهمیت آلودگی همزمان در پویایی بیماری سوختگی باکتریایی (CBB) و پژمردگی باکتریایی (BW) را نشان می‌دهد.

واژگان کلیدی: پژمردگی باکتریایی لوبیا، بلایت باکتریایی معمولی لوبیا، آلودگی همزمان، شدت بیماری، جمعیت باکتری