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Research Article

Unravelling The Diversity of Cherry Tomato (*Solanum lycopersicum* var. *cerasiforme*) Seed Microbes and Their Effect on Seed Health

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ABSTRACT

Healthy seeds are the foundation of healthy plants. Planting healthy seeds contributes to securing crop productivity and seed germplasm conservation. In this study, we have identified microbes associated with seeds of three cherry tomato genotypes and demonstrated their negative effect on general seed health. Through a combined morpho-cultural and molecular characterisation (using multi-loci analysis of the ITS, β-tubulin, tef1α, and gapdh gene regions for fungi and 16s rDNA for bacteria), we have identified three fungi (*Nigrospora sphaerica*, *N. lacticolonia*, and *Curvularia aeria*), and two bacteria (*Citrobacter freundii*, and *Stenotrophomonas maltophilia*) from healthy-looking tomato seeds. These fungi and bacteria, through seed-soaked-inoculation, caused seed discoloration, lesions, and low germination. To our knowledge, these are the first reports of *Nigrospora sphaerica*, *N. lacticolonia*, *Curvularia aeria*, *Citrobacter freundii*, and *Stenotrophomonas maltophilia* on tomato seeds and demonstrated their negative impact on seed health. Seed treatment and interventions are needed to negate the possible effect of these microbes. Future studies on possible seed transmission are warranted.

INTRODUCTION

The cherry tomato (*Solanum lycopersicum* var. *cerasiforme*) is a small round tomato genotype and a genetic mixture among wild currant-type tomatoes and domesticated garden tomatoes (Chanthini et al. 2019). In the Philippines, they have become cash crops or moneymakers for the farmers (Sarian 2018) compared to table tomatoes. It is used in many dishes, such as salads, garnishes, and toppings, e.g., pizza and pasta.

Quality seeds are indispensable in crop production and an essential farming input for smallholder farmers, including those that plant tomatoes. Healthy seeds are crucial to producing a yield that meets market demands. Quality seeds are also a prerequisite for successful seed germplasm conservation. Storing diseased and nonviable seeds can result in the loss of germplasm materials over time, and seeds contaminated with pathogenic microbes may contaminate the storage facilities, possibly

contaminating other healthy seeds.

Seeds harbour various fungi and bacteria that may be pathogenic or saprophytic (Utobo et al. 2011). Bacteria enter stomata and hydathodes, especially in wounds, which thrive in the apoplast (intercellular space). Fungi directly enter the plant's epidermal cells or spread hyphae on, between, or through plant cells (Nallathambi et al. 2020). These microbes can diminish seed quality and weaken germination, producing abnormal and diseased seedlings (Islam & Borthakur 2012). However, some of these microbes may be inactive or quiescent during a period. In such conditions, the seeds may not show any disease symptoms, or the pathogen does not show any sign of growth.

This study aimed to determine the diversity of microorganisms inhabiting cherry tomato seeds (*Solanum lycopersicum* var. *cerasiforme*). Specifically, this study aimed to identify and to characterise fungi and bacteria in healthy-looking cherry tomato seeds and determine the effect of these microbes on general seed health (cosmetic and germination).

MATERIALS AND METHODS

Microbial assay of cherry tomato seeds

Seeds of three open-pollinated cherry tomato genotypes 'Elmundo,' 'Betty,' and 'Cherrys' from the Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines Los Baños) were placed equidistantly in Petri dishes containing potato dextrose agar (PDA) medium (Himedia Laboratories Ltd., India). Two experiments were performed simultaneously, one with surface-sterilised seeds and the other with non-surface-sterilised seeds. Both experiments were replicated three times and performed twice. Each replicate plate contained ten and six seeds in Trials 1 and 2. The surfaced-sterilised seeds were obtained as follows: first, seeds were immersed in 10% (v/v) commercial sodium hypochlorite (NaClO) bleach for 1 min, then seeds were rinsed thrice in sterile distilled water, and, finally, air-dried in sterile tissue paper before transferring onto PDA medium. Petri plates containing the seeds were incubated at room temperature (28.5 °C) for three days. Seeds were examined for growing microbes, which were isolated, purified, and maintained in PDA (for fungi) or Nutrient Agar (NA) medium (for bacteria).

Microbe Characterisation

Five mm of the fungal mycelial plug from each seven-day-old pure culture was transferred to a new PDA medium and incubated. Cultural characteristics, i.e., mycelial colour and form, were recorded seven days after incubation. Conidia length and width were measured from 30 randomly selected conidia of the seven-day-old fungal cultures under Olympus CX22 (Japan) microscopes. Photomicrographs were measured using the ImageJ software Version 1.51s (Wayne Rasband, National Institutes of Health, USA).

Bacterial isolates were streaked onto the NA medium to obtain a pure culture. The cultural characteristics were examined on their respective agar medium. Colony growth was examined, in terms of colour, shape, form, texture, size, and margin, after 48 hours of incubation.

Polymerase Chain Reaction (PCR) Assay

The bacterial genomic DNA of 2-day-old pure cultures of MBCTB01a and MBCTB01b isolates were extracted using Chen and Kuo's (1993) extraction method. The 16s rDNA gene region using the bacterial isolates was amplified by PCR using the 27F and 16s 1492R primers

(Suzuki & Giovannoni 1996). The fungal genomic DNA was extracted using CTAB (Cullings 1992; Doyle & Doyle 1987). The extracted genomic DNA of each isolate was used in subsequent PCR assays to amplify several fungal gene regions. The internal transcribed spacer (ITS), transcription elongation factor1-alpha (*tef1*), and partial β-tubulin (*tub2*) gene regions were amplified using primers ITS5/ITS4 (White et al. 1990), EF1-728F/EF1-986R (Carbone & Kohn 1999), and Bt2a/Bt2b (Glass & Donaldson 1995) and were used for isolates MBCTA01 and MBCTC02A. For isolate MMBCV02, ITS using primers ITS5/ITS4 (White et al. 1990) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using primers GDF and GDR were used. Amplifications were performed in a MyCycler™ (Thermal Cycler System #1709703, Bio-Rad Laboratories, Inc., USA) using the different PCR conditions per gene loci (White et al. 1990; Carbone & Kohn 1999; Glass & Donaldson 1995). The PCR products were resolved by gel electrophoresis [1.5% Agarose (Vivantis) 0.5X TAE (Tris-Acetate-EDTA) buffer containing two µL GelRed solution (Biotium) (PowerPac™ and Sub-Cell GT, (Bio-Rad Laboratories)] and viewed using the GelDoc™ XR+ with Image Lab software (Bio-Rad Laboratories Inc., USA). PCR products were purified and sequenced with the primers specified above at Apical Scientific Sdn. Bhd. (Malaysia).

Phylogenetic Analysis

The consensus sequences were assembled from the forward and reverse sequences using the Geneious sequence editing software. The BLASTN search program (Zhang & Madden 1997; Zhang 2000) was carried out to determine the isolates' closest fungal and bacterial genera based on the highest percent similarity e-value and highest query cover. Then, phylogenetic analyses were performed using the Maximum likelihood (ML) method in MEGA-X software (Kumar et al. 2018). Sequences were aligned using CLUSTALW. The concatenated sequences of the MBCTA01 and MBCTC02A isolates' ITS, tef1α, and *tub2* genes were assembled and compared with the sequences of other *Nigrospora* species (Table 1; Wang et al. 2017). *Arthrinium kogelbergense* (CBS 113333) (Crous & Groenewald 2013) was used as an outgroup. The ML tree was generated using the HKY (Hasegawa-Kishino-Yano) model (Hasegawa et al. 1985) with gamma-distributed and invariants sites (G+I). The concatenated ITS and GAPDH genes of isolate MBCV02 were compared with the sequences of *Curvularia* species (Table 2) and other members of Pleosporaceae, *i.e.*, *Bipolaris maydis* (CBS 136.29) and *B. panici-miliacei* (CBS 199.29) were used as outgroup. The ML tree was generated using the Kimura-2 parameter model (Kimura 1980) with gamma-distributed and invariants sites (G+I).

Bacterial isolates MBCTB01A and MBCTB01B were aligned with the species 16s rRNA sequences of their genera. MBCTB01a isolate was compared and aligned with sequences of *Citrobacter* species and other Enterobacteriaceae, *i.e.*, *Klebsiella pneumonia* (DSM 30104), *Proteus vulgaris* (ATCC 29905), and *Serratia marcescens* subsp. *marcescens* (ATCC 13880) (Table 3). MBCTB01b was compared with sequences of *Stenotrophomonas* species and other Xanthomonadaceae, *i.e.*, *Pseudoxanthomonas taiwanensis* (CB-2660), *Xanthomonas campestris* (ATCC 33913), *Xylella fastidiosa*. (PCE-FF) (Table 4). The ML tree was generated using the Kimura-2 parameter model (Kimura 1980) with gamma-distributed and invariants sites (G+I). Support values of all trees were evaluated with 1000 bootstrap replicates.

Table 1. *Nigrospora* species used in phylogenetic analysis and their corresponding accession numbers.

Species	Isolate*	Host	Country	ITS	β-tubulin	*tef1*
Nigrospora aurantiaca	CGMCC 3.18130*=LC 7302	*Nelumbo* sp. (leaf)	China	KX986064	KY019465	KY019295
N. bambusae	CGMCC 3.18327*=LC 7114	Bamboo (leaf)	China	KY385307	KY385319	KY385313
N. camelliae-sinensis	LC 3287	*Camellia sinensis*	China	KX985975	KY019502	KY019323
N. chinensis	CGMCC 3.18127*=LC 457	*Machilus breviflora*	China	KX986023	KY019462	KY019422
N. gorlenkoana	CBS 480.73	*Vitis vinifera*	Kazakhstan	KX986048	KY019456	KY019420
N. guilinensis	CGMCC 3.18124*=LC 3481	*Camellia sinensis*	China	KX985983	KY019459	KY019292
N. hainanensis	CGMCC 3.18129*=LC 7030	*Musa paradisiaca* (leaf)	China	KX986091	KY019464	KY019415
N. lacticolonia	LC 7009	*Musa paradisiaca* (leaf)	China	KX986087	KY019594	KY019454
N. musae	CBS 319.34*	*Musa paradisiaca* (fruit)	Australia	KX986076	KY019455	KY019419
N. sphaerica	LC 4303	*Rhododendron arboreum*	China	KX986004	KY019528	KY019345
N. oryzae	LC 6759	*Oryza sativa*	China	KX986054	KY019572	KY019374
N. osmanthi	CGMCC 3.18126*=LC 4350	*Osmanthus* sp.	China	KX986010	KY019461	KY019421
N. pyriformis	CGMCC 3.18122*=LC 2045	*Citrus sinensis*	China	KX985940	KY019457	KY019290
N. rubi	CGMCC 3.18326*=LC 2698	*Rubus* sp.	China	KX985948	KY019475	KY019302
N. vesicularis	CGMCC 3.18128*=LC 7010	*Musa paradisiaca* (leaf)	China	KX985948	KY019463	KY019294
N. zimmermanii	CBS 290.62*	*Saccharum officinarum* (leaf)	Ecuador	KY385309	KY385317	KY385311
Arthrinium kogelbergense	CBS 113333	*Restionaceae*	South Africa	KF144892	KF144984	KF145026

*CGMCC= China General Microbiological Culture Collection, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China; CBS= Culture Collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; LC= working collection of Lei Cai, housed at the Institute of Microbiology, Chinese Academy of Sciences, Beijing, China. Reference: Wang et al. (2017), Crous et al. (2013).

Table 2. *Curvularia* species used in phylogenetic analysis and their corresponding accession numbers.

Species	Isolate*	Host	Country	ITS	GAPDH	References
Bipolaris maydis	CBS 136.29	*Zea mays*	Japan	KJ909769	KM034845	Manamgoda et al. 2014
B. panici-miliacei	CBS 199.29	*Panicum miliaceum*	Japan	KJ909773	KM042896	Manamgoda et al. 2014
Curvularia aeria	BRIP:61232b	*Oryza sativa*	Australia	KU552200	KU552162	Khemmuk et al. 2016
C. affinis	CBS 154.34	Unknown	Indonesia	KJ909780	KM083608	Manamgoda et al. 2015
C. akaii	CBS 317.86	*Themada triandra*	Japan	KJ909782	KM230402	Manamgoda et al. 2015
C. alcornii	BRIP:61672a	*Oryza* sp.	Queensland	KU552202	KU552157	Khemmuk et al. 2016
C. arcana	CBS 127224	-	-	MN688801	MN688828	Marin-Felix et al. 2020
C. asianensis	MFLUCC 10-0711	*Panicum* sp.	Thailand	JX256424	JX276436	Manamgoda et al. 2012b
C. australiensis	IMI 53994	*Oryza sativa*	Australia	JN601026	KC747744	Manamgoda et al. 2012a
C. australis	BRIP 12521	*Sporobolus carolii*	Australia	KJ415541	KJ415405	Tan et al. 2014
C. austriaca	CBS 102694	Nasal cavity of patient with sinusitis	Austria	MN688802	MN688829	Marin-Felix et al. 2020
C. bannonii	BRIP 16732	*Jacquemontia tamnifolia*	USA	KJ415542	KJ415404	Tan et al. 2014
C. borreriae	AR5176r	*Sorghum bicolor*	South Africa	KP400637	KP419986	Manamgoda et al. 2015

Table 2. Contd.

Species	Isolate*	Host	Country	ITS	GAPDH	References
C. bothriochloae	BRIP 12522	*Bothriochloa bladhii*	Australia	KJ415543	KJ415403	Tan et al. 2014
C. buchloës	CBS 246.49	*Buchloë dactyloides*	USA	KJ909765	KM061789	Manamgoda et al. 2014
C. cactivora	CBS 580.74	Cactaceae	Suriname	MN688803	MN688830	Marin-Felix et al. 2020
C. canadiensis	CBS 109239	Overwintered grass	Canada	MN688804	MN688831	Marin-Felix et al. 2020
C. clavata	BRIP 61680	*Oryza rufipogon*	Australia	KU552205	KU552167	Khemmuk et al. 2016
C. coicis	CBS 192.29	*Coix lacryma*	Japan	JN192373	JN600962	Manamgoda et al. 2015
C. crustacea	BRIP 13524	*Sporobolus* sp.	Indonesia	KJ415544	KJ415402	Tan et al. 2014
C. dactyloctenii	BRIP 12846	*Dactyloctenium radulans*	Australia	KJ415545	KJ415401	Tan et al. 2014
C. ellisii	CBS 193.62	Air	Pakistan	JN192375	JN600963	Manamgoda et al. 2011
C. gladioli	ICMP 6160	*Gladiolus* sp.	New Zealand	JX256426	JX276438	Manamgoda et al. 2012a
C. geniculata	CBS 187.50	Unknown seed	Indonesia	KJ909781	KM083609	Manamgoda et al. 2015
C. graminicola	BRIP 23186a	-	Australia	JN192376	JN600964	Manamgoda et al. 2012a
C. harveyi	BRIP 57412	*Triticum aestivum*	Australia	KJ415546	KJ415400	Tan et al. 2014
C. hawaiiensis	BRIP 11987	*Oryza sativa*	USA	KJ415547	KJ415399	Tan et al. 2014
C. heteropogonicola	BRIP 14579	*Heteropogon contortus*	India	KJ415548	KJ415398	Tan et al. 2014
C. heteropogonis	CBS 284.91	*Heteropogon contortus*	Australia	JN192379	JN600969	Manamgoda et al. 2012
C. hominis	Cu_RgMdu	*Luffa acutangula*	India	MK737953	MK737951	Balamurugan et al., 2020
C. inaequalis	CBS 102.42	Sand dune soil	France	KJ922375	KM061787	Manamgoda et al. 2014
C. lunata	DMCC2087	*Zea mays*	USA	MG971304	MG979801	Garcia-Aroca et al., 2018
C. miyakei	CBS 197.29	*Eragrostis pilosa*	Japan	KJ909770	KM083611	Manamgoda et al. 2014
C. muehlenbeckiae	BRIP:61671	*Oryza* sp.	Australia	KU552201	KU552163	Khemmuk et al., 2016
C. neergaardii	BRIP 12919	*Oryza sativa*	Ghana	KJ415550	KJ415397	Tan et al. 2014
C. neoindica	IMI129790	*Brassica nigra*	India	MH414910	MH433649	Tan et al. 2018
C. nicotiae	BRIP 11983	Soil		KJ415551	KJ415396	Tan et al. 2014
C. nodulosa	CBS 160.58	*Eleusine indica*	USA	JN601033	JN600975	Manamgoda et al. 2015
C. oryzae	CBS 169.53	*Oryza sativa*	Vietnam	KP400650	KP645344	Manamgoda et al. 2015
C. ovariicola	BRIP 15882	*Eragrostis interrupta*	Australia	JN192384	JN600976	Manamgoda et al. 2012a
C. papendorfii	CBS 308.67	*Acacia karroo*	South Africa	KJ909774	KM083617	Manamgoda et al. 2014
C. pallescens	CBS 156.35	Air	Indonesia	KJ922380	KM083606	Manamgoda et al. 2015
C. perotidis	CBS 350.90	*Perotis rara*	Cape York	JN192385	HG779138	Manamgoda et al. 2011; Madrid et al. 2014; Manamgoda et al. 2015;
C. protuberata	CBS 376.65	*Deschampsia flexuosa*	UK	KJ922376	KM083605	Manamgoda et al. 2014
C. ravenelii	BRIP 13165	*Sporobolus fertilis*	Australia	JN192386	JN600978	Manamgoda et al. 2012a
C. richardiae	BRIP 4371	*Richardia brasiliensis*	Australia	KJ415555	KJ415391	Tan et al. 2014
C. robusta	CBS 624.68	*Dichanthium annulatum*	USA	KJ909783	KM083613	Manamgoda et al. 2014
C. ryleyi	BRIP 12554	*Sporobolus creber*	Yetman	KJ415556	KJ415390	Tan et al. 2014
C. sorghina	BRIP 15900	*Sorghum bicolor*	Australia	KJ415558	KJ415388	Tan et al. 2014
C. spicifera	CBS 274.52	-	-	JN192387	JN600979	Manamgoda et al. 2011
C. subpapendorfi	CBS 656.74	Desert soil	Egypt	KJ909777	KM061791	Manamgoda et al. 2015

Table 2. Contd.

Species	Isolate*	Host	Country	ITS	GAPDH	References
C. tsudae	ATCC 44764	*Chloris gayana*	Japan	KC424596	KC747745	Deng et al. 2014
C. trifolii	ICMP 6149	*Setaria glauca*	New Zealand	KM230395	JX276457	Manamgoda et al. 2015
C. tripogonis	BRIP 12375	-	Australia	JN192388	JN600980	Manamgoda et al. 2011
C. tropicalis	BRIP 14834	*Coffea arabica*	India	KJ415559	KJ415387	Tan et al. 2014
C. verruculosa	CPC 28792	-	Thailand	MF490825	MF490847	Marin-Felix et al. 2017

*ATCC- American Type Culture Collection, University Boulevard, Manassas; BRIP- The Plant Pathology Herbarium, Department of Agriculture, Fisheries and Forestry Indooroopilly, Queensland; CBS: Culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; CPC- Culture collection of Pedro Crous; ICMP- International Collection of Microorganisms from Plants Landcare Research, Auckland Mail Centre, Auckland; IMI= Culture Collection of CABI Europe UK Centre, Egham, UK.

Table 3. *Stenotrophomonas* species used in phylogenetic analysis and their corresponding accession numbers.

Species	Isolate*	Host	Country	*16s*	References
Pseudoxanthomonas taiwanensis	CB-226	hot spring	Taiwan	NR_025198	Chen et al. 2002
Stenotrophomonas acidaminiphila	AMX 19 16S	anaerobic sludge blanket	Mexico	NR_025104	Assih et al. 2002
Stenotrophomonas bentonitica	BII-R7	clay	Spain	NR_157765	Sánchez-Castro et al. 2017
Stenotrophomonas chelatiphaga	LPM-5	sewage sludge	Russia	NR_116366	Kaparullina et al. 2009
Stenotrophomonas daejeonensis	MJ03	sewage	South Korea	NR_117259	Lee et al. 2011
Stenotrophomonas ginsengisoli	DCY01	ginseng field	South Korea	NR_115687	Kim et al. 2010
Stenotrophomonas humi	R-32729	soil	Belgium	NR_042568	Heylen et al. 2007
Stenotrophomonas koreensis	TR6-01	compost	South Korea	NR_041019	Yang et al. 2006
Stenotrophomonas maltophilia	K13M1Y001	coastal dune	India	MK106330	Shet & Garg 2021
Stenotrophomonas maltophilia	OsEnb_HZB_H21	*Oryza sativa*	India	MN889407	Sahu et al. 2021
Stenotrophomonas maltophilia	ATCC 13637T	polluted urban soil	Japan	NR_040804	Iizuka et al. 1998
Stenotrophomonas maltophilia	IAM 12423	plastic industry soil	Pakistan	MN240936	Javaid et al. 2020
Stenotrophomonas maltophilia	S11-5	soil	USA	MN733007	Lopez et al. 2020
Stenotrophomonas nitritireducens	L2	biofilters	-	NR_025305	Finkmann et al. 2000
Stenotrophomonas panacihumi	MK06	soil	South Korea	NR_117406	Yi et al. 2010
Stenotrophomonas pavanii	ICB 89	*Saccharum officinarum* stalk	Brazil	NR_116793	Ramos et al. 2011
Stenotrophomonas pictorum	LMG 981	-	-	NR_041957	Hauben et al.1999
Stenotrophomonas rhizophila	e-p10	-	Germany	NR_028930	Minkwitz & Berg 2001
Stenotrophomonas terrae	R-32768	soil	Belgium	NR_042569	Heylen et al. 2007
Stenotrophomonas tumulicola	T5916-2-1b	yellow-cream viscous gel biofilm	Japan	NR_148818	Handa et al. 2016
Xanthomonas campestris	ATCC 33913	-	-	NR_074936	da Silva et al. 2002
Xylella fastidiosa	PCE-FF	-	-	NR_041779	Chen et al. 2000

*ATCC- American Type Culture Collection, University Boulevard, Manassas; LMG- LMG Bacteria Collection BCCM/LMG, Ghent University, Laboratory for Microbiology, Gent; ICB- Departamento de Patologia/ICB Campus Universitario, Estrada do Contorno, Manaus, Amazonas.

Table 4. *Citrobacter* species used in phylogenetic analysis and their corresponding accession numbers.

Species	Isolate	16s	References
Citrobacter amalonaticus	CECT 863	NR_104823	Yarza et al. 2013
Citrobacter cronae	XY1017	MW793480	Cui 2021
Citrobacter europaeus	CIP:106467	NR_156052	Ribeiro et al. 2017
Citrobacter farmeri	DP4R2A60	MH972183	Nimonkar et al. 2019
Citrobacter freundii	ATCC 8090 = MTCC 1658	NR_028894	Spröer et al. 1999
Citrobacter freundii	JCM 1657	NR_113340	Yarza et al. 2013
Citrobacter freundii	BAB-173	KF535108	Joshi et al. 2013
Citrobacter freundii	SS1KSU	MH973163	Tankrathok & Karnmongkol 2018
Citrobacter freundii	BAB-161	KF535107	Joshi et al. 2013
Citrobacter freundii	MD2	MZ047972	Joy et al. 2021
Citrobacter gillenii	CIP 106783	KM515970	Clermont et al. 2015
Citrobacter koseri	CDC-8132-86	NR_104890	Yarza et al. 2013
Citrobacter murliniae	HOP4	MT664058	Yang et al. 2020
Citrobacter rodentium	ATCC 51459	AB045737	Okutani et al. 2001
Citrobacter sedlakii	YL090822	GU726186	Wei et al. 2010
Citrobacter werkmanii	CIP 104555	KM515974	Clermont et al. 2015
Citrobacter youngae	GTC 1314	NR_041527	Nhung et al. 2007
Klebsiella pneumoniae	DSM 30104	NR_036794	Ludwig et al. 1995
Proteus vulgaris	ATCC 29905	NR_115878	Pignato et al. 1999
Serratia marcescens subsp. *marcescens*	ATCC 13880	NR_041980	Spröer et al. 1999

*ATCC- American Type Culture Collection, University Boulevard, Manassas; BAB- Instituto Nacional de Tecnologia Agropecuaria, Instituto de Recursos Biologicos, Castelar, Buenos Aires; CECT- Coleccion Espanola de Cultivos Tipo Edificio de Investigacion, Campus de Burjasot, Burjasot; DSM- Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstraße 7 B, 38124 Braunschweig; GTC- Gifu Type Culture Collection, Department of Microbiology, Gifu University School of Medicine, Gifu; JCM- Japan Collection of Microorganisms, Hirosawa, Wako, Saitama

Seed Germination assay

Two independent seed germination assays of the three cherry tomato genotypes ('Elmundo,' 'Betty,' and 'Cherrys') were performed to evaluate the effect of the isolated bacteria and fungi on percent seed germination. Fifteen seeds of each genotype, replicated three times, were soaked for 24 hours with a 3 mL suspension of the two identified bacteria. The turbidity and optical density of the suspension of both bacterial isolates were adjusted to OD_{600}= 0.3 in a spectrophotometer (SPECTRO 23 RS, LaboMed, Inc.), bringing a concentration of 10^8 CFU/mL. The three fungal isolates were scraped and strained in Muslin cloth. Seeds were soaked in 3 mL spore suspension (10^8 spores mL^{-1}). After 24 hours of seed soaking in the bacterial and fungal spore suspension, seeds were plotted equidistantly in Petri dishes and incubated (Memmert Incubator IN750, Memmert GmbH + Co. KG) (28.5 °C) for seven days. Germination percentages (%) were recorded seven days after incubation, and the appearance of the seeds was examined.

Statistical analysis

All statistical analyses were performed in SPSS statistical software (ver. 26, IBM, Armonk, NY). Data were analysed using one-way analysis of variance (ANOVA). Post hoc analyses of means were evaluated using Tukey's Honest Significant Difference (HSD) test (Tukey 1951) with a 95% significance level.

RESULTS AND DISCUSSION

Seed-borne microbes in cherry tomato seeds

A higher incidence of bacteria was observed and recorded compared to fungi (Table 1). All fungal isolates were obtained from non-surface-sterilised seeds of all three genotypes (Table 1). Both bacterial isolates MBCTB01A and MBCTB01b were found in non-surfaced-sterilised seeds of three tomato genotypes (Table 5). Only the bacterial isolate MBCTB01A was consistently found in all three tomato genotypes in surfaced-sterilized seeds. Bacterial isolate MBCTB01b was found in surfaced -sterilised seeds of genotype 'Cherrys' but not in 'Elmundo' and 'Betty.' A significant percent reduction in the incidence of fungi and bacteria ($p= 0.002$) was recorded in surface-sterilised seeds.

Table 5. Percent (%) incidence of seed-borne microbes found in three cherry tomato seed cultivars.

Cultivar	Isolate	Identity	Non-surfaced-sterilised (%)	Surfaced-sterilised (%)
'Elmundo'	MBCTB 01A	*Citrobacter freundii*	45.83	10.42
	MBCTB 01b	*Stenotrophomonas maltophilia*	18.75	0.00
	MBCT A01	*Nigrospora lacticolonia*	8.33	0.00
'Betty'	MBCTB 01A	*Citrobacter freundii*	95.83	18.75
	MBCTB 01b	*Stenotrophomonas maltophilia*	8.33	0.00
	MBCV0 2	*Curvularia aeria*	4.17	0.00
'Cherrys'	MBCTB 01A	*Citrobacter freundii*	60.42	2.08
	MBCTB 01b	*Stenotrophomonas maltophilia*	22.92	8.33
	MBCT C02A	*Nigrospora sphaerica*	8.33	0.00

Morphological and cultural characteristics

Fungal isolate MBCTA01 (Figure 1A) is floccose, creamy-white with a dark greenish-brown patch on the obversed centre and reversed. Conidia are smooth, dark brown to black, solitary, aseptate, globose to ellipsoidal-shaped, measuring an average of 134.21 µm² (30 conidia, ranging from 93.51 to 176.81 µm²), observed from the seven-day-old culture. Fungal isolate MBCTC02A (Figure 1B) is a floccose, grayish white colony. Conidia are smooth, dark brown to black, solitary, aseptate, globose to ellipsoidal-shaped (identical to conidia of MBCTA01 isolate), measuring an average of 140.85 µm² (n=30 conidia, ranging from 107.32 to 179.71 µm²), observed from the seven-day-old culture. Based on previous reports, the morpho-cultural characteristics resemble *Nigrospora* sp. (Wang et al. 2017; Taguiam et al. 2020). Whereas, fungal isolate MBCV02 is black with a velvety texture and smooth margins, and grew rapidly on PDA, reaching the edge of the Petri plate on the seventh-day post-incubation. Conidia are straight to pyriform-shaped, smooth-walled, four-celled, and obliquely septate with mainly three septa, and pale brown to dark brown-coloured with the middle two cells being darker than the end cells, measuring an average of 6.30 µm × 2.52 µm (n=30 conidia, ranging

from 4.64 to 8.32 µm × 1.93 to 3.06 µm). Based on these morphological features and previous reports (Khemmuk et al. 2016), MBCV02 is identified as *Curvularia* sp.

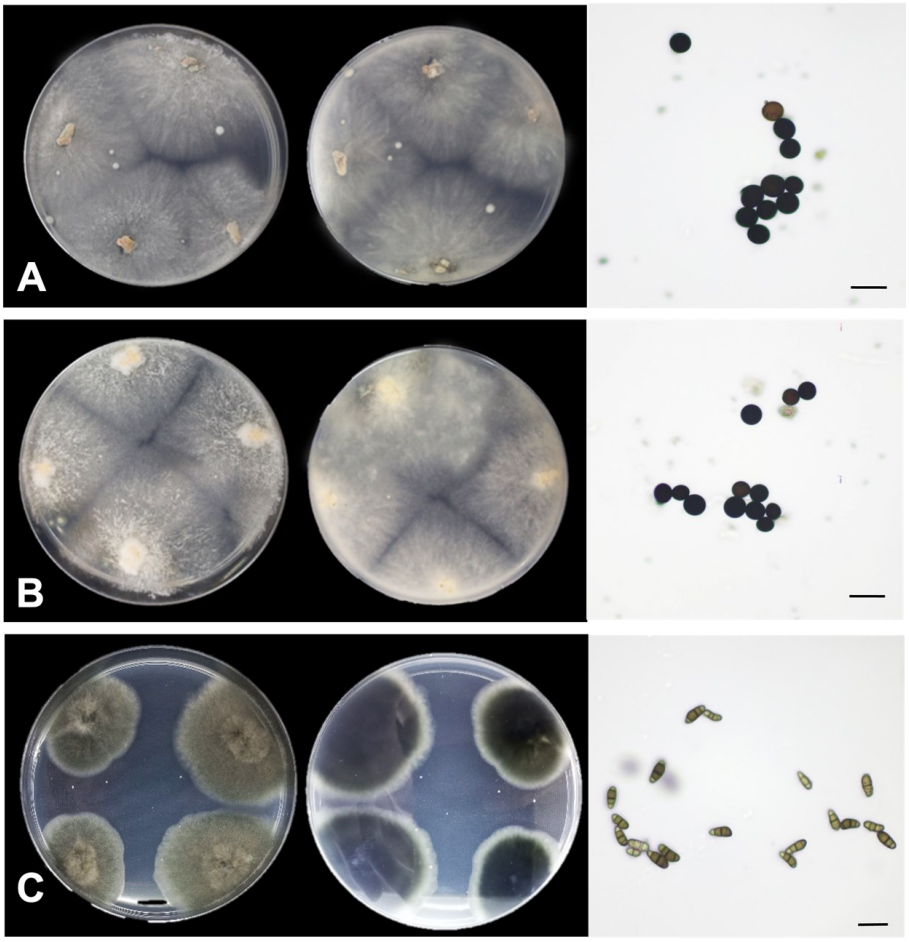

Figure 1. Cultural and conidial morphology of fungal isolates from cherry tomato seeds at 7 days after incubation (DAI). a. MBCTA01, b. MBCTC02, c. MBCV02. Scale bar = 20µm.

Bacterial isolate MBCTB01A produced bacterial colonies that are small, mucoid, and round-shaped with an entire (smooth) margin and convex elevation. Bacterial isolate MBCTB01B produced bacterial colonies that are dry, small, flat, round-shaped, and off-white-coloured with undulated to lobate margins (Figure 2).

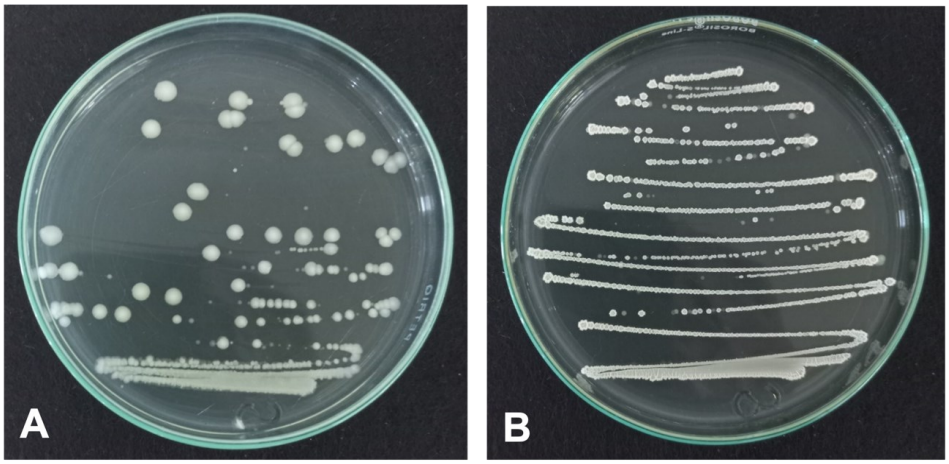

Figure 2. Cultural characteristics of bacteria found in cherry tomato seeds at 2 days after incubation (DAI). a. MBCTB01A, b. MBCTB01B.

Molecular identity

In the initial BLASTN analysis of the fungal isolates, the ITS gene sequences of MBCTA01, MBCTC02A, and MBCV02 isolates showed a 100% similarity to *Nigrospora lacticolonia* isolate KoRLI047323 (MN341462) and 14 *N. lacticolonia* strains, 100% similarity to *Nigrospora sphaerica* strain SX 4-1 (MH393359) (and 95 other *N. sphaerica* strains), and *Curvularia aeria* isolates B3153 (MT043775), and other 47 *C. aeria* isolate, respectively. The *tef1* gene of MBCTA01 and MBCTC02A isolates showed 99.55% similarity to *Nigrospora lacticolonia* strain LC12061 (MN264024) and 13 other *N. lacticolonia* strains, 99.54% similarity to *Nigrospora sphaerica* culture MFLUCC:18-0895 (MN995332), and other 105 *N. sphaerica* strains, respectively. The *tub2* gene of MBCTA01 and MBCTC02A isolates showed 100% *Nigrospora lacticolonia* strain LC12059 (MN329947) and 13 other *N. lacticolonia* 100% similarity to *Nigrospora sphaerica* isolate PC KS4A1 C R2 (MK408565) and 105 other *N. sphaerica* strains, respectively. The GAPDH gene of the MBCV02 isolate showed 98.31% similarity to *Curvularia* sp. isolate USJCC-0002 (MN053011). All isolates' sequences (Table 6) were deposited in NCBI GenBank.

Using the concatenated sequences of the ITS, *tef1*, and *tub2* gene regions, the phylogenetic analysis of fungal isolate MBCTA01 showed the isolate was grouped with *N. lacticolonia* LC7009 clade with 100 % ML support (Figure 3) and MBCTC02A grouped with the *N. sphaerica* LC4303 clade with 100% ML support (Figure 3). The fungal isolate MBCV02 grouped with the *C. aeria* BRIP:61232b clade (Figure 4) with 85% ML support.

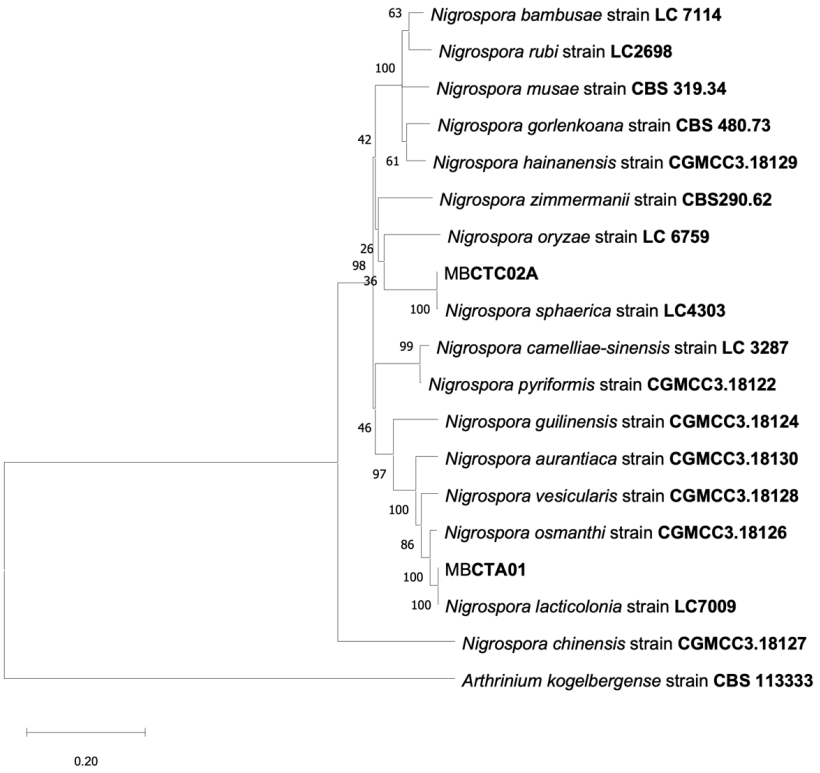

Figure 3. Phylogenetic tree generated by maximum likelihood analysis of the concatenated sequences of ITS, tef1α, and tub2 genes of *Nigrospora* species. ML (%) bootstrap support values are indicated near the nodes. Isolates MBCTA01 and MBCTC02A used in this study are in black arrowheads. The tree is rooted with *Arthrinium kogelbergense* CBS 113333. Bar = 0.20 indicates substitutions per nucleotide position.

Table 6. GenBank accession numbers of microbes isolated from tomato seeds.

Isolate	Identity	ITS	TUB	TEF	GAPDH	16sRNA
MBCTA01	*N. lacticolonia*	OR256266	OR271208	OR271206	-	-
MBCTA02A	*N. sphaerica*	OR256267	OR271209	OR271207	-	-
MBCV02	*C. aeria*	OR256268	-	-	OR271210	-
MBCTB01A	*C. freundii*	-	-	-	-	OR256216
MBCTB01B	*S. maltophilia*	-	-	-	-	OR256217

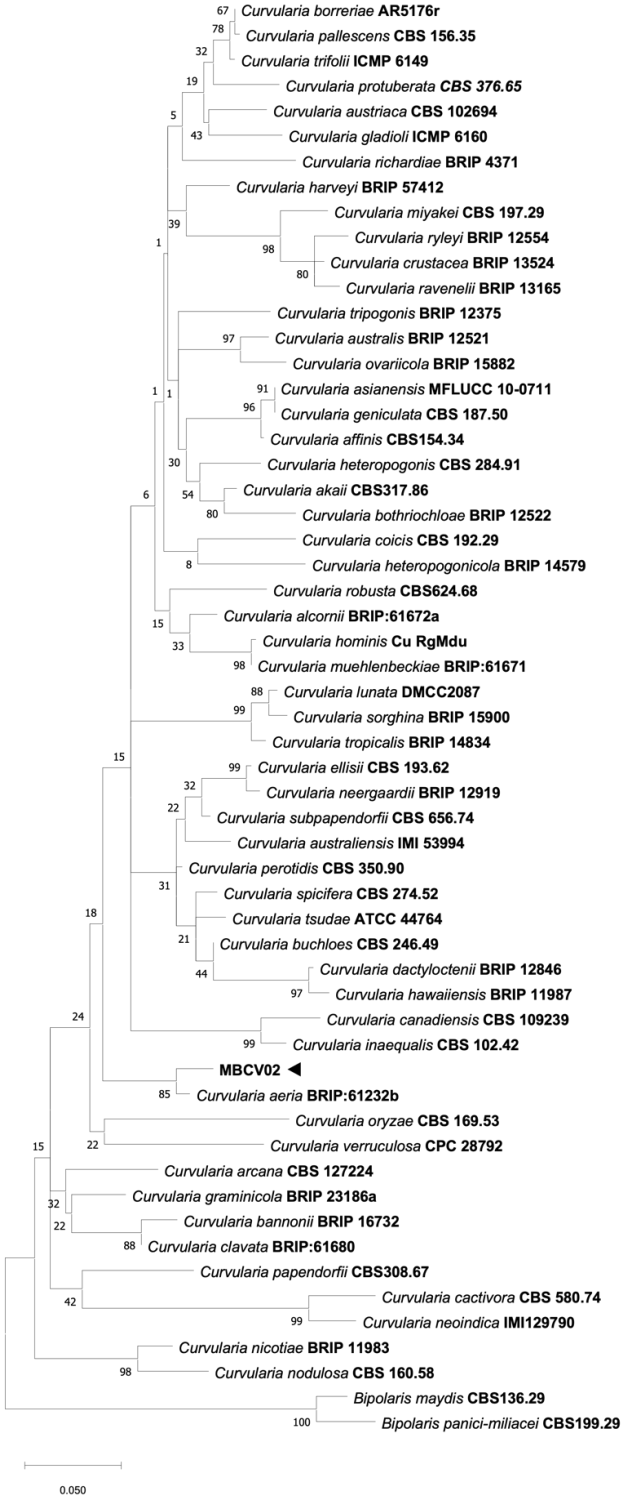

Figure 4. Phylogenetic tree generated by maximum likelihood analysis of the ITS and GAPDH gene of *Curvularia* species. ML (%) bootstrap support values are indicated near the nodes. Isolate MBCV02 used in this study is in black arrowhead. The tree is rooted with *Bipolaris maydis* CBS 136.29 and *Bipolaris panici-miliacei* CBS 199.29. Bar = 0.050 indicates substitutions per nucleotide position.

The initial BLASTN analysis of the bacterial isolates, using the 16s gene region, revealed that MBCTB01A was 99.44% similar to *Citrobacter freundii* strain BAB-173 (KF535108) and further supported with high similarity to 20 other *C. freundii* strains. The MBCTB01b isolate was 99.64% similar to *Stenotrophomonas maltophilia* strain K13M1Y001 (MK106330) and 33 other *S. maltophilia* isolates deposited in GenBank. The phylogenetic analyses confirmed this with isolate MBCTB01a grouped with the *Citrobacter freundii* clade (Figure 5) and the MBCTB01B isolate grouped with the *Stenotrophomonas maltophilia* clade (Figure 6).

Seed germination assay

Seeds soaked in fungal and bacterial suspension had significantly lower germination ($p < 0.001$) as compared to the controls (Figure 7). However, no significant variation in germination rate within inoculated seeds was observed among cherry tomato genotypes ($p = 0.673$). Rotting, discoloration, and lesions on the testa, cotyledons, and radicle of the roots and shoots were observed in the cherry tomato seeds that sprouted and germinated compared to controls (Figure 8). Similar symptoms were observed in both fungi and bacteria-treated seeds.

DISCUSSION

This study isolated and identified three fungi (*Nigrospora sphaerica*, *N. lacticolonia*, and *Curvularia aeria*) and two bacteria species (*Citrobacter freundii* and *Stenotrophomonas maltophilia*) from seeds of three cherry tomato genotypes ('Elmundo,' 'Betty,' and 'Cherrys'). These microbes are

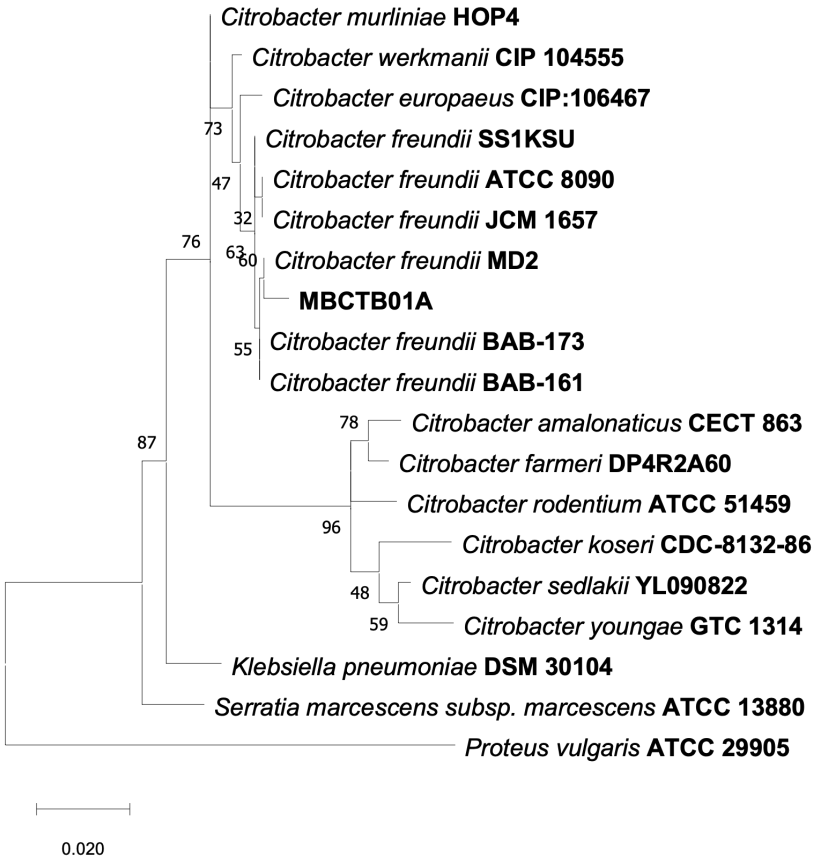

Figure 5. Phylogenetic tree generated by maximum likelihood analysis of the 16s RNA gene of *Citrobacter* species. ML (%) bootstrap support values are indicated near the nodes. Isolate MBCTB01A used in this study is in black arrowhead. *Klebsiella pneumoniae* DSM 30104, *Serratia marcescens* subsp. *marcescens* ATCC 13880, and *Proteus vulgaris* ATCC 29905 were used as an outgroup. Bar = 0.020 indicates substitutions per nucleotide position.

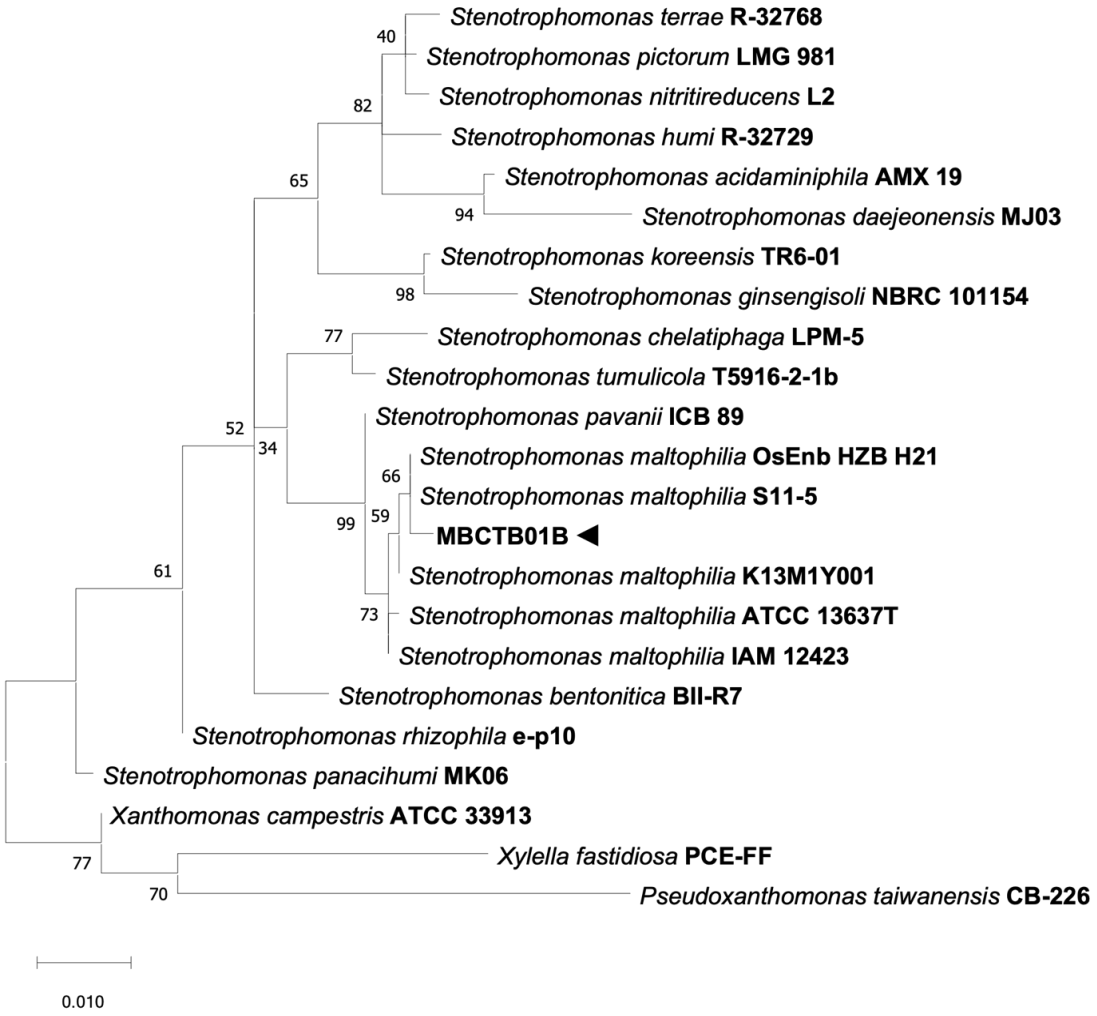

Figure 6. Phylogenetic tree generated by maximum likelihood analysis of the 16s rRNA gene of *Stenotrophomonas* species. ML (%) bootstrap support values are indicated near the nodes. Isolate MBCTB01B used in this study is in black arrowhead. *Xylella fastidiosa* PCE-FF and *Pseudoxanthomonas taiwanensis* CB-266 strains were used as an outgroup. Bar = 0.010 indicates substitutions per nucleotide position.

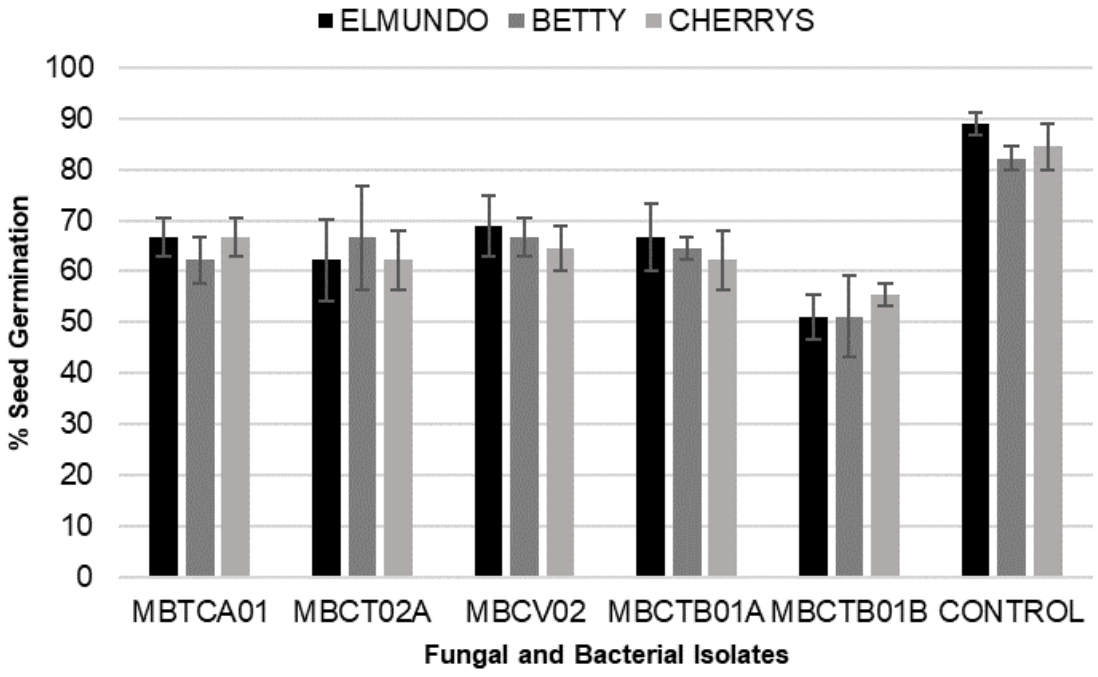

Figure 7. Mean percent (%) seed germination of cherry tomato genotypes at 7 days after sowing, soaked-inoculated with fungal and bacterial isolates.

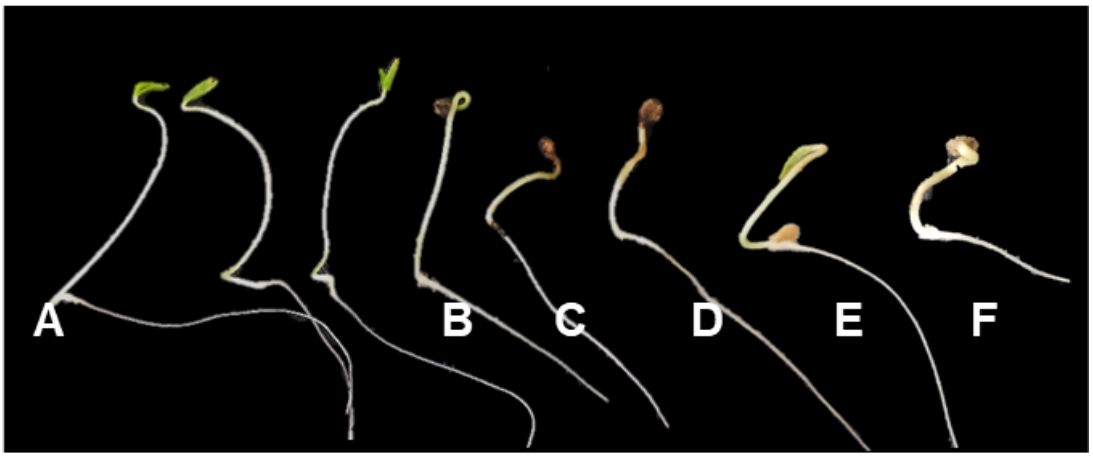

Figure 8. Germinated seedlings of cherry tomatoes soaked-inoculated by seed-borne fungi and bacteria at 7dpi; a. control, b. MBCTA01, c. MBCTC02A, d. MBCV02, e. MBCTB01A, f. MBCTB01B.

reported for the first time in association with tomato seeds. These microbes reduce the number of healthy seeds (low germination) and have a noticeable negative effect on root and shoot growth. The results demonstrate that pathogens lurking in apparently-healthy seeds, initially as endophytes, could become pathogenic when introduced to its host externally and at high inoculum pressure.

Curvularia aeria (Bat., J.A. Lima & C.T. Vasconc.) Tsuda 1994 (Nakada et al. 1994), being a plurivorous species, has been reported recently on *Etlingera linguiformis* in India (Kithan & Daiho 2014), *Ficus religiosa* in Pakistan (Nayab & Akhtar 2016), *Helianthus annuus* in Mexico (Valázquez-del Valle et al. 2017), *Lactuca sativa* in Thailand (Pornsuriya et al. 2018), *Oryza sativa* in Queensland (Khemmuk et al. 2016), and *Cyperus rotundus* (Ferreira & Barreto 2020) among others. *Curvularia species* such as *C. lunata* had been previously reported in seeds of *Coix lacryma-jobi* (Kim & Lee 1998), *Andropogon* sp. (Santos et al. 2018), *Dalbergia sissoo* (Gupta et al. 2017), and *Solanum lycopersicum* in Pakistan (Iftikhar et al. 2016). This study first reported *C. aeria* associated from seeds of cherry tomatoes (*Solanum lycopersicum* var. *cerasiforme*).

Nigrospora lacticolonia Wang et al. (2017) is a filamentous fungus from the ascomycetes. It has been reported in *Hylocereus polyrhizus* in Malaysia (Kee et al. 2019; Hao et al. 2020), *Phoenix dactylifera* in Oman (Al-Nadabi et al. 2020), *Bougainvillea spectabilis* (Li et al. 2022), *Camellia sinensis*, *Saccharum officinarum*, and *Musa* × *paradisiaca* in China (Wang et al. 2017; Raza et al. 2019). *Nigrospora sphaerica* (Sacc.) E.W. Mason 1927, an endophyte, saprobe, and plant pathogen with a relatively larger number of reported hosts than *N. lacticolonia*, has been reported in about 386 plant species (Farr & Rossman 2022). Among these are *Actinidia deliciosa* (Chen et al. 2016), *Hylocereus undatus* in China (Liu et al. 2016), *Solanum tuberosum* in Brunei Darussalam (Peregrine & Ahmad 1982), *H. megalanthus*, *H. undatus*, and *H. polyrhizus* (Taguiam et al. 2020), and *Saccharum officinarum* (Teodoro 1937) in the Philippines. This species has been reported and transmitted in seeds of *Heliocarpus americanus* in Brazil (Bernardi et al. 2022). Other *Nigrospora* species, e.g., *N. oryzae*, have been confirmed to be transmitted in rice, maize, and soybean seeds (Vasantha et al. 1987; Soesanto et al. 2020). Our work adds cherry tomatoes as a host of *N. sphaerica* and *N. lacticolonia*.

Stenotrophomonas maltophilia (Hugh) Palleroni & Bradbury (1993) is a bacterial pathogen that has been lately increasingly associated with tomatoes in various studies, viz., tomato seeds in Zimbabwe along with

Xanthomonas species (Sibiya et al. 2003), tomato roots and rhizosphere (Marquez-Santacruz et al. 2010), bald seeds of tomato (Stoyanova et al. 2018), tomato fruits (Stoyanova & Bogatzevska 2012). It has been isolated from the soil, rhizosphere, and waters and reported as an endophyte in plants (Ryan et al. 2009). *Citrobacter freundii* (Braak 1928) Werkman & Gillen, 1932, a bacterium of the Enterobacteriaceae and an opportunistic food-borne pathogen (Liu et al. 2020) has been recently reported from *Zingiber officinale* in China (Zhao et al. 2021) and *Morus alba* in Iran (Allahverdi et al. 2020). Our study reports *S. maltophilia* and *C. freundii* from cherry tomato seeds in the Philippines. Nonetheless, only *C. fruendii* was consistently isolated from surfaced sterilized seeds. This bacterium could pose more problems if contaminated seeds are sown without intervention, e.g., seed treatment.

Several studies have reported on microbial diversity in seeds of different plants, e.g., maize (Brito et al. 2022), orchid (Gao et al. 2019), cauliflower (Dhekle et al. 2013), cacao (de Araujo et al. 2019) lima bean (Mota et al. 2017), common bean (Parsa et al. 2016), and rice (Fisher & Petrini 1992). Our study looked into the microbes associated with cherry tomato seeds. Here, we provide baseline knowledge on the diversity of seed pathogens that impacted tomato seed health. The fungi and bacteria isolated from this study resulted in a reduced seed germination rate. They also caused discoloration and lesions on the germinated seeds' testa, cotyledons, and radicle. Seed-borne pathogens may cause the weakening or death of embryos and gradually kill the embryos of the seeds they invade (Christensen 1962; Bewley & Black 2012). Moreover, seed-borne pathogens are also accountable for plant morphology variation and yield loss in the field. The bacteria and fungi isolated from non-surfaced sterilised seeds may be considered epiphytic or surface contaminants (Vishnuvat & Shukla 1979; Khare 1996) as most were isolated frequently in the non-surfaced-sterilised seeds. Their incidence was significantly reduced when seeds were chemically surfaced-sterilised, leaving *C. freundii* as the only microbe isolated from the three tomato seeds. Hence, it demonstrates that intervention, e.g., surface sterilisation, can reduce pathogenic seed microbes' inoculum (and even eliminate it). Nonetheless, the chemical seed treatment choice must not affect seed germination or subsequent plant development. Moreover, the choice of farming practice, e.g., organic or inorganic, should be considered when identifying chemical seed treatments or any intervention.

Proper seed sterilisation methods before sowing and planting are recommended. They should be communicated to tomato growers and vegetable farmers, especially in small farming communities, to ensure pathogen-free and healthier plants that will contribute to a better yield. Management of these pathogens while still in seeds is an early and practical disease control approach. Healthy seeds are the foundation of healthy plants, contributing to better and higher plant yields. Moreover, seeds used for germplasm conservation should be free of any microbes that may negatively impact seed germination in the future. Seed treatment and interventions are needed to negate the possible impact of these microbes.

CONCLUSIONS

This study isolated and identified *N. sphaerica*, *N. lacticolonia*, *C. aeria*, *S. maltophilia*, and *C. freundii* from cherry tomato seeds 'Elmundo,' 'Betty,' and 'Cherry.' These microbes were frequent in non-surfaced-sterilised seeds. However, their presence was significantly reduced (or eliminated) in surface-sterilized seeds. At high inoculum pressure, these microbes

caused discoloration and lesions on the testa, cotyledons, and radicle and have proved to diminish the cherry tomato seeds' germination. Future studies to determine if these microbes affect other plant organs (leaves, stems, and fruits) are warranted to quantify the scale of the impact of these microbes on tomato health and yield. Furthermore, future studies on possible seed transmission are warranted.

AUTHOR CONTRIBUTION

H.D.A. conceived the work, conducted the experiments, collected and analysed he data, and drafted the manuscript. J.B. edited the manuscript. M.A.B. conceived the work, contributed to the experimental design, and edited the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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