A journey of discovery: *Curtobacterium flaccumfaciens* pv. *allii* is a new potential threat for maize

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Abstract

In the current scenario of globalized markets and climate change, phytosanitary risks are greater than ever before: the former facilitates the spread of pests over long distances, while the latter can create environmental conditions favourable for the establishment of pests in new areas.

In 2022, symptoms including chlorotic streaks, stunted growth and wilting where seen on maize plants in Northern Italy. Diagnostic procedures to identify known pathogens associated with these symptoms gave negative results but allowed the isolation of bacterial colonies found only in symptomatic plants and therefore identified as the putative causative agent of the symptoms. As the pathogen initially seemed to be *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* but was not detected by specific assays for this pathogen, several assays were carried out using molecular biology techniques and whole genome sequencing to identify the taxonomy of these four bacterial isolates and biological assays to characterize their pathogenicity, allowing to determine their taxonomical identity as *Curtobacterium flaccumfaciens* pv. *allii*.

Subsequent pathogenicity assays allowed to verify Koch's postulates by inoculating the isolates on maize, observing the development of symptoms, and re-isolating the bacteria from the infected plants, confirming the status of *C. flaccumfaciens* pv. *allii* as a newly reported pathogen of maize.

To date there is no information on this organism and the damage it may cause in the field. Further studies are therefore needed, in particular to investigate its epidemiology, pathways and even possible host range.

Keywords: *Zea mays*, *Pantoea stewartii*, maize chlorotic streak, Illumina sequencing, onion bulb rot

1. Introduction

Maize is one of the major crops worldwide, accounting for the 13% of the global production of the primary crops in 2021 and with a production increased from 0.59 billion tonnes in 2000 to 1.61 billion tonnes in 2021. The largest maize producing country are the United States, with 32% of global production, followed by China, Brazil, and the European countries which, as a whole, produce around 9% of maize in the world (FAOSTAT, 2022; FAOSTAT, 2023). Despite being the fourth largest producer, demand for maize in Europe is very high, leading to imports of this crop (European Commission, 2024), a situation that is particularly acute in Italy.

Lombardy is Italy's leading maize-producing region, with an area of around 300,000 hectares dedicated to maize cultivation (ISTAT, 2023). This crop is very important for human consumption and to support the dairy sector, which is the main contributor to the regional agricultural gross domestic product. In this context, maize is a priority crop for the Lombardy Plant Protection Service and, in accordance with European Union Regulation (Reg (EU) 2016/2031, Reg (EU) 2017/625; Reg (EU) 2019/2072), special attention is paid to the possible introduction, establishment and spread of quarantine pests. For this reason, intense monitoring activities of the territory are carried out through General surveillance and Specific surveillance actions (FAO-ISPM).

Phytosanitary risks are amplified by the current world economic system, affected by rapid and intense changes in processes and products. The consolidation on the global market of emerging production areas, rapid transportation, the removal of customs barriers, tourism, and the decentralization of production, have led to a sharp increase in the international movement of people and goods. In turn, this increase in international movement has exponentially increased the risk of exporting fearsome quarantine pests, previously confined by geographic isolation of continents or natural barriers, to new territories.

In addition, it is also necessary to consider as in the overall context the influence of climate change, which can negatively affect the potential production of maize (Shrestha et al., 2013). This may

67 occur not only due to the direct effect of abiotic factors such as increasing temperatures, decreasing
68 precipitation or increasing extreme weather events, but these same factors may also have an indirect
69 effect by influencing adaptation and epidemiological development of new pests (Anderson et al.,
70 2004; Janse, 2012; Jones et al., 2012).

71 At present, there are several bacterial diseases of maize with variable distribution worldwide and
72 different host range. The gram-negative bacteria *Pantoea stewartii* subsp. *stewartii* (EPPO code:
73 ERWIST; abbreviated as PSS in the manuscript), the etiological agent of Stewart's vascular wilt and
74 leaf blight of maize and sweet corn (Coplin et al., 2002), currently considered a quarantine pest in
75 Europe. Typical symptoms are longitudinal leaf streaks with irregular or wavy margins that run
76 parallel to the veins and can extend across the entire leaf. These lesions are initially green-yellow
77 coloured, but turn brown as the disease progresses, resulting in leaf blight (Roper, 2011).

78 Considering instead the ability of a pathogen to adapt to new hosts that were previously unknown,
79 an example can be made regarding the species *Pantoea agglomerans*. *P. agglomerans* was
80 originally reported as plant pathogen on pearl millet in India in 1958 and in Zimbabwe in 1997
81 (Rajagopalan et al., 1958; Frederickson et al., 1997), and reported later as responsible for several
82 diseases on different host plants like onion in the USA and Chinese cabbage in China (Tho et al.,
83 2015; Guo et al., 2020). It was also reported to cause leaf blight, bacterial wilt in maize (Morales-
84 Valenzuela et al., 2007).

85 Cases such as these illustrate the complexity and the importance of research into new potential
86 diseases and the correct identification of new or already known organisms as potential etiological
87 agents, especially in agriculturally important crops such as maize.

88 When dealing with new or unexpected pathogenic organisms, traditional diagnostic methods, both
89 culture-based and molecular, can lead to confusing or unconclusive results. In these cases, the
90 solution to correctly identify the pathogen can come from sequencing of the putative pathogen's

whole genome, as analyses based on the whole genomic sequence can determine the species of an organism where single gene or multilocus sequencing approaches fail (Koutsoumani et al., 2019).

2. Description of the 2022 outbreak

In May 2022, in Northern Italy (Lombardy region) maize plants were found with symptoms that could be associated to some of the bacterial diseases described above. **S**amples of chopping corn were received at the Plant Protection Service laboratory; plants were sampled by a private company technical service in the province of Cremona (Lombardy region) from demonstration fields. The symptoms seen on plants at pre-flowering stage in field included longitudinal chlorotic streaks on the leaves, stunted growth, and – in the most severe cases – wilting of the plant (**Figure 1**). The chlorotic streaks can later degenerate into necrotic lesions (**Figure 1c**). Root system of the affected plants showed no evident sign of damage but, in some cases, a reduced development.

3. Characterization of the pathogen

3.1. Initial analyses: ruling out the involvement of regulated pests

In the lab, each plant sample was divided into three sections: rhizosphere, consisting of the earth still attached to the roots; root, consisting of root tissue after removal of rhizosphere; stalk, consisting of the first 5 cm of the stalk above root crown. The rhizosphere samples were accurately mixed, and three 0.5 g aliquots were weighed from each sample for microorganism isolation.

For both root and stalk samples, a superficial washing and sterilization step was carried out: the plant material was first washed with tap water to remove soil and other possible contaminants, then the material was immersed in a sterilizing solution (3% sodium hypochlorite, 20% ethanol) for 3 minutes and then washed three times in sterile distilled water to remove the sterilizing agents. Aliquots of the last washing bath (1 mL) were plated on plate-count agar (PCA) and incubated at 22 °C for 7 days to check the absence of microbial growth and therefore the effectiveness of the superficial sterilization.

115 With a sterile scalpel, sterilized roots and stalk samples were cut and weighed to obtain three 0,5 g
116 portions of plant material that were used for microorganism isolation from each sample.

117 Each 0,5 g sample of rhizosphere, root, and stalk was ground in 5 mL of sterile PBS to obtain a
118 homogeneous suspension. This suspension was further diluted serially in sterile PBS to obtain three
119 concentrations: undiluted, 1:10, and 1:100.

120 From each dilution of the suspension, a 100 µL aliquot was plated on one Petri dish containing
121 PCA, potato dextrose agar (PDA), nutrient saccharose agar (NSA), or yeast peptone glucose agar
122 (YPGA) media. After inoculation, each plate was incubated at 22 °C and monitored for growth of
123 microorganisms, known fungal or bacterial pathogens that might be associated with the symptoms.

124 This initial isolation procedure identified several fungi in the rhizosphere and root samples, mostly
125 belonging to the *Trichoderma*, *Fusarium*, and *Rhizopus* genera according to a morphological
126 evaluation at the microscope. These fungi were found in both symptomatic and asymptomatic plants
127 and were therefore excluded as etiological agents of the disease.

128 The only clear difference between symptomatic and asymptomatic plants was found when analysing
129 the stalk samples plated on YPGA plates, on which some bright yellow bacterial colonies were
130 found only in samples obtained from symptomatic plants. As such, these four isolates,
131 morphologically identical but obtained from four different symptomatic plant samples, were
132 selected for characterization and labelled as C8-5L, C8-6L, C8-JL, C8-KL and were further
133 investigated as the putative etiological agent of the disease.

134 These four isolates were cultivated in liquid culture in Luria-Bertani broth overnight at 22 °C with
135 shaking (150 rpm) and then 1 mL aliquots were used for nucleic acid extraction using a CTAB
136 method (Wilson, 2001).

137 The DNA samples were used to determine if the colonies could belong to PSS using the following
138 methods: Real-time PCR according to Tambong et al. (2008) and PCR modified from Coplin et al.

(2002). Both methods used gave negative results on all four isolates, excluding the involvement of this regulated pest.

Then, a PCR was carried out using the general 16S primers pA/pH (Edwards, et al., 1989; Bruce et al., 1992; Greisen et al., 1994) to amplify 1.5 kb of rDNA. The amplicons were sequenced in both senses (5X coverage per base position) by a commercial service (Eurofins Genomics, Germany). Nucleotide sequences were compiled in FASTA format, assembled by employing the Contig Assembling Program of the software BioEdit version 7.2 (Hall, 1999). Each sequence was compared against the NCBI database using the BLAST online tool with standard settings. The results of this analysis were the same for all the four isolates: BLAST analysis returned identity above 97% and 100% coverage with *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (CFF). Since this quarantine pest was previously reported on maize, but never associated with symptoms, a specific PCR assay for CFF was carried out), as reported in the EPPO PM7/102 using primers CF4 and CF5 (Guimaraes et al., 2003) to confirm this result. Despite the identification on the 16S gene as CFF, all isolates gave negative results when subjected to these pathogen-specific assays.

3.2. Molecular taxonomical characterization of the pathogen

The obtained 16S sequences, as well as 16S sequences of different *Curtobacterium* strains obtained from NCBI, were used to generate a database, aligned and used to compile a phylogenetic tree using the Mega XI software (Tamura et al., 2021). The sequences obtained from the sequencing of the 16S gene of colonies C8-5L, C8-6L, C8-JL, C8-KL are available in GenBank under accession numbers PQ006086 –PQ006089. All our isolates clustered in the same branch as CFF and CFA but this analysis did not allow discrimination between them (**Figure 2**). It is important to note that on different databases online, including NCBI, this pathovar is still reported as *C. allii*, a separate species from *C. flaccumfaciens*, based on the original report (Khanal et al., 2023a) but it has since been re-classified as *Curtobacterium flaccumfaciens* pv. *allii* (CFA) (Osdaghi et al., 2024).

Having reached no conclusive evidence on the taxonomy of these isolates, the next step was to sequence their whole genome and use them to achieve a more precise taxonomical attribution. DNA obtained from all four isolates samples were quality checked using Tape Station instrument (Agilent Technologies, Santa Clara, CA, USA) and Qubit BR dsDNA. WGS libraries for all samples were prepared with KAPA HyperPrep PCR-free kit protocol (Roche, Basel, Switzerland), starting from 500 ng of genomic DNA that was mechanically sheared using Covaris instrument. A final Size Selection was added to obtain longer insert size (0.75x using Ampure XP). Libraries were first quantified using the Qubit BR dsDNA assay kit (ThermoFisher, Waltham, Massachusetts, USA) and then by Real-Time PCR against a standard curve with the KAPA Library Quantification Kit (Kapa-Biosystems, Wilmington, MA, USA). Sequencing was conducted on a NovaSeq 6000 Illumina platform (Illumina, San Diego, CA, USA) on 150 paired-end mode generating an average of 2.6 million fragments per sample.

The quality of reads was assessed using FastQC v.0.11.9 (Andrews, 2010). For each of the four samples, raw reads were taxonomically classified with Kraken v.2.1.2 (Wood et al., 2019) to a custom database collecting bacterial genomes derived from National Center for Biotechnology Information (NCBI) repository (ftp://ftp.ncbi.nlm.nih.gov/genomes/, accessed on 15 May 2024) including *Curtobacterium allii* genome (RefSeq GCF_021271025). Such classification generated four reports listing taxonomic assignations. As shown in **Figure 3**, over 98% of each sample raw reads were classified as bacterial. For each sample, reads classified as belonging to *Curtobacterium allii* were extracted with KrakenTools v.1.2 (https://github.com/jenniferlu717/KrakenTools) and then assembled with MaSuRCA v.4.0.9 (Zimin et al., 2013). Subsequently, each assembly was inspected with assembly-stats v.1.01 (https://github.com/sanger-pathogens/assembly-stats). The sequences utilized in the assemblies are available in GenBank in BioProject under ID PRJNA1135786. Moreover, the use Pavian v.1.2.0 (Breitwieser et al., 2020) on Kraken reports allowed a graphical representation of taxonomical classification for each isolate. The results

obtained for the C8-5L isolate are reported in **Figure 4** as example, but the result was the same for all four isolates. The reads that can be attributed specifically map to the *Curtobacterium* genus and, within the genus, the two main species identified are *C. flaccumfaciens* and *C. allii*. Since, as previously stated, *C. allii* is now recognized as a pathovar of *C. flaccumfaciens*, this analysis identified CFA as the most likely classification of the isolates.

3.3. Biological confirmation of the pathogen belonging to *C. flaccumfaciens* pv. *allii*

The molecular characterization suggested a strong hypothesis but did not remove all possible doubts regarding the taxonomic characterization of the isolates. Therefore, a biological assay of pathogenicity on onion was carried out as, according to the original report of CFA pathogenicity (Khanal et al., 2023b). Briefly, five onion bulbs per isolate were inoculated with 500 µL of bacterial suspension in sterile PBS using a syringe. The bulbs were then incubated at 25 °C in the dark for five days, after which they were cut in half with a knife and the presence of rot was visually assessed. In addition to the four bacterial isolates, a healthy control in which only sterile PBS was mock inoculated in the bulbs (negative control) was prepared.

As reported by this study, CFF does not cause symptoms on onion, while CFA was first described as the etiological agent of onion bulb rot.

The results of the inoculation with the isolates from maize on onion (**Figure 5**) show clear rotting symptoms on inoculated bulbs, while the control bulbs that underwent a mock inoculation showed no symptoms. These results, compounded with the evidence from the different molecular characterizations, confirm that the four isolates are CFA.

CFA was suggested as a novel species of *Curtobacterium* first identified in Texas, USA, as the etiological agent of onion bulb rot. Starting from symptomatic plant material, the first genetic analyses carried out by Khanal et al. 2023a, using not only 27F/1492R primers but also 534R for the sequencing of 16S rRNA gene, defined the similarity with CFF. Subsequently, CFA was shown

212 to be different from other *Curtobacterium* strains by physiological and chemotaxonomic analyses
213 carried out by the same authors. CFA is described as being Gram-positive, non-spore-forming, non-
214 motile, obligately aerobic, oxidase negative and catalase positive, forming smooth and cream-
215 yellow colonies on NBYA and YPGA medium with optimal growth at 25°C

216 **4. Fulfilment of Koch's postulates on maize**

217 Isolates were tested for their ability to reproduce field symptoms when inoculated on maize, under
218 experimental conditions, fulfilling Koch's postulates and demonstrating their role as etiological
219 agents of the disease.

220 Based on the information available on CFF, known to be a seedborne pathogen, inoculation of the
221 bacteria was carried out on seeds. Maize seeds (B73 inbred line) were surface sterilized using a
222 sterilizing solution (3% sodium hypochlorite, 20% ethanol) for 3 minutes and then washed three
223 times in sterile distilled water to remove the sterilizing agents. Aliquots of the last washing bath (1
224 mL) were plated on plate-count agar (PCA) and incubated at 22 °C for 7 days to check the absence
225 of microbial growth and therefore the effectiveness of the superficial sterilization.

226 After sterilization, the seeds were placed in a bacterial suspension in sterile PBS (10^6 CFU/mL) and
227 left to soak either for 65 minutes in normal conditions (Inf-S), or under vacuum conditions for 5
228 minutes to facilitate the entrance of the bacteria inside the seed and then other 60 minutes without
229 vacuum (Inf-V). The healthy control plants were subjected to the same treatments but using only
230 sterile PBS, without added bacteria.

231 After the treatment, all seeds were left to soak in tap water overnight and then sowed in commercial
232 potting soil. A total of 45 seeds were prepared: 15 mock inoculated (8 without vacuum, 7 with
233 vacuum), 15 Inf-S, and 15 Inf-V. In the results, the mock inoculated plants are reported as a single
234 "Mock" treatment, since there were no differences between the presence of vacuum or not. This
235 experiment was repeated four times, using a different colony for infection each time.

The plants were monitored for 14 days after emergence of the coleoptile, when early symptoms that matched those seen in field could be detected. For each isolate and condition the germination percentage and incidence of the disease were registered.

At 14 days post emergence, plants that emerged from seeds that were either soaked in the bacterial suspension (Inf-S) or soaked in the bacterial suspension and subjected to vacuum conditions (Inf-V) showed the early symptoms of the disease, developing the chlorotic streaks on the leaves (**Figure 6a**). Only in a minority of the Inf-V plants, also the severe symptoms with greatly reduced growth (**Figure 6b**) and wilting (**Figure 6c**) were registered. No such symptoms were seen on any of the non-infected control plants (**Figure 6d**).

The mock inoculation with PBS and Inf-S plants showed 100% germination, while the Inf-V plants showed a reduced germination percentage, around 70-75%. This lack of germination might be a symptom of the infection and not caused by the vacuum, since it was not registered in the mock-inoculated plants that underwent the 5 minutes of vacuum (**Table 1**).

While both inoculation methods were effective, the application of vacuum facilitates the infection process, raising symptom incidence from 17% to 79%. Still, as vacuum is not a realistic infection condition in nature, it is reasonable to conclude that the real ability of the pathogen to infect maize seeds is closer to that shown in the Inf-S condition than in the Inf-V one. Regardless, further investigations in natural conditions will be necessary to ascertain the real ability of the pathogen to infect maize.

Table 1 Table reporting the germination percentage and symptom incidence in the maize bioassay

	Mock		Inf-S		Inf-V	
	Germination	Symptom Incidence	Germination	Symptom Incidence	Germination	Symptom Incidence
C8-5L	100 %	0 %	100 %	25 %	75 %	80 %
C8-6L	100 %	0 %	100 %	7 %	71 %	82 %
C8-JL	100 %	0 %	100 %	14 %	71 %	75 %
C8-KL	100 %	0 %	100 %	23 %	73 %	79 %

Average	100 %	0 %	100 %	17 %	73 %	79 %

From symptomatic plants, stalks were collected and processed to isolate the bacteria as previously described. Nucleic acids were extracted from isolates, used for 16S amplification and sequencing, allowing to determine that the isolated colonies are indeed indistinguishable from the initial inoculum and thus fulfilling all of Koch's postulates.

5. *C. flaccumfaciens* pv. *alli*: a new threat for maize?

Following the identification of a non-specific symptomatology on maize plants in Northern Italy (Lombardy region), the Phytosanitary Service implemented all necessary actions to determine the cause and exclude the presence of quarantine pests. Once the presence of quarantine pests had been excluded, the material was subjected to all tests necessary to identify the cause of the symptoms observed and, therefore, the etiological agent. The exclusion of CFF as a potential pathogen was particularly complicated since the obtained results were seemingly contradictory: the 16S sequencing suggested CFF as a pathogen, but the specific PCR assay gave negative results. Even after the report of the new pathogen, CFA, the different molecular biology approaches yielded no definitive answer, and biological pathogenicity assays were necessary to offer conclusive demonstration of the hypothesis of this taxonomical attribution.

Based on this study, it's possible to affirm that CFA is the etiological agent of the symptoms observed. This not only is the first report of this bacterium outside the country where it was first discovered, but it is also the first time in association with maize plants.

In general, considering that little information is available on this pathogen both in its first reported host (onion) and that the outbreak described in this study is, to the best of our knowledge, the only case on maize thus far there are several points that remain obscure regarding the pathogen and its biology. It is currently unknown: how is CFA transmitted in nature; whether a vector is involved or

not in its epidemiology; which are the actual environmental conditions that can allow its life cycle and therefore in which areas it can be present; if it has other secondary hosts might be infected.

The difficulties encountered in the identification of this pathogen, requiring the use of several tests, indicate the need for the development of a suitable diagnostic method. This would allow differentiation from CFF and therefore an easier detection procedure for CFA.

Due to its isolated occurrence and the limited amount of information available, it has not been possible to classify it as a regulated pest. Therefore, no phytosanitary measures had to be adopted in this case. If other symptoms are detected on plants, and should new outbreaks be identified, they will make it possible to better understand the biology of the pathogen and the real threat it poses.

Author's Contributions

Conceptualization: Paola Casati, Piero Attilio Bianco, Massimo Delledonne; **Methodology**: Alessandro Passera, Massimo Delledonne, Francesca Gaffuri; **Data curation**: Irene Ferraris, Luca Angheben; **Investigation**: Alessandro Passera, Alessia Follador, Irene Ferraris, Alessia Travaglino, Francesca Gaffuri; **Formal Analysis**: Alessandro Passera, Irene Ferraris, Antonina Rita Limongi; **Writing – Original draft preparation**: Alessandro Passera; **Writing – review and editing**: all authors; **Funding acquisition**: Piero Attilio Bianco, Massimo Delledonne; **Resources:** Beniamino Cavagna, Massimo Delledonne; **Supervision:** Paola Casati. All authors read and approved the final manuscript.

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Data Availability

All data produced in this study is available in the manuscript or at the Accession Numbers provided for the GenBank database

Conflict of Interest The authors declare that they have no conflict of interest.

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436 **Figure Captions**

437 **Fig. 1 Symptoms seen on maize plants.** Pictures showing the symptoms seen on the maize plants:
438 a) chlorotic stripes, b) reduced growth of plants and chlorotic leaves, c) chlorotic stripes becoming
439 necrotic; d) stunted growth and wilting

440

441 **Fig. 2 Taxonomical classification of the isolates based on 16S.** Phylogenetic tree obtained with
442 Neighbor-Joining Algorithm showing the taxonomical position of the four isolates compared to
443 other *Curtobacterium* isolates based on the sequence of 16S gene. Sequences from this study are
444 indicated only as the code of the isolate (C8-5L, C8-6L, C8-JL, C8-KL), while sequences from
445 GenBank are reported as species name followed by the accession number of the sequence

446

447

448 **Fig. 3 Sequencing reads classification.** Image representing general classification in all samples

449

450 **Fig. 4 Taxonomical classification of isolates based on sequencing reads.** Graph reporting the
451 output obtained from processing the Kraken2 reports for isolate C8-5L through Pavian v.1.2.0

452

453 **Fig. 5 Symptoms on onion.** Pictures showing the results of the pathogenicity bioassay on onion
454 bulbs. a) healthy control bulb, mock inoculated with sterile PBS; b) infected bulb inoculated with
455 CFA isolates, showing rot symptoms

456

457 **Fig. 6 Symptoms on inoculated maize plants.** Pictures showing the results of the pathogenicity
458 bioassay on maize: symptoms developed after inoculation with CFA isolates a) chlorotic stripes, b)
459 reduced growth of plants, c) wilting; d) healthy control plants, mock inoculated with sterile PBS,
460 with or without vacuum

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