copLAB gene prevalence and diversity among Trinidadian *Xanthomonas* spp. black-rot lesion isolates with variable copper resistance profiles

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Background: There has been limited exploration of *copLAB* genotypes and associated copper resistance phenotypes in *Xanthomonas* sp. in the southern Caribbean region. An earlier study highlighted a variant *copLAB* gene cluster found in one Trinidadian *Xanthomonas campestris* pv. *campestris* (Xcc) strain (BrA1), with <90% similarity to previously reported *Xanthomonas copLAB* genes. With only one report describing this copper resistance genotype, the current study investigated the distribution of the BrA1 variant *copLAB* gene cluster and previously reported forms of copper resistance genes in local *Xanthomonas* sp.

Methods: *Xanthomonas* sp. were isolated from black-rot infected lesions on leaf tissue from crucifer crops at intensively farmed sites with high agrochemical usage in Trinidad. The identity of morphologically identified isolates were confirmed using a paired primer PCR based screen and 16s rRNA partial gene sequencing. MGY agar amended with $CuSO_4.5H_2O$ up to 2.4mM was used to establish MIC's for confirmed isolates and group strains as sensitive, tolerant, or resistant to copper. Separate primer pairs targeting the BrA1 variant *copLAB* genes and those predicted to target multiple homologs found in *Xanthomonas* and *Stenotrophomonas* sp. were used to screen copper resistant isolates. Select amplicons were sanger sequenced and evolutionary relationships inferred from global reference sequences using a ML approach.

Results: Only four copper sensitive/tolerant *Xanthomonas* sp. strains were isolated, with 35 others classed as copper-resistant from a total population of 45 isolates. PCR detection of *copLAB* genes revealed 2 PCR negative copper-resistant resistant strains. Variant *copLAB* genes were only found in Xcc from the original source location of the BrA1 strain, Aranguez. Other copper-resistant strains contained other *copLAB* homologs that clustered into three distinct clades. These groups were more similar to genes from *X. perforans* plasmids and *Stenotrophomonas* sp. chromosomal homologs than reference Xcc sequences. This study highlights the localisation of the BrA1 variant *copLAB* genes to one agricultural community and the presence of 3 distinct *copLAB* gene groupings in Xcc and related *Xanthomonas* spp. with defined $CuSO_4.5H_2O$ MIC. Further characterisation of these gene groups and copper resistance gene exchange dynamics on and within leaf tissue between Xcc and other *Xanthomonas* species are needed as similar gene clusters showed variable copper sensitivity profiles. This work will serve as a baseline for copper resistance gene characterisation in Trinidad and the wider Caribbean region and can be used to boost already lacking resistant phytopathogen management in the region.

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Abstract

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Methods.

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Results.

Only four copper sensitive/tolerant *Xanthomonas* sp. strains were isolated, with 35 others classed as copper-resistant from a total population of 45 isolates. PCR detection of *copLAB* genes revealed 2 PCR negative copper-resistant resistant strains. Variant *copLAB* genes were only found in Xcc from the original source location of the BrA1 strain, Aranguez. Other copper-resistant strains contained other *copLAB* homologs that clustered into three distinct clades. These groups were more similar to genes from *X. perforans* plasmids and *Stenotrophomonas* sp.

chromosomal homologs than reference Xcc sequences. This study highlights the localisation of the BrA1 variant *copLAB* genes to one agricultural community and the presence of 3 distinct *copLAB* gene groupings in Xcc and related *Xanthomonas* spp. with defined $CuSO_4.5H_2O$ MIC. Further characterisation of these gene groups and copper resistance gene exchange dynamics on and within leaf tissue between Xcc and other *Xanthomonas* species are needed as similar gene clusters showed variable copper sensitivity profiles. This work will serve as a baseline for copper resistance gene characterisation in Trinidad and the wider Caribbean region and can be used to boost already lacking resistant phytopathogen management in the region.

Keywords: *Xanthomonas campestris* pv. *campestris*, *Xanthomonas* sp., copper resistance, *copLAB*.

Introduction

Copper salts have been commonly used in chemical-based management schemes for bacterial and fungal diseases in agriculture for more than a century (Ayres 2004; Lamichhane et al. 2018). Their consistent use was quickly followed by reports of reducing disease management efficacies from as early as the 1980s. This was shortly followed by the discovery of copper-resistant phytopathogens (Adaskaveg and Hine 1985; Cooksey 1987; Sundin and Bender 1993; Goto et al. 1994). Among these were *Xanthomonas* spp. (Xsp) that form a major group of bacterial phytopathogens that affect multiple vegetable crops worldwide and are now known to have a high occurrence of copper resistance (Lamichhane et al. 2018). *Xanthomonas campestris* pv. *campestris* (Xcc), the causal agent of black-rot in crucifers (Vicente and Holub 2013), is of particular concern due to the lack of up-to-date studies concerning this species in the Caribbean region. This pathogen causes significant yield loss in cruciferous vegetables in Trinidad and the southern Caribbean. Disease management in this country still depends heavily on chemical usage with little or no rotation of diverse chemicals. Continued application of chemical formulations with copper salts has been met with reduced disease management outcomes in Trinidad. Furthermore, the mono-cropping of solanaceous and cruciferous crops has

83 exacerbated chemical exposure and pathogen enrichment in these farming districts (Ali,
84 Ramsubhag, and Jayaraman 2021). These agricultural practices are not unique to Trinidad and
85 have contributed to the dire consequence of the global occurrence of copper-resistant
86 *Xanthomonas* spp. across six continents (Lamichhane et al. 2018).

87 Copper resistance phenotypes in *Xanthomonas* spp. are attributed to the presence of a
88 plasmid-borne *cop* operon and survival at >0.8 mM $CuSo_4.5H_2O$ (Marin et al. 2019). These
89 plasmid-borne operons and homologs have been well described in numerous publications since
90 the 1990s (Lim and Cooksey 1993; Voloudakis, Bender, and Cooksey 1993; Behlau et al. 2011).
91 Several studies have shown the presence of homologs of these *cop* genes and their extensive
92 horizontal transfer among bacterial phytopathogens (Behlau et al. 2012; Behlau et al. 2013).
93 Copper-resistant *Xanthomonas* spp. and closely related *Stenotrophomonas* spp., are known to
94 share ~90% nucleotide similarity among the *copLAB* genes implying horizontal transfer within
95 these genera (Teixeira et al. 2008; Behlau et al. 2012). While *cop* operon gene content is not
96 consistent in all plasmids, the *copLAB* genes are common and account for the major copper
97 resistance phenotype (Behlau et al. 2011). The *copL* gene functions as a transcriptional activator
98 of downstream *cop* gene expression in the presence of elevated intracellular copper ions (Pontel
99 and Soncini 2009; De Freitas et al. 2019). The *copA* gene encodes a multicopper oxidase and
100 *copB* is predicted to bind Cu ions but may be involved in removal from the periplasm (Teixeira
101 et al. 2008; Behlau et al. 2013). While the relationship between diverse *copLAB* genes and
102 copper resistance levels is yet to be fully explained, understanding the changes in *copLAB* gene
103 diversity within *Xanthomonas* spp. and related species in relation to disease management
104 practices are of paramount importance.

105 In Trinidad, copper-resistant Xcc was first reported by Lugo et al. (2013), who noted a
106 positive correlation between resistant isolates and monoculture of cruciferous vegetables at
107 agricultural sites. One of the copper-resistant Xcc isolates (BrA1) from the farming district of
108 Aranguez contained a plasmid-borne *copLAB* gene cluster with very low nucleotide homology to
109 published Xcc *copLAB* genes (Behlau et al. 2017). The report by Behlau et al. (2017) also
110 highlighted the inability of current PCR amplification and nucleic acid hybridization-based
111 diagnostic strategies to detect this "variant" form of copper resistance. The prevalence of the
112 BrA1 variant *copLAB* gene cluster in Xcc populations on the island has not been established, nor

are there detailed reports on the diversity of copper resistance genes among strains of this pathogen as a whole. The genomes of some local copper resistance Xcc and other Xsp were reported in (Ramnarine, Jayaraman, and Ramsubhag 2022), which revealed copper responsive elements further characterised in ongoing works (Ramnarine, Jayaraman, and Ramsubhag, forthcoming). The current study aimed at determining the presence of the BrA1 variant *copLAB* genes identified from local draft genomes and, attempt to capture other forms using primers designed against Xsp and *Stenotrophomonas* spp. homologs. Xcc and other Xsp were obtained from black-rot infected leaf tissue sampled at crucifer farms in four farming districts in Trinidad, inclusive of Aranguez where Xcc BrA1 was first isolated. Crop cultivation at these locations has been almost consistent for >30 years. PCR-based detection and sequence analysis coupled with evolutionary analysis was employed to determine the *copLAB* genetic diversity in these bacterial isolates. The *copLAB* genes characterized by Behlau et al. (2017) are distinguished in this study with the label "Variant" and are compared to previously reported genes contained in the NCBI database from *Stenotrophomonas* spp. and *Xanthomonas* spp.

Methods

Agricultural sites involved in the study with varying intensities of cultivation.

Leaf samples of cabbage and cauliflower showing symptoms of black-rot infection were collected from two fields at each location indicated in Figure 1. The farming districts of Maloney and Aranguez have >30-year history of agricultural land use, whereas Navet and, Bon Air have a shorter history (<15 years). Each agricultural district has been associated with crucifer cultivation, with a greater incidence of monoculture in the Navet district.

Sample collection and bacterial isolation.

Leaf samples were stored at 4 °C, and bacteria were isolated within 24 hours according to Lugo et al. (2013). Isolates similar to or with matching morphologies to *Xanthomonas* (yellow, convex shaped colonies) were selected after 48hrs incubation on nutrient agar (NA). These isolates were labelled as the lesion-associated population. Further investigation into the distribution of copper-resistant bacteria in the Aranguez district was carried out in 2017 to determine the distribution of variant BrA1 *copLAB* genes between bacteria at an active and abandoned crucifer field. Phylloplane-associated and soil-borne bacteria were isolated from plant material and soil respectively from an active cauliflower field and abandoned plot approximately 300m away. Isolates were obtained by plating dilution washes on NA. Morphologically unique colonies were selected after 48hrs and labelled as the environmental reference population. In total 45 lesion-associated, and 199 environmental population isolates were retained and stored at -80 °C in 25% glycerol/Nutrient Broth.

Taxonomic assignment of bacterial isolates in the lesion-associated and environmental reference population.

Lesion-associated isolates displaying similar morphologies to *Xanthomonas* were identified as XCC using primers (XCF-CGATTCGGCCATGAATGACT, XCR-CTGTTGATGGTGGTCTGCAA) targeting a highly conserved region of the Xcc *hrpF* gene (Park et al. 2004) or as a member of the *Xanthomonas* genus (Xsp) using primers targeting the *hrpB6* gene (RST2-AGGCCCTGGAAGGTGCCCTGGA, RST3-ATCGCACTGCGTACCGCGCGCGA) (Leite et al. 1994), or using RT 16 F/R primers targeting the *gumL* gene (Pandey et al. 2016). Isolates with expected amplicons for XCF/R and RT 16 F/R primers were labelled as Xcc while those with RST2/3 and or RT 16 F/R bands were labelled as Xsp. Strains without amplification with any primer were not used in further analysis. Classification using this scheme was corroborated using partial 16s rRNA sequencing (Macrogen, Korea), which was also applied to environmental isolates. Sequences with QV >16 were selected and aligned using blast (blastn, https://blast.ncbi.nlm.nih.gov/) against the NCBI 16s rRNA database. Only copper-resistant environmental isolates were identified using 16s rRNA sequencing. While species-level identification was obtained for most isolates (Coverage

>90% and %ID >98%), only identified genera were considered for consistency across the environmental dataset. WGS characterised local Xcc and *Xanthomonas melonis* (Xmel) isolates (Ramnarine, Jayaraman, and Ramsubhag 2022) were included as controls. Partial 16s rRNA Sanger-generated sequences can be accessed at https://doi.org/10.5281/zenodo.6795859.

Copper sensitivity profiling.

Copper sensitivity screening was carried out according to Ramnarine, Jayaraman and Ramsubhag (2022) using $CuSO_4.5H_2O$ concentrations ranging from 0 – 2.4mM amended into Mannitol Glutamate Yeast Agar. Briefly, the copper sensitivity of isolates was categorized based on the MIC of $CuSo_4.5H_2O$ of isolates after 48hrs incubation on amended media at 28^0C. Copper-sensitive isolates only grew in <0.6mM $CuSo_4.5H_2O$, copper tolerant strains survived concentrations between 0.6 – 0.8mM while copper resistant isolates survived >0.8 mM. Amended media was prepared using a filter sterilized $CuSo_4.5H_2O$ stock solution both prepared fresh on the day of use. 48hr cultures of bacterial isolates were suspended in sterile water and 5 µL spot plate onto solid amended agar in triplicate.

***copLAB* gene detection in lesion-associated and environmental isolates.**

Primers were designed for the BrA1 variant *copLAB* gene sequences (Behlau et al. 2017) using reference homologs (similarity >= 60%) from the *Xanthomonas* and *Stenotrophomonas* genera available from GenBank. These were aligned using ClustalW in BioEdit v7.0.5.3 (Hall 1999). Variant-specific primers targeting unaligned regions unique to the BrA1 variants were manually designed and screened for specificity using PrimerBlast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Those predicted to amplify variant genes unambiguously were further screened using gDNA of Xcc strain BrA1, and the amplicons were Sanger sequenced (Macrogen, Korea) before cross-referencing to the draft BrA1 genome contig containing the variant genes (GCA_002806765.1). Primers targeting aligned regions from reference homologs (similarity >= 60%) from the *Xanthomonas* and *Stenotrophomonas* genera

199 available from GenBank were designed to target previously reported sequences. Primer pairs
200 were screened for specificity using PrimerBlast (https://www.ncbi.nlm.nih.gov/tools/primer-
201 blast/). These primers were in-silico verified to amplify those from plasmids, characterised
202 genes from copper resistant Xsp and those from *Stenotrophomonas* chromosomes with identical
203 synteny to *copLABMGF* copper resistant operons. All primers were synthesized at IDT,
204 sequences and amplicon length are listed in Table 1.

205 Total genomic DNA was isolated from all lesion-associated and copper-resistant
206 environmental isolates according to Wilson (2001) and quality was assessed using standard gel
207 electrophoresis methods (1% agarose). PCR amplification using designed primers (Table 1) and
208 molecular reagents from Bioland Scientific were carried out in 25 µL reactions as outlined in
209 Ramnarine, Jayaraman and Ramsubhag (2022).

210

211 **_copLAB_ gene sequencing and evolutionary analysis of variants.**

212

213 Amplicons generated from primers targeting both variant and previously reported genes
214 were gel purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and,
215 sequenced on an ABI3730XL platform (Macrogen, Korea). An overall QV score threshold of
216 <16 was set for sequence rejection and end trimming. Pairwise blast using the blastn and tblastn
217 (https://blast.ncbi.nlm.nih.gov/) algorithms were used to verify the homology with annotated
218 *copLAB* reference sequences against the entire NCBI GenBank database. Final curated
219 sequences, GenBank references, and full gene sequences from the Xcc BrA1 and other local Xsp
220 genomes (Supplemental Table 1) were submitted to ngphylogeny (https://ngphylogeny.fr/) for
221 evolutionary analysis. Using this server, multiple alignment and curation were carried out using
222 MAFFT (Katoh and Standley 2013) and BMGE (Criscuolo and Gribaldo 2010) respectively.
223 Alignments were then submitted to FastTree (Price, Dehal, and Arkin 2010) for maximum
224 likelihood phylogenetic reconstruction with 1000 bootstrap branch support (Felsenstein 1985).
225 The bootstrap consensus tree for each dataset was visualized and edited in TreeGraph 2 (Stöver
226 and Müller 2010). Alignments of *copLAB* sequences were also analysed in MEGA 11
227 (https://www.megasoftware.net/) to identify variable sites. Sanger-generated sequences for
228 amplified *copLAB* genes can be accessed at https://doi.org/10.5281/zenodo.6795859.

Statistical Inference.

Statistical analysis was carried out on lesion associated and environmental isolate datasets using RStudio (2023.03.0+386) and R version 4.2.3. Statistical tests included Anova, TukeyHSD post-hoc test and Pearson's Chi-squared Test from the R stats package (v4.2.3).

Results

copLAB gene prevalence in copper-resistant lesion-associated *Xanthomonas* spp.

The 45 lesion-associated isolates were distributed by agricultural district as follows: Aranguez = 12, Maloney = 5, Navet = 24, Bon Air = 4. These are represented in Table 2 which includes species-level identification, copper sensitivity profiles and max CuSo4.5H2O MIC. Most isolates from this population were copper resistant (78%) but only 31% were Xcc. There were <7% tolerant and only one sensitive isolate obtained but profiles of 5 isolates could not be determined. Most (80%) of the copper-resistant isolates grew in the presence of 2.4 mM $CuSO_4.5H_2O$ with the lowest MIC for this category being 1.2mM. Among the *Xanthomonas* species there were significant differences in CuSo4.5H2O MIC means ($P = 0.0176$) but only between Xsp and Xcc ($P = 0.0134$). However, no significant difference was observed between MIC's, *cop* genotype and sample location.

Figure 2 only represents copper resistant isolates (35) which survived four $CuSo_4.5H_2O$ concentrations represented in Table 2, 1.2 mM (2), 1.6 mM (4), 2 mM (1) and 2.4 mM (28). Only isolates from Aranguez (11) contained all three variant BrA1 *copLAB* genes (Variant, Figure 2). Previously reported *copLAB* genes (Traditional, Figure 2) were found in Xsp, Xmel and Xcc strains from Maloney, Bon Air and Navet. The BrA1 Variant *copLAB* primers were able

to amplify individual *copLAB* genes in WGS characterised local Xcc. Primers targeting Xanthomonad *copLAB* genes amplified those in characterised Xmel strains described in (Ramnarine, Jayaraman, and Ramsubhag 2022). No significant difference in MIC's were observed between variant and previously reported *cop* genotypes. However, there was a significant difference in *cop* genotypes (P = <2e-16) by location, specifically when comparing Aranguez to the other three districts.

Two Xcc and one Xsp from Navet and, two Xsp from Maloney were characterised as copper resistant, but PCR amplification was not able to detect any *copLAB* gene. Only one sensitive (Xcc CaNP1C) and three tolerant (Xcc Ar1PC2 and CNP4A and Xmel CaNP1D) strains were isolated and were all *copLAB* PCR negative. All 45 isolates and associated isolation location and PCR prevalence data are given in Supplemental Table 2. In brief, most of the 45 *Xanthomonas* isolates were copper resistant (80%) up to 2.4 mM $CuSo_4.5H_2O$. From these isolates, only Xcc strains from the Aranguez district contained the BrA1 variant *copLAB* gene cluster. Previously reported *copLAB* genes were also identified in copper resistant isolates from other agricultural districts using the primers designed in this study. However, four copper resistant strains, including Xcc, were PCR negative for *copLAB* genes. Significant differences in CuSo4.5H2O MIC were observed between Xcc and Xsp strains and with the BrA1 variant *cop* genotype in Aranguez compared to the other districts.

BrA1 variant *copLAB* gene prevalence among copper resistant environmental phylloplane and soil associated bacteria from the Aranguez district.

Differences in the proportions of isolates characterised as copper sensitive, tolerant and resistant in the environmental population are summarised in Figure 3. Full isolate profiles are given in Supplemental Table 3. Greater proportions of resistant isolates were obtained from the phylloplane and soil of the cultivated field, with the highest proportions from the latter environment (57%) (Figure 3 B). While copper-resistant isolates were found in all environments and higher proportions were observed among soil isolates (Figure 3 B). Copper tolerant and sensitive isolate proportions were higher from the phylloplane and soil of the abandoned plot

respectively (20 vs 48% and 21 vs 49%). Overall, of the 199 isolates screened, 78 were copper sensitive, 46 tolerant and 75 were resistant. Pearson's Chi-squared test showed a significant association between copper sensitivity and, the sampling environment and location ($P = 0.0006$). Using Pearson residuals, a positive correlation was observed between copper tolerant bacterial strains and the phylloplane environment of the abandoned field. This was also seen with copper resistant strains and the soil environment of the cultivated field. In summary, there were statistically significant differences in copper sensitive isolates from the phylloplane and soil of a cultivated and abandoned field. From this there were greater positive correlations with copper resistant isolates and the cultivated field.

The 75 copper-resistant isolates from the environmental population were screened for the variant *copLAB* genes and identified using 16s rRNA gene sequencing (Table 3). Interestingly, no copper-resistant Xsp were identified within this sub-group of environmental isolates. Notable is the higher diversity in soil and phylloplane from the cultivated field and the presence of *Stenotrophomonas* spp. isolates (Table 3, *) in all locations except the phylloplane of the abandoned field. More isolates from this genus were obtained from the cultivated field which also had a greater number of *Pseudomonas* spp., *Achromobacter* spp. and unidentified isolates. However, no copper-resistant isolate in the environmental population contained the BrA1 variant *copLAB* gene.

Evolutionary analysis of *copLAB* genotypes

Sequences from local *X.* sp. isolates were similar (>96%) to previously reported *cop* sequences from *Xanthomonas* plasmids or *Stenotrophomonas* spp. chromosomes. Furthermore, these sequences were not clustered with *coh* chromosomal homologs further establishing confidence in their designation as *cop* sequences. In line with established similarity patterns in global strains, *copA* (Figure 4 B) and *copB* (Figure 4 C) genes from local isolates appear to be more closely related than *copL* (Figure 4 A). In each phylogeny, three distinct groupings of PCR amplified *copLAB* genes from local isolates were observed. Group 1 and 3 *copL* sequences, were most distantly placed when compared to all other sequences. Both groups consisted of genes

~93.5% similar to each other. Isolates in these groups were all from Navet. Group 1 strains had a CuSo4.5H2O MIC of 400ppm with Group 3 being 600ppm. Notably, these identical Group 1 sequences were obtained from two *Xanthomonas* species (Xcc and Xsp). When compared to *X. eu* pLMG930.1, four variable sites in Group 1 *copL* sequences from both Xcc and Xsp were identified. Group 3 sequences were identical for the aligned region but did not cluster with a previously reported sequence. However, when compared to Group 1, there were 22 variable sites in the aligned sequences in Group 3. Interestingly, in the BrA1 variant Group 2, four variable sites were detected when compared to the BrA1 draft genome *copL* sequence. However, these variations were only present in Xcc Ca3B and Cf6A1. Overall, the *copL* sequences in all 3 groups contained 147 variable sites.

The *copA* sequences formed 2 distinct groups, with Group 1 consisting of the BrA1 variant genes. While Group 2 comprised Xsp and Xcc isolates from Navet, isolates in both groups had a CuSo4.5H2O MIC of 600ppm. The Xcc CaNP6B sequence in this group almost entirely (99%) aligned with 99.8% similarity to multiple *copA* homologs in *Stenotrophomonas* spp. and *Xanthomonas* spp. plasmids. The Xsp sequences in Group 2 were identical to a partial *copA* CDS from *X. perforans* (MK616431) while local Xcc variant sequences clustered with the BrA1 variant and a *S.* sp. *copA* gene. These variant sequences in Group 1 did not show the same trend as *copL* (Figure 4 A Group 2) as all sequences were identical. Furthermore, when compared to other sequences in Group 2, Xcc CaNP6B shared 15 variable sites with the Xsp sequences in this group (which were all identical). Overall, both groups shared 153 variable sites.

Previously reported *copB* sequences clustered into two closely related groups, 2 and 3 (Figure 4 C). Group 2 contained a single sequence from Xcc CaNP6B which was similar to sequences from the local Xmel CaNP6A genome but identical to multiple *Stenotrophomonas* spp. homologs (Similar to Figure 4 B Group 2). Notably, the *copL* and *copA* genes from this Xcc strain did not show this clustering pattern. Group 3 sequences clustered with the well-referenced *copL* sequence from *X. euvesicatoria citrumelonis* (HM579937) showing 99.5% similarity at 99% coverage. Isolates from all groups had a CuSo4.5H2O MIC of 600ppm. In Group 1, the BrA1 variant sequences were all identical (as seen in Figure 4 B Group 1). Despite their separate group designation, the closely placed Xcc sequence and Xsp sequences in Groups 2 and 3 (Figure 4 C) were compared. Xcc CaNP6B *copB* shared 46 variable sites with Group 3

sequences. Within Group 3, however, two groupings based on 4 variable sites were observed. These consisted of PNP25 and PNP34 and, PNP54 and PNP62. Overall, the sequences from all three groups shared 210 variable sites. As noted with the previous Xcc BrA1 study, variant sequences from local strains clustered separately from others. These variant sequences also clustered with and were identical to *cop* homologs from *Stenotrophomonas* sp. WZN-1 (CP021768.1). Variant *copLAB* sequences are represented in Group 2 Figure 4 A and Group 1 Figure 4 B and 4 C respectively in blue boxes.

Discussion

This study demonstrated that 70% of Xcc strains and 78% of the overall *Xanthomonas* population had copper resistance up to a high MIC of 600ppm. Drastically, only 1 Xcc strain was copper sensitive. Furthermore, three *cop* genotypes were identified and associated with different copper resistant MIC. One previous study established copper sensitivity profiles of Xcc strains isolated between 1999 and 2003 (Lugo et al. 2013). Copper sensitive and resistant (59% of isolates) strains were obtained in that study. This trend indicates that copper resistance and tolerance levels in Xcc have increased since 2003, in line with expectations given that no drastic change in copper-based chemical usage guidelines have taken place since. In Trinidad, the agricultural use of chemicals is not strictly regulated beyond the point of sale. Application rates and concentration depend heavily on the discretion of farmers at the field level. In one case, the indiscriminate and monotonous use of copper-based fungicides was previously reported at most of the farming sites in Trinidad (Ali, Ramsubhag, and Jayaraman 2021). Lugo et al. (2013) noted the correlation of higher proportions of copper resistant Xcc with long-term use of copper sprays in Trinidad. With some overlap in sample locations, this study further evaluated *Xanthomonas* isolates from Aranguez, Maloney, Navet and Bon Air to determine *copLAB* genotypes. Copper resistance has been linked to plasmid-borne *copLAB* genes in *Xanthomonas* spp. in numerous studies (Pontel and Soncini 2009; Behlau et al. 2013; Bondarczuk and Piotrowska-Seget 2013). Thus, all copper resistant *Xanthomonas* isolates in this study were expected to contain plasmid-borne *copLAB* homologs.

Sequencing partial *copLAB* genes of the BrA1 variant and previously reported forms in local *Xanthomonas* strains illustrated separate groupings in the deduced phylogenies. The BrA1 variant genes from local Xcc strains clustered separately from other previously reported sequences except for one *Stenotrophomonas* chromosomal homolog (CP021768.1). Apart from the latter observation in this study, this clustering pattern was noted by Behlau et al (2017). Overall, the closest related reference clades to local sequences originated from *Xanthomonas* spp. plasmids and *Stenotrophomonas* spp. chromosomes, but notably, did not include Xcc. These Xcc reference sequences were most distantly placed in all phylogenies. This possibly indicates an origin of *copLAB* genotypes from other Xsp and *S.* sp. within the local Xcc ecosphere. Horizontal transfer and the homology of *copLAB* genes between *Xanthomonas* spp. and *Stenotrophomonas* spp. have been documented in other studies (Behlau et al. 2013; Lai, Lin, et al. 2021). Notably, diverse *copLAB* genotypes within Xcc species are not well-reported and often rely on homology-based searches in reference to established forms from other species.

Interestingly, all copper resistant Xcc from Aranguez contained the BrA1 variant. This genotype appears to be strongly associated only with this species and only at this location. Of note is the 100% identity to a global *S.* sp. chromosomal operon but the absence of this variant in any copper resistant *Stenotrophomonas* strains from environmental isolations. Also remarkable is the apparent lack of drift into other agricultural districts. However, this will need to be reassessed with a newer and more extensive sampling population. The gene neighbourhood of the BrA1 variant *copLAB* operon and the identical reference both contain mobile elements (Ramnarine, Jayaraj and Ramsubhag, forthcoming), and its localization on a plasmid further adds evidence of interaction with another bacterial species and mobile elements. That is, the movement of this variant into Xcc appears to be indirect.

Two major groups of *copA* and *B* sequences were observed in local *Xanthomonas* and Xcc strains. With *copL* sequences, three groups were observed. Furthermore, there are also at least 2 unique *copLAB* clusters within local copper resistant Xcc. Two strains from Navet did not produce amplicons and thus potentially contain a novel variant. Another strain from Navet contained diverse *copL* and *A* genes with partial operon homology to more than one reference sequence. The latter case implies some measure of recombination which was not fully assessed due to the length of sequences obtained. Studies have noted that environmental exposure to

copper chemicals can impact bacterial diversity and the persistence of copper resistance genes in strains (Araújo et al. 2012). One study noted that the continuous selection pressure of copper ions was linked to the presence of *cop* genes in environmental *Xanthomonas* sp. (Roach et al. 2020)□. Copper resistance is a complex trait in different bacterial strains and the persistence of resistance genes can be attributed to horizontal transfer of plasmid-borne copper resistance elements (Altimira et al. 2012) or enrichment of existing stress response factors within strains (Teelucksingh, Thompson, and Cox 2020).

While it is largely unknown what advantages these variant genes may have in the local *Xanthomonas* population, it can be presumed that the persistence of certain *copLAB* genotypes provides advantages over others within this species. This was observed as most genotype groups survived CuSo4.5H2O MIC up to 600ppm except for one group of Xcc and Xsp which survived up to 400 ppm. The impact of *cop* genotype on these MIC values can also be investigated via variable sites in the *copA* and *B* genes. The *copL* gene is not predicted to be protein coding and its exact function in regulation is largely unknown but *copA* and *B* are copper binding proteins. The amplified sequences used in this study covered copper binding motifs from both genes (Ramnarine, Jayaraj and Ramsubhag, forthcoming). It is thus assumed that these variable sites may play a role in MIC differences due to changes in copper binding capacity. One study evaluated mutations in the *copA* gene binding motifs and demonstrated that nucleotide changes affected overall Xcc strain MIC against copper (Hsiao et al 2011). In the wider environment, consistent application of copper appears to enrich for copper tolerant and resistant bacteria in the soil. Interestingly, the persistence of resistant and tolerant environmental bacteria was even noted in soil and plant material from an abandoned agricultural field. Not only are there *cop* genotypes in *Xanthomonas* strains allowing for survival up to 600ppm CuSo4.5H2O, after agricultural activity has stopped resistant strains persist in the environment. While older studies noted that fields without a consistent use of copper-based chemicals contained copper susceptible *Xanthomonas* (Adaskaveg and Hine 1985, Lugo et al 2013), this relationship appears to not revert once agricultural activity has ceased. Thus, for environments like those sampled in districts like Aranguez, the resistance phenotype is maintained in abandoned fields. Unfortunately, this adds to the complex nature of copper resistance in bacterial populations under the influence of human activity. The persistence of copper resistance further impacts disease and

433 resistance management, proper agrochemical consumption in a largely unregulated space and,
434 future agricultural land use.

435 Anecdotal data from local farmers indicates that black-rot in cabbage usually begins late
436 in the cropping stage and the progression slows with copper sprays but eventually overwhelms
437 the plant despite continuous applications. Understanding the different *cop* genotypes present in
438 local *Xanthomonas* strains is an essential first step in resistance management as at least one
439 group has been associated with a lower CuSo4.5H2O MIC in this study. Furthermore, the
440 potential for novel *cop* genotypes exists at least in one agricultural district. The occurrence of
441 novel variants within specific agricultural areas in Trinidad and the barriers which prevent their
442 spread to other areas and related genera needs to be explored further. This is especially important
443 in the Aranguez and Navet districts in the face of changing land use and far-reaching impacts of
444 climate change.

445

446 **Conclusion**

447

448 This is the first study to report on the distribution of *Xanthomonas* spp. *copLAB* genes
449 both in Xcc and Xsp. isolates from agrochemical contaminated sites in Trinidad. The variant
450 *copLAB* genes first identified in a single local Xcc strain from Aranguez (Behlau et al. 2017)
451 were seen to only occur in Xcc and only those from that same farming district. Two Xcc strains
452 from another district did not yield amplicons for primers designed to target multiple *cop*
453 homologs from Xsp plasmids and *S.* sp chromosomes and another contained genes more similar
454 to other local Xsp genotypes and worldwide plasmid sequences. Phylogenetic clustering for each
455 individual *copLAB* gene indicated at least three potential gene clusters where one is unique in its
456 gene complement homology. The closely related Xsp also serves as a benchmark for local
457 copper resistant *copLAB* gene diversity necessary in future studies on the impact of disease
458 management on resistance, shifts in *Xanthomonas* populations and changes in *copLAB* gene
459 dynamics. Furthermore, all identified *cop* genotypes were associated with a max $CuSo_4.5H_2O$
460 MIC. This updated evaluation on copper sensitivity of Xcc and Xsp from leaf lesions indicated
461 an increase in copper resistant and tolerant strains in agricultural fields from 2003-2017. Lesion-
462 associated strains were seen to survive $CuSo_4.5H_2O$ concentrations up to 2.4mM. The increase is

not surprising given unchanged and non-standardized copper-based chemical usage in Trinidad. More concerning is the persistence of copper-resistant environmental bacteria observed in abandoned field soil, pointing to populations enriched for resistance after chemical applications have ceased. This study serves as an important record of changing copper sensitivities, not only among Xcc but also with lesion-associated Xsp. Furthermore, it is the first attempt at characterising copper resistance *copLAB* genotypes among these isolates and has demonstrated the tight association of a variant cluster to its original isolate's location. Furthermore, despite 100% identity to a gene cluster in a global *S.* sp strain, this cluster was not found in local isolates of this genus. Additionally, the potential for more diverse *copLAB* genotypes is possible in at least 2 Xcc strains. The clustering of local and worldwide *copLAB* sequences and, the lack of the variant cluster in the related *Stenotrophomonas* genus further highlights the complex dynamics behind the origin of Xcc *copLAB* genotypes.

Acknowledgements

We thank Mr Omar Ali (University of the West Indies, St. Augustine) for his critical manuscript review and advice on statistical methods.

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Table 1(on next page)

Primers designed for PCR amplification of previously reported *copLAB* genes.

Primers	Primer Sequence (5'-3')	Amplicon size (bp)	Target
SMXCOPLF	GGTGGTCGGATCAGATGAGG	405	Xanthomonad *copL*
SMXCOPLR	GCCCGTGTCAGCCTC		
XEGVCOPAF	GTGCAAATGGATGGGATG	514	Xanthomonad *copA*
XEGVCOPAR	GGATCATGGGTCGATGGAT		
XCCCOPBGF	GACCACAGTCAGATGGGACAC	744	Xanthomonad *copB*
XCCCOPBGR	GGTACGAAGGCCGATGACCA		
copLSRF2	TGGCATCCCATCCTCTTTCG	311	BrA1 *copL*
copLSRR2	ACAGGAATAGACGCACAGGC		
copASRF9	TGTCCTTAGTGGGACCGAGT	306	BrA1 *copA*
copASRR9	CCTTGCTGAACCGTAAAGCG		
copBSRF2	TACCGACCTCAACCGTCTCT	533	BrA1 *copB*
copBSRR2	GAACCAAGTGCGTAGACCGA		

1

Table 2(on next page)

Local *Xanthomonas* copper sensitivity profiles, PCR-based identification, and *cop* genotype.

Xmel (*Xanthomonas melonis*). * - Copper-resistant isolate with no detected *copLAB* genes for primers used. *cop* genotype – Traditional = similarity to previously reported *copLAB* sequences, Undetected = PCR negative result, Variant = BrA1 variant *copLAB* genes. CuSo4.5H2O MIC – represented as ppm and mM, max concentration as determined using media screens. Cu Sensitivity – R = Resistant, T = Tolerant, S = Sensitive.

Isolate	Location	Species	*cop* genotype	CuSo4.5H2O MIC (ppm)	CuSo4.5H2O MIC (mM)	Cu Sensitivity
AR1PC2	Arangue z	Xcc	Undetected	200	0.8	T
Cf4B			Variant	-	-	-
Ar1BCA 2				400	1.6	R
BrA1				300	1.2	
Ca3B				600	2.4	
Cf1A				600	2.4	
Cf3A1				600	2.4	
Cf3C				600	2.4	
Cf4B1				600	2.4	
Cf5A2				600	2.4	
Cf5B				600	2.4	
Cf6A1				600	2.4	
FRL2D3	Bon Air	Xcc	Traditional	-	-	-
FRL2G4				-	-	
FRL1C1		Xsp		400	1.6	R
FRL2G5				600	2.4	
DMCE	Maloney	Xmel	Traditional	-	-	-
DMCA				600	2.4	R
DMCX				600	2.4	
DMCK*			Undetected	600	2.4	
CCB4		Xsp	Traditional	-	-	-
CaNP6B	Navet	Xcc	Traditional	500	2	R
CNP1E				300	1.2	
CNP2A*			Undetected	400	1.6	

Isolate	Location	Species	*cop* genotype	CuSo4.5H2O MIC (ppm)	CuSo4.5H2O MIC (mM)	Cu Sensitivity
CNP3C*				400	1.6	
CaNP1C				100	0.4	S
CNP4A				200	0.8	T
CaNP6A		Xmel	Traditional	600	2.4	R
CaNP5B				600	2.4	
CaNP1D			Undetected	200	0.8	T
CaNP3B		Xsp	Traditional	600	2.4	R
PNP25				600	2.4	
PNP26				600	2.4	
PNP34				600	2.4	
PNP39				600	2.4	
PNP44				600	2.4	
PNP49				600	2.4	
PNP54				600	2.4	
PNP58				600	2.4	
PNP62				600	2.4	
PNP63				600	2.4	
PNP64				600	2.4	
PNP72				600	2.4	
PNS3*			Undetected	600	2.4	

1

Table 3(on next page)

Copper-resistant bacterial isolate counts from an environmental population originating in the Aranguez agricultural district.

Bacterial counts for each identified genus from a sub-group of the environmental isolate population is shown is this table.

	Phylloplane		Soil	
Genus	Abandoned plot	Cultivated Field	Abandoned plot	Cultivated Field
Achromobacter	0	0	0	3
Acinetobacter	0	4	0	3
Bacillus	3	5	0	0
Enterobacter	0	0	7	0
Klebsiella	0	0	3	0
Pseudomonas	0	9	4	4
Serratia	0	1	0	3
Shigella	0	0	0	1
Sphingomonas	1	0	0	0
Stenotrophomonas (*)	0	3	2	6

1

Figure 1

Agricultural districts sampled in Trinidad, Trinidad and Tobago, W.I.

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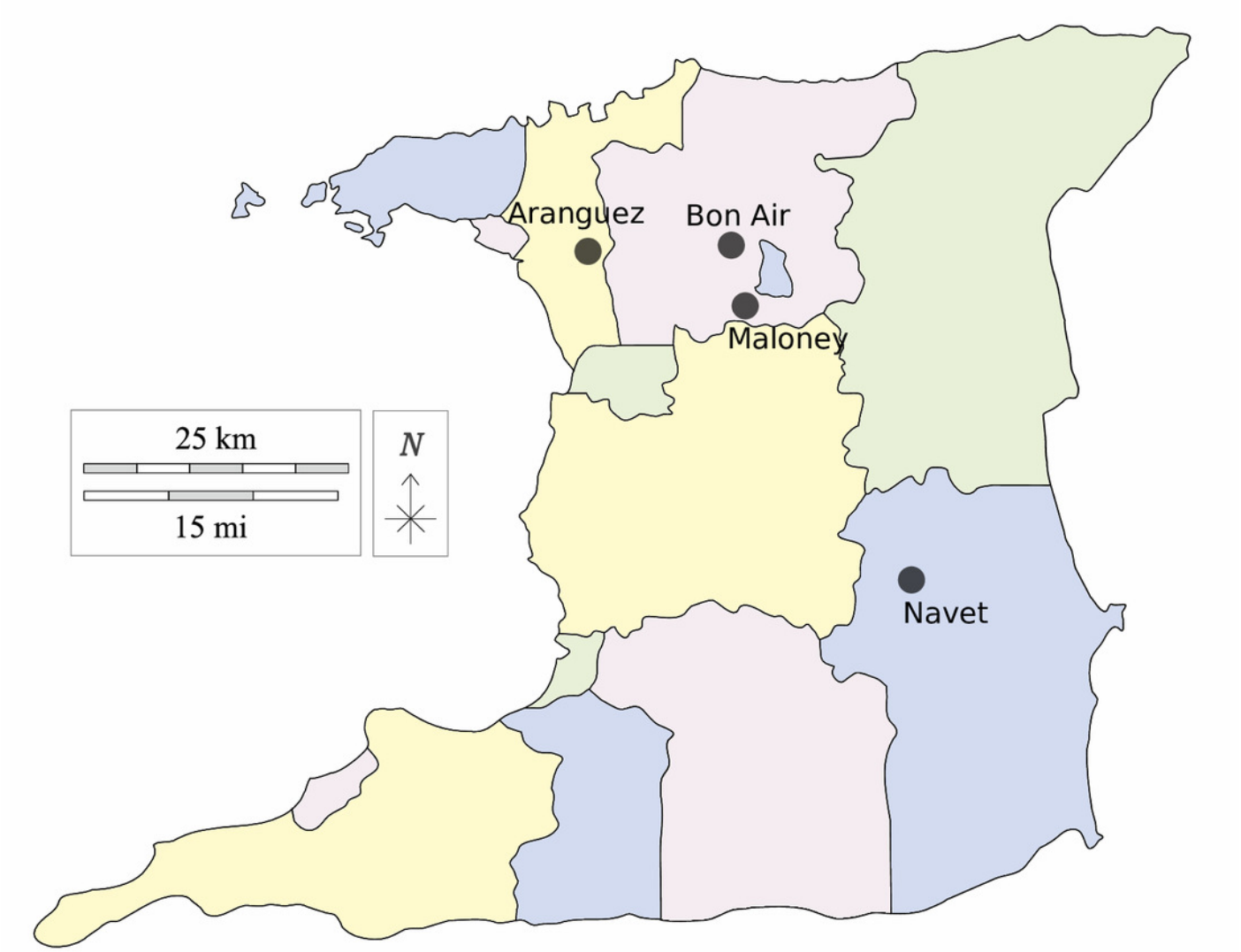


Figure 2

copLAB gene prevalence in local copper resistant *Xanthomonas*.

Traditional refers to previously reported *copLAB* genes. Only copper-resistant isolates (35) are represented in this figure. WGS characterised Xcc and Xmel strains are also represented here. Copper resistant sample sizes: Aranguez = 10, Maloney = 3, Navet = 20, Bon Air = 2.

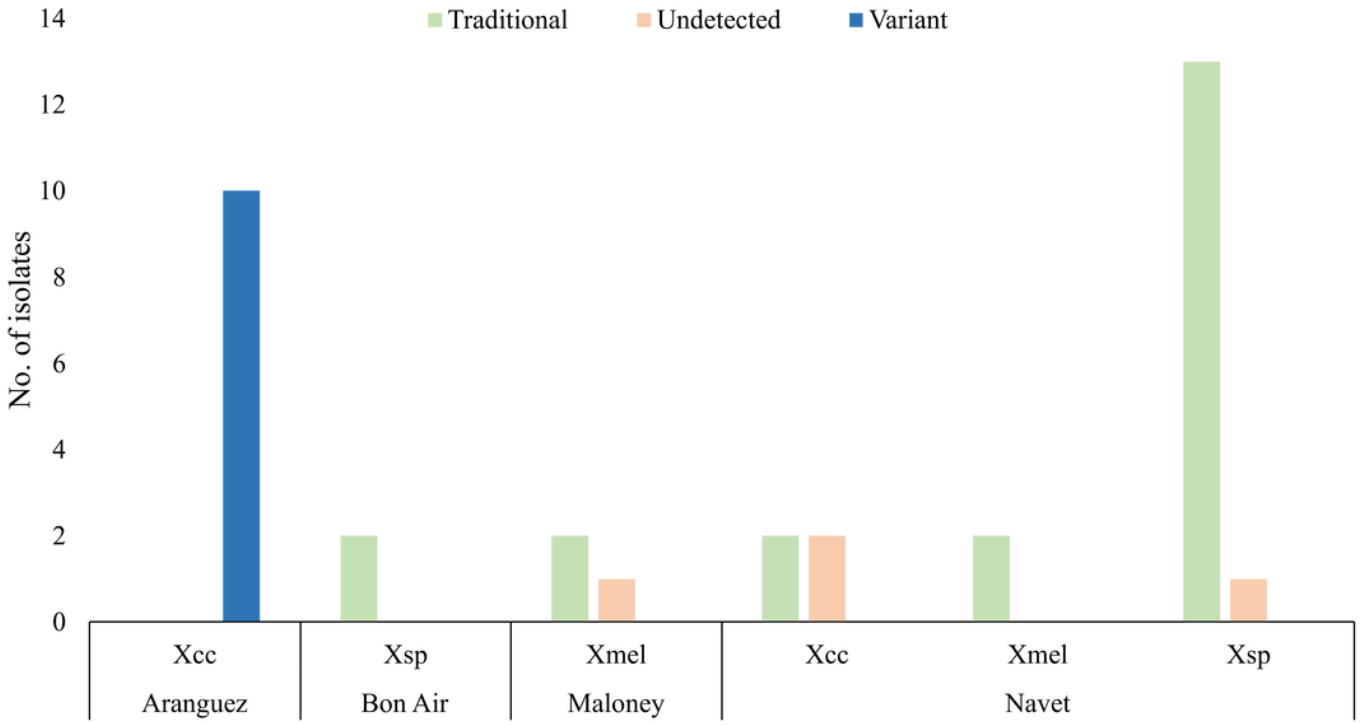


Figure 3

Proportional differences in copper sensitivity of phylloplane (A) and soil (B) environmental bacterial isolates from a cultivated and an abandoned field in the Aranguez agricultural district.

Proportions are given as percentages of total isolates per field per environmental sample.

The number of isolates is given in () above each pie chart.

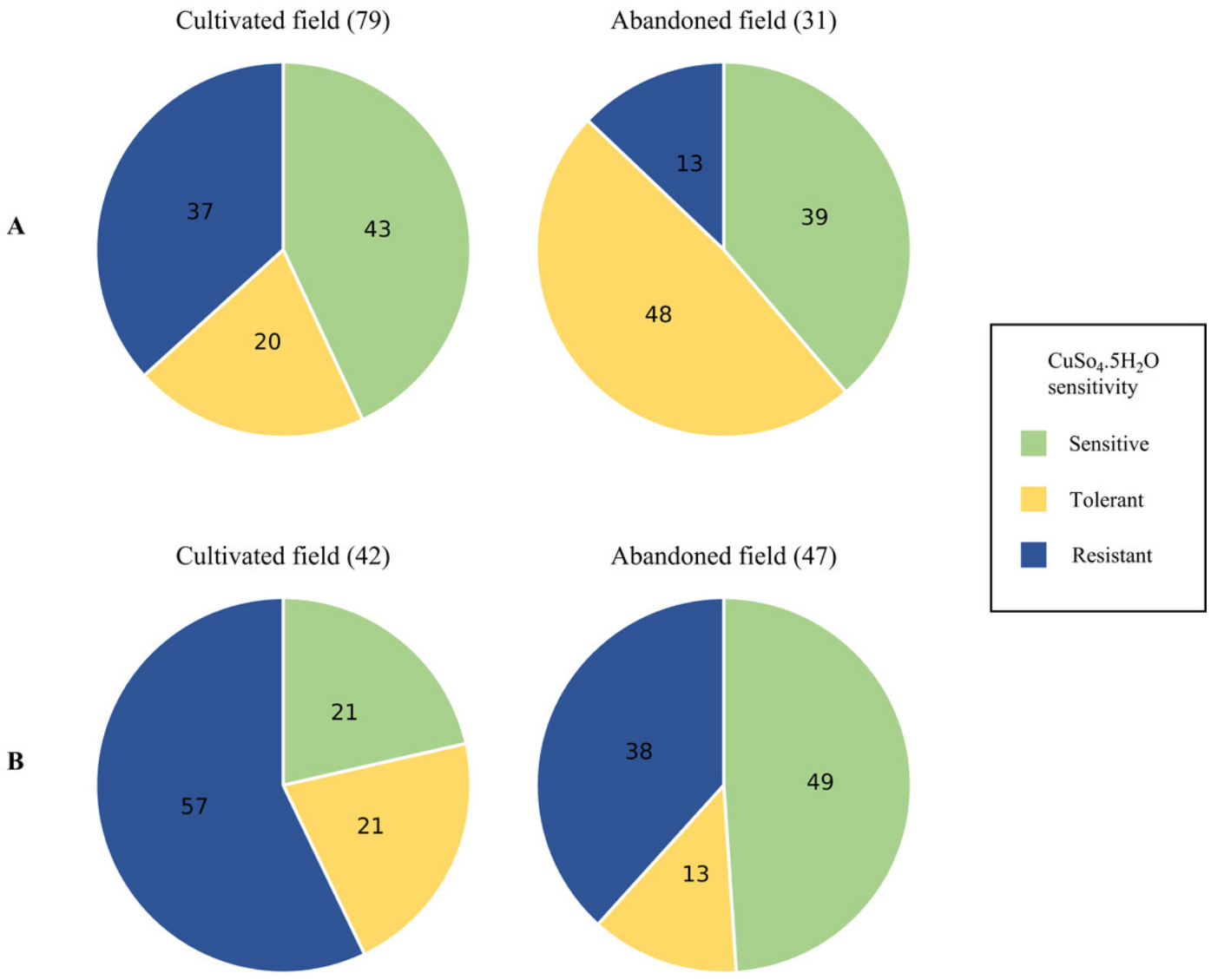
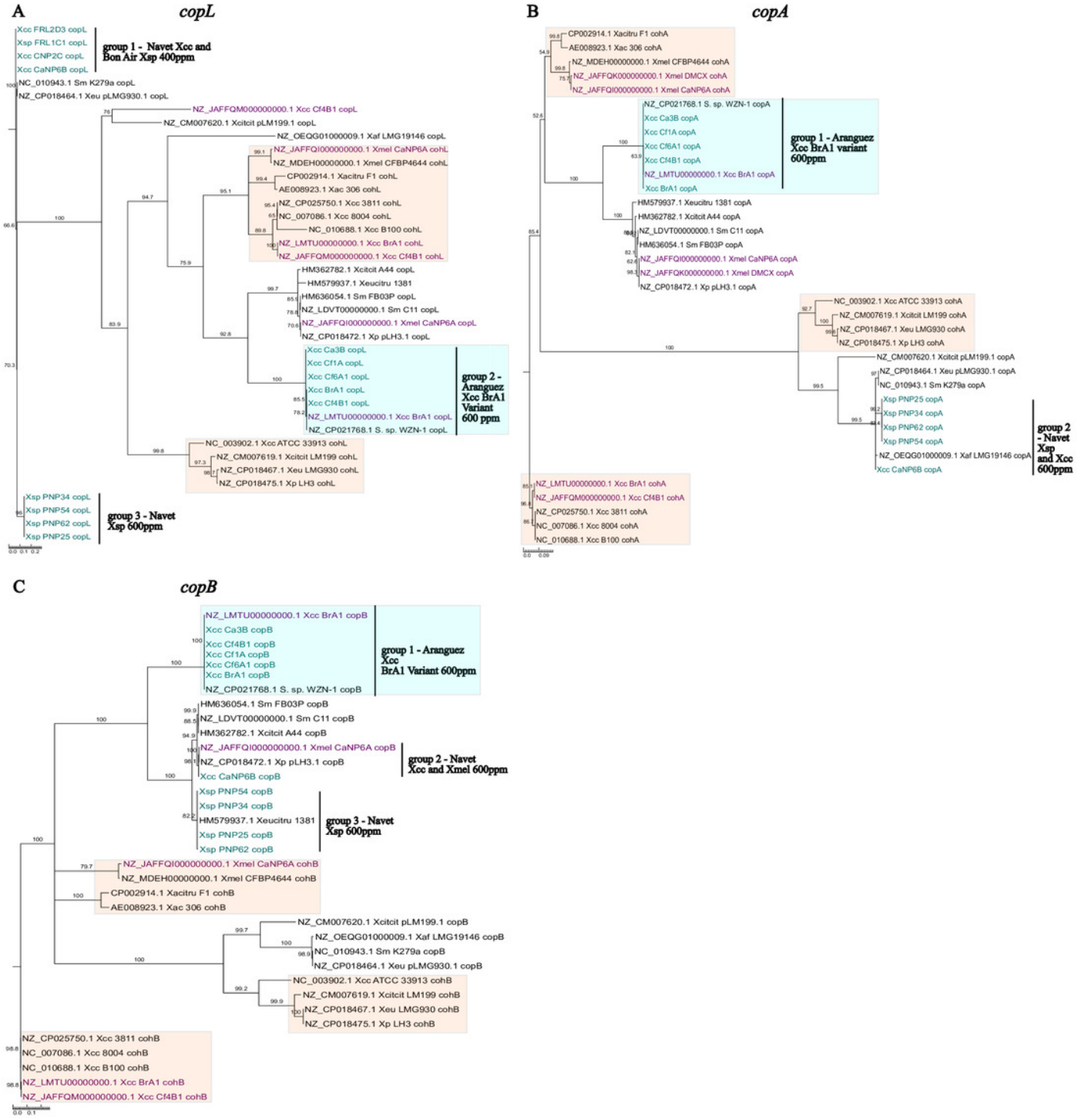


Figure 4

Phylogenetic reconstruction of amplified partial *copL* (A), *copA* (B) and *copB* (C) sequences from local copper-resistant *Xanthomonas* isolates.

Sequences from PCR amplifications are highlighted in blue, those from local isolate draft genomes are given in purple, and other sequences originate from GenBank reference genomes of *Xanthomonas* spp., *Stenotrophomonas* spp. and *Xanthomonas* plasmids. Chromosomal *copLAB* homologs (*coh*) are highlighted in orange boxes while the BrA1 variant *cop* genes are in blue boxes.


A
copL
Xcc FRL2D3 copL
Xsp FRL1C1 copL
Xcc CNP2C copL
Xcc CaNP6B copL
group 1 - Navet Xcc and Bon Air Xsp 400ppm
NC_010943.1 Sm K279a copL
NZ_CP018464.1 Xeu pLMG930.1 copL
NZ_JAFFQM000000000.1 Xcc Cf4B1 copL
NZ_CM007620.1 Xcitcit pLM199.1 copL
NZ_OEQG01000009.1 Xaf LMG19146 copL
NZ_JAFFQI000000000.1 Xmel CaNP6A cohL
NZ_MDEH00000000.1 Xmel CFBP4644 cohL
CP002914.1 Xacitru F1 cohL
AE008923.1 Xac 306 cohL
NZ_CP025750.1 Xcc 3811 cohL
NC_007086.1 Xcc 8004 cohL
NC_010688.1 Xcc B100 cohL
NZ_LMTU00000000.1 Xcc BrA1 cohL
NZ_JAFFQM000000000.1 Xcc Cf4B1 cohL
HM362782.1 Xcitcit A44 copL
HM579937.1 Xeucitru 1381
HM636054.1 Sm FB03P copL
NZ_LDVT00000000.1 Sm C11 copL
NZ_JAFFQI000000000.1 Xmel CaNP6A copL
NZ_CP018472.1 Xp pLH3.1 copL
Xcc Ca3B copL
Xcc Cf1A copL
Xcc Cf6A1 copL
Xcc BrA1 copL
Xcc Cf4B1 copL
NZ_LMTU00000000.1 Xcc BrA1 copL
NZ_CP021768.1 S. sp. WZN-1 copL
group 2 - Aranguez Xcc BrA1 Variant 600 ppm
NC_003902.1 Xcc ATCC 33913 cohL
NZ_CM007619.1 Xcitcit LM199 cohL
NZ_CP018467.1 Xeu LMG930 cohL
NZ_CP018475.1 Xp LH3 cohL
Xsp PNP34 copL
Xsp PNP54 copL
Xsp PNP62 copL
Xsp PNP25 copL
group 3 - Navet Xsp 600ppm
B
copA
CP002914.1 Xacitru F1 cohA
AE008923.1 Xac 306 cohA
NZ_MDEH00000000.1 Xmel CFBP4644 cohA
NZ_JAFFQK000000000.1 Xmel DMCX cohA
NZ_JAFFQI000000000.1 Xmel CaNP6A cohA
NZ_CP021768.1 S. sp. WZN-1 copA
Xcc Ca3B copA
Xcc Cf1A copA
Xcc Cf6A1 copA
Xcc Cf4B1 copA
NZ_LMTU00000000.1 Xcc BrA1 copA
Xcc BrA1 copA
group 1 - Aranguez Xcc BrA1 variant 600ppm
HM579937.1 Xeucitru 1381 copA
HM362782.1 Xcitcit A44 copA
NZ_LDVT00000000.1 Sm C11 copA
HM636054.1 Sm FB03P copA
NZ_JAFFQI000000000.1 Xmel CaNP6A copA
NZ_JAFFQK000000000.1 Xmel DMCX copA
NZ_CP018472.1 Xp pLH3.1 copA
NC_003902.1 Xcc ATCC 33913 cohA
NZ_CM007619.1 Xcitcit LM199 cohA
NZ_CP018467.1 Xeu LMG930 cohA
NZ_CP018475.1 Xp LH3 cohA
NZ_CM007620.1 Xcitcit pLM199.1 copA
NZ_CP018464.1 Xeu pLMG930.1 copA
NC_010943.1 Sm K279a copA
Xsp PNP25 copA
Xsp PNP34 copA
Xsp PNP62 copA
Xsp PNP54 copA
NZ_OEQG01000009.1 Xaf LMG19146 copA
Xcc CaNP6B copA
group 2 - Navet Xsp and Xcc 600ppm
NZ_LMTU00000000.1 Xcc BrA1 cohA
NZ_JAFFQM000000000.1 Xcc Cf4B1 cohA
NZ_CP025750.1 Xcc 3811 cohA
NC_007086.1 Xcc 8004 cohA
NC_010688.1 Xcc B100 cohA
C
copB
NZ_LMTU00000000.1 Xcc BrA1 copB
Xcc Ca3B copB
Xcc Cf4B1 copB
Xcc Cf1A copB
Xcc Cf6A1 copB
Xcc BrA1 copB
NZ_CP021768.1 S. sp. WZN-1 copB
group 1 - Aranguez Xcc BrA1 Variant 600ppm
HM636054.1 Sm FB03P copB
NZ_LDVT00000000.1 Sm C11 copB
HM362782.1 Xcitcit A44 copB
NZ_JAFFQI000000000.1 Xmel CaNP6A copB
NZ_CP018472.1 Xp pLH3.1 copB
Xcc CaNP6B copB
group 2 - Navet Xcc and Xmel 600ppm
Xsp PNP54 copB
Xsp PNP34 copB
HM579937.1 Xeucitru 1381
Xsp PNP25 copB
Xsp PNP62 copB
group 3 - Navet Xsp 600ppm
NZ_JAFFQI000000000.1 Xmel CaNP6A cohB
NZ_MDEH00000000.1 Xmel CFBP4644 cohB
CP002914.1 Xacitru F1 cohB
AE008923.1 Xac 306 cohB
NZ_CM007620.1 Xcitcit pLM199.1 copB
NZ_OEQG01000009.1 Xaf LMG19146 copB
NC_010943.1 Sm K279a copB
NZ_CP018464.1 Xeu pLMG930.1 copB
NC_003902.1 Xcc ATCC 33913 cohB
NZ_CM007619.1 Xcitcit LM199 cohB
NZ_CP018467.1 Xeu LMG930 cohB
NZ_CP018475.1 Xp LH3 cohB
NZ_CP025750.1 Xcc 3811 cohB
NC_007086.1 Xcc 8004 cohB
NC_010688.1 Xcc B100 cohB
NZ_LMTU00000000.1 Xcc BrA1 cohB
NZ_JAFFQM000000000.1 Xcc Cf4B1 cohB