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KAVHIZA NYASHA JOHN

Analysis of biological properties and improvement of molecular genetic methods for diagnosing the phytopathogen *Xanthomonas euvesicatoria* pv. *allii*

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Zargar Meisam

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КАВИЗА НЬЯША ДЖОН

Анализ биологических свойств и совершенствование молекулярно-генетических методов диагностики фитопатогена *Xanthomonas euvesicatora pv. allii*

Специальности 4.1.3 Агрохимия, агропочвоведение, защита и карантин растений

Диссертация на соискание
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Научный руководитель:
Заргар Мейсам

Доктор сельсхозяйственных наук, доцент

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Contents

Introduction

Relevance of the topic: The influx of new plant pathogens and new strains of already existing pathogens give rise to the emergence of plant diseases. Some pathogens associated with emerging diseases are categorised as quarantine organisms. These organisms have an invasive action and have a tendency to spread before their threat to agriculture is recognized by farmers. This situation is further exacerbated by the lack of appropriate diagnostic technologies (Robene et al., 2015).

Bacterial blight of onion (BBO) caused by *Xanthomonas euvesicatoria* pv. *allii* (Kadota et al. 2000) is a plant disease that has high agronomic and economic implications on onion production. Though it is a foliar disease it results in stunted growth as well as undersized bulbs thus reducing yields by 50% or more (Gagnevin et al., 2014). The disease is most severe during the juvenile vegetative stages of the plant. The bacterium gains entry into the plant through the stomata and rapidly multiplies especially during phases of high humidity (Nga et al., 2021). The seed-borne nature of this bacteria can significantly reduce onion seed marketability. To curb further introduction of *X. euvesicatoria* pv. *allii* into new territories and regions it has been designated as a quarantine pathogen on the EPPO A1 list.

Management of BBO is primarily based on copper-based bactericides usually applied with ethylenebis dithiocarbamate (EDBC) group of fungicides. However, the wide-spread existence of copper resistance genes in the genus *Xanthomonas* limits the effectiveness of this control method, (Richard et al., 2017; Richard et al., 2016; Behlau et al., 2011). In addition to the operon conferring resistance to cupric compounds, copper tolerance genes known as *cop* genes have also been discovered (Arguello et al., 2013); Marin et al., 2019). Moreover, reliance on antibiotics in crop production can lead to horizontal transmission of antibiotic resistance genes from plant-associated bacteria to human pathogens, (Nga et al., 2021).

Efforts are constantly being made to find sustainable ways of managing the bacterial blight of onion (BBO). Phuong et al. (2022) reported that 1% nanoemulsion formulations of essential oils of *Piper aduncun* and *Cymbopogon nardus*, with a strain of MRSNR 3.1 of *Bacillus thuringensis* exhibited bactericidal properties effective in controlling *X. euvesicatoria pv. allii*. Use of bacteriophages poses promising control method. Nga et al., (2021) promulgated that using phages specific to *X. euvesicatoria* pv. *allii* at concentrations 10^7 - 10^8 plaque-forming units per milliliter (PFU/ml) under field conditions in welsh onions (*A. fistulosum*) significantly suppressed disease symptoms comparable to a chemical bactericide Starner (oxolinic acid).

The development of a robust, sensitive, and highly reliable diagnostic protocol is indispensable to effective control of *X. euvesicatoria* pv. *allii*. PCR based detection and identification of Xanthomonads in plant material has been proved to be highly effective (Robene-Soustrade et al., 2006). Robene-Soustrade et al. (2010) developed a nested PCR assay based on the *avrRxv* gene and the pilus (*pilW* and *pilX*) assembly genes. When the PCR was tested for exclusivity in bacteria of the genus *Xanthomonas*, the majority of the strains did not produce amplicons besides 9 strains from *X. axonopodis* subgroup 9.1 and 9.2 which are not pathogenic to onion. The subgroup 9.1 includes pvs. *spondiae* and *begonia*, and the subgroup 9.2 includes pvs. *ricinni, citrumelo, vesicatoria, alfalfa, betae,* and the target group of pv. *allii*. The rationale behind the development of this multiplex PCR protocol was to enable detection of genetically heterogenous strains of *X. euvesicatoria* pv. *allii*. This research has also demonstrated enhanced sensitivity and specificity through the use of nested PCR. Prior to the development of the PCR assay pathogenicity tests usually coupled with molecular typing carried subsequent to pathogen isolation on selective media were the main identification methods (Picard et al., 2008; Gent et al., 2004). Such methods are inefficient as false negative results could be obtained at low concentrations of the bacteria. Moreover, the methods are time-consuming (Robene-Soustrade, 2010). To complement the conventional nested PCR, a real-time PCR

(qPCR) was developed to enable high throughput testing for both quarantine and research purposes (Robene et al., 2015). Optimizing this qPCR can bring significant improvements, such as high specificity, sensitivity, rapidity, possibility of quantification, and reduction of post-amplification handling.

The degree of development of the topic. The main means of *X. euvesicatoria* pv. *allii* transmission to new regions is through seed trade. Therefore, to effectively curb transboundary transmission of this quarantine pathogen molecular-based detection and identification techniques have to be developed and constantly optimized as a response to mutating pathogens. This therefore culminated in the analysis of the biological features of this pathogen as well as optimizing the molecular diagnostic method i.e. PCR. Moreover, important information about the bacteria's pathogenicity was also generated so as to improve ways of managing the pathogen.

Objective and specific tasks of the study- To analyze various biological properties and optimize molecular genetic methods for diagnosing the phytopathogen *Xanthomonas euvesicatoria* pv. *allii*

To achieve this goal the following tasks had to be completed:

1. Collect and analyze information on the systematics, pathogenicity and biology of *X. euvesicatoria* pv. *allii*.

2. To study the cultural properties and growth characteristics of *X. euvesicatoria* pv. *allii* on various nutrient media.

3. Test and optimize existing diagnostic methods.

4. Search for an alternative probe to the MGB probe.

5. Assess the efficacy of DNA extraction methods on the PCR assay for *X. euvesicatoria* pv. *allii*

The scientific novelty of the work:

1. The correlation between the DNA isolation method and PCR assay performance was unraveled. This led to the enhancement of the PCR assay as a result of identifying the optimum DNA extraction method.
2. The effect of *X. euvesicatoria* pv. *allii* in reducing the germination percentage and germination rate of onion seed was unraveled.
3. Testing the BHQ™ (Black Hole Quencher™) probe as an alternative to the Applied Biosystems TaqMan minor groove binder probes (MGB) probe which is not available on the Russian market, showed compatibility with both AVR and PIL primers. Henceforth, the BHQ probe was designated as an effective substitute of MGB.
4. Exploring disease effects in-planta on various parts of onion plants grown from different propagation material under field conditions exhibited that the bacteria multiplied more vigorously and prolonged persistence in the bulb apex.
5. The study revealed the effect of temperature on the growth of *X. euvesicatoria* pv. *allii* on the Onion Extract Medium (OEM).

Theoretical and practical significance. The improved and optimized PCR based diagnostic protocol for *X. euvesicatoria pv. allii* is suitable for Russia as well as developing nations. All aspects of the PCR assay such as sensitivity, specificity, repeatability and reproducibility were evaluated and validated. The identification of an optimized bacterial DNA extraction method highly adaptable to the PCR significantly enhanced assay efficiency consequently enabling faster and more accurate pathogen detection and identification. Carrying out the study led to the obtainment of an alternative to the MGB probe by evaluating the suitability of the BHQ probe. The study further contributed to knowledge of the biology of the bacterium through the trials on host selectivity and pathogen cultural properties on various growth media. The identification of the bulb apex as the plant part with the most pronounced pathogen abundance and/or persistence, unraveled knowledge about plant propagules that serve as potential sources of secondary inoculum and

origins of epidemics. The research findings are vital to the operations carried out by plant quarantine agencies.

Basic provisions for defense

1. Validate and optimize classic and real-time PCR for *X. euvesicatoria* pv. *allii*.
2. Evaluation and confirmation of the black hole quencher (BHQ) as an effective alternative probe to the Minor Groove Binder (MGB) probe.
3. Identification of Probe GS as the best DNA extraction method followed by the PCR assay of *X. euvesicatoria* pv. *allii*.
4. Assessment of the germination parameters of *Allium cepa* affected by *X. euvesicatoria* pv. *allii*.

Approbation of the work. The dissertation research culminated in the publication of 7 papers including 6 in scientific jounals indexed in the SCOPUS database and one in the Higher Attestation Commission of the Russian Federation (*BAK*)- listed journal.

Personal contribution of the Author. The Author participated in setting the aim and specific objectives of the study; collected and analyzed the obtained material, processed and interpreted the data, as well as prepared the publications in co-authorship.

Structure and volume of the dissertation. The dissertation constists of an introduction, three chapters, conclusion, and references. It contains 136 pages, the information is presented together with 32 tables and 15 figures. The reference list comprises of 149 literature sources.

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Chapter 1

Literature Review

1.1. Onion production and importance of the crop

The onion, *Allium cepa* L., belongs to the Clade Monocot, Order Asparagales, Family Amaryllidaceae, which consists of 75 botanical genera (Christenhusz and Byng, 2016). The genus *Allium* encompasses about 800 species that produce bulbs in the soil and is present in different regions of the world. The *Allium* species are mostly distributed in the Northern hemisphere (Wheeler et al. 2013; Sabiu et al. 2019; Jimenez et al. 2020). Asia is the most probable centre of origin and domestication of the genus *Allium*, with the second centre of domestication and diversity in North America (Choi and Oh, 2011; Jimenez et al. 2020).

Onion is one of the most consumed and cultivated vegetables in the world, with great relevance for the national and international market (Teshika et al. 2019). An essential food in the preparation of different national dishes, onion is mainly consumed fresh, in the form of salad, but also as a condiment in the preparation of soups, stews and sauces (Adeoti et al. 2021). About 66% of the world's onion production is concentrated in the Asian continent, especially China and India, which are the world's largest producers, (FAO. 2021).

The onion crop has always been an important vegetable historically. The Latin word from which the noun onion is derived means, “large pearl”, this not only refers to the aspects of shape and appearance but also points to the nutritional importance of this plant. Modern onion cultivars are characterized by various bulbs, colours and tastes making the crop an indispensable ingredient in many different onions across the globe (Nikus and Mulugeta, 2010). Onion breeding and genetics has evolved over the years. For the onion wild relatives are of paramount importance since they are a source of source pest and disease resistance genes. Studies on interspecific breeding and crossability have revealed that only a few wild species can be directly

crossed with onion. In the year 1925 male sterility was discovered in onion and this trait has been crucial in the production of F_1 hybrid seed (Shigyo and Kik, 2008).

In onion culture, symptoms caused by different pests and diseases may show similarities, and can be confused with abiotic causes such as phytotoxicity, water and nutritional deficiency. Incorrect diagnoses trigger, in many cases, the application of pesticides in situations where such management would not be necessary (Wordell and Boff, 2006).

Water deficiency is the most relevant abiotic factor for onion cultivation, can lead to large production losses, as onions are more susceptible to drought stress than other crops (Marouelli et al. 2005; Tosta et al. 2014). In the event of water deficit, the symptom observed in plants is necrosis of the apex of the leaves, also known as dry point (Wordell and Boff, 2006). In this situation, the symptoms are due to a physiological disorder caused by an abiotic factor, however, such a factor can predispose the plant to attack by pests and pathogens (Carvalho Bispo et al. 2018).

1.2. Bacterial pathogens that cause diseases in onion

Among the phytopathogenic bacteria that affect the onion crop are those that cause symptoms of bulb rot - scale rot of onion: *Burkholderia cepacia*, *B. gladioli*, *Pseudomonas aeruginosa*, *Pantoea ananatis*, *Klebsiella sp*., and *Enterobacter sp*. (Zaid et al. 2012; Abd-Alla and Bashandy, 2012; Stoyanova et al. 2012; Leach et al. 2020). The complex of bacterial species that causes scale rot in onions can cause losses of up to 50% in production (Wordell Filho and Boff, 2006). A common plant pathogen - *Pectobacterium carotovorum* subsp. *carotovorum* causes soft rot in several vegetable species including onion bulbs (Beriam, 2007; Marconatto et al. 2017).

1.3. Bacterial Taxonomy

Taxonomy (from Greek: taxis, “arrangement” and nomia, “method”) is the biological discipline of defining groups of organisms based on their shared characteristics, and giving names to the different groups. Cowan stated in 1968

(Cowan, 1968) that taxonomy is divided in three parts: (1) classification, arranging the organisms into groups based on similarity; (2) nomenclature, labelling the groups defined by classification; (3) identification, assigning an unknown organism to an known taxonomic group. Modern taxonomy also includes phylogeny and population genetics as an integral part of the classification process (Vandamme et al., 1996). Prokaryote classification is the most recent among the different classifications of all living organisms. The taxonomic classification system (also called the Linnaean system after its inventor Carl Linnaeus, a Swedish botanist, zoologist and physician) uses a hierarchical model. There, the basic unit of biological classification, the species, was named according to the Linnaean binomial system consisting of two parts: a noun (substantive) in the nominative case, which correspond to the "genus", followed by the epithet (adjective) that indicates the "species" in that genus. Together these form the scientific name that identifies the species. This binominal system was applied both to plants and animals. In 1786, the Danish naturalist Friedrich Müller described several bacterial species and attempted the first bacterial classification. Initially, bacterial species were defined according to the damage they produce. Plant pathologists assigned a new specific epithet to bacteria causing diseases on plants from which bacterial plant pathogens had not previously been isolated and/or diseases that looked different than other bacterial diseases on a particular host. This common practice was reflected in the "new host – new species" concept by Starr (1981). The number of species resulting from this practice grew rapidly, resulting in complex genera consisting of hundreds of species. The initially bacterial taxonomy evolved into a more objective one, after the release of the canonical Bergey's Manual for Determinative Bacteriology in 1923, which represented a modern identification key for bacteria. At that time there was no common agreement on prokaryotic classification (Staley & Krieg, 1989), this manual and the later editions became the reference work on bacterial classification. These publications provided formal description of all bacterial taxa and keys for the identification of new isolates (Murray & Holt, 2005). Meanwhile, more flexible

approaches such as numerical taxonomy and chemotaxonomy aimed to sort individual strains into species, genera and higher groupings (Rosselló-Mora & Amann, 2001). The need for a more formalized bacterial taxonomy led to the formation of an International Committee on Systematic Bacteriology (ICSB), now known as the International Committee on Systematics of Prokaryotes (ICSP). Between 1970 and 1980, the ICSB adopted the International Code of Nomenclature of Bacteria (Bacteriological Code; 1990 Revision (Lapage et al., 1992) and an Approved Lists of Bacterial Names (Skerman et al., 1980). The decision to recognize species as valid only if they were represented by a legitimate name, a species description and a type strain required pathologists to admit that many species differed only in host range, a character not considered to form part of a species description in terms of the Bacteriological Code. With these restrictions, many pathogens were considered to be members of the same species. This problem was solved by recognizing the infrasubspecific term "pathovar" for populations of pathogens within species and by creating the International Standards for Naming Pathovars of Phytopathogenic Bacteria (The Standards (Dye et al., 1980), and the adaptation of its own "Comprehensive List of Names of Plant Pathogenic Bacteria" (Bull et al., 2010).

The Gold Standard of DNA-DNA homology. A new DNA homology-based species concept was created in 1987, defining it as a group of strains, including the type strain, sharing 70% or greater DNA-DNA relatedness with 5°C or less ΔTm (difference in melting temperature in degrees Celsius between the homologous and heterologous hybrids under standard conditions (Wayne et al., 1987). Although this concept was considered the "gold standard" (Stackebrandt & Goebel, 1994) several practical problems existed because DNA-DNA hybridization (DDH) was time-consuming and because different methods were used to determine the level of DDH and these did not always show the same results (Gevers et al., 2005). Therefore, the value of 70% DNA relatedness was considered indicative rather than absolute. A

first alternative solution for this problem was provided with the appearance of 16S ribosomal RNA gene (16S rRNA gene) sequencing (Woese, 1987). Since the 16S rRNA is present in all bacteria, is functionally constant and is composed of conserved and variable regions, it has consistently served as a good taxonomic marker for deriving taxonomic relationships (Vandamme et al., 1996). Therefore, it was suggested that strains sharing at least 97% 16S rRNA gene sequence identity, should be considered members of the same species (Stackebrandt & Goebel, 1994). However, the resolution of 16S rRNA gene was often insufficient to elucidate affiliations between closely related species (Fox et al., 1992; Gevers et al., 2005) and sometimes it was impossible to draw a conclusion based on this threshold of 97% sequence identity. Stackebrandt and Ebers (2006) proposed to shift this 97% value to the new 98.7-99%, but this has not yet been widely adopted and was only recently started to be used (Yarza et al., 2014). The current consensus in bacterial taxonomy is to use a polyphasic approach for characterizing and classifying bacteria. A range of genotypic and phenotypic techniques are applied to characterize a bacterial species in the most comprehensive way possible (Vandamme et al., 1996; Moore et al., 2010). In practice, a species is defined as "a group of strains characterized by a certain degree of phenotypic consistency, by a significant degree (50 to 70%) of whole genome DNA relatedness and over 97% 16S ribosomal RNA gene sequence identity" (Coenye et al., 2005). In general, phenotypic techniques are very useful in characterizing an organism and chemotaxomic methods might help in drawing a picture of high-level taxonomy. But phylogeny mostly cannot be determined based on phenotype alone. Therefore, genotypic methods, such as 16S rRNA gene sequencing and rRNA homology are mostly applied. However, the current species concept is criticized by some researchers as being too conservative, leading to an underestimation of the real diversity (Rosselló-Mora & Amman, 2001). Genomics based taxonomy. Currently, focus in bacterial taxonomy is on whole-genome sequencing (WGS), which might contribute to unravel evolutionary relationships between prokaryotes and to result in a workable, satisfying species

concept (Coenye et al., 2005; Gevers et al., 2005; Konstantinidis & Tiedje, 2005). As stated earlier, 16S rRNA gene sequencing suffers from lack of resolution for closely related strains, and alternatives were found in the analysis of housekeeping genes. Such approach is known as Multi Locus Sequence Typing (MLST) or Multi Locus Sequence Analysis (MLSA). MLSA schemes often provide higher resolution than 16S rRNA gene sequencing, allowing differentiation at the species level (Moore et al., 2010). Two of the most recent methods to delineate bacterial species are Average Nucleotide Identity (ANI) and Average Amino acid Identity (AAI) (Richter & Rosselló-Mora, 2009). Both parameters rely on the pairwise comparison of whole genome sequences to determine a set of orthologous genes conserved among both genomes. Preliminary results have shown that ANI and AAI-values correlated extremely well with experimentally determined DDH-values, and the comparison between these techniques resulted in a threshold value of 95% ANI and 95-96% AAI for species delineation, comparable to the 70% DNA-relatedness value. Therefore, it has been suggested that ANI could be a more practical replacement for DDH within the current species concept (Konstantinidis & Tiedje, 2005; Goris et al., 2007). However, it has also been observed that strains with a 95% ANI value could still have up to 20% difference in gene content, leading to a stricter ANI cut-off (98-99%) to obtain a higher predictive value for species delineation than the 70% DDH (Konstantinidis et al., 2006).

1.4. Genus *Xanthomonas*

Xanthomonas is one of 22 currently acknowledged genera (http://www.bacterio.net) within the family of Xanthomonadaceae, order Xanthomonadales of the class of Gammaproteobacteria (Garrity et al., 2005). The type species is *X. campestris*, and the type strain is *X. campestris* pv. *campestris* LMG 568 (equivalent strain numbers: ATCC 33913, CCRC 12846, CCUG 47691, CECT 97, CFBP 5241, DSM 3586, JCM 13371, KACC 10913, NCPPB 528, PDDCC13). The genus Xanthomonas comprises 27 (Constantin, 2017), plant associated bacterial species and although

most members of the genus are thought to have a narrow host range, Xanthomonas as a genus is able to infect a broad range of plants, covering at least 124 monocotyledonous and 268 dicotyledonous species (Leyns et al., 1984). The genus was first proposed by Dowson (1939), who described 60 species. Although several efforts were made by different research groups to reclassify members of Xanthomonas (De Vos & De Ley, 1983; Swings et al., 1983; Van Den Mooter & Swings 1990; Yang et al., 1993; Vauterin et al., 1995), the taxonomy and classification in the genus is still undergoing revision because of phytopathogenic diversity (Vauterin et al., 2000; Rademaker et al., 2005; Schaad et al., 2005) and more recent in-depth genomic characterization. Xanthomonas taxonomy continues to be controversial.

1.5. History of genus *Xanthomonas*

In 1883 Wakker reported for the first time a disease termed as "Yellow disease of hyacinthi" also known as the *Bacterium hycinthi*. The yellow colonies were later ascribed to the genus Pseudomonas and subsequently to Phytomonas which housed all yellow pigmented plant pathogenic bacteria (Constantin, 2017).

In the year 1930 Burkholder conducted a comparative study at grand scale whereby he sorted out the major bacterial groups previously compiled into Phytomonas. Dowson (1939) further classified phytopathogenic bacteria into 3 main genera namely, (i) Bacterium Ehrenberg 1828 constituting of Gram-negative bacteria that have peritrichous flagella, (ii) Pseudomonas Migula 1897- consisting of green fluorescent bacteria that have polar flagella and (iii) *Xanthomonas*- constituting of Gram-negative bacteria with yellow pigment and single polar flagellum. Moreover, Dowson distinguished 19 species belonging to this new genus (Van den Mooter and Swings, 1990).

Initially in the *Xanthomonas* genus any variant exhibiting a different host range or inducing different disease symptoms was designated as a separate species (Starr, 1981). Nonetheless this culminated in an excessively large number of nanospecies

later classified based on the classical nomenclature. Burkholder and Starr (1948) unravelled the limitations associated with the method of distinguishing the Xanthomonas species based on physiological and biochemical characteristics alone without taking into account their hosts. Furthermore, they also made criticism of the new host- new species theory. Owing to the fact that the "distinguished" species were almost indifferent, the number of species ultimately reduced to only 5 i.e. *X. albilineans, X. axonopodis, X. campestris, X. ampelina* and X. *fragariae*. The result was the large-scale merging of the species into one viz. *Xanthomonas campestris* which was subdivided into various pathovars (Dye et al. 1974).

Amendments were made to the genus, for instance the transference of the species *X. ampelina* to a new genus Xylophilus becoming *Xylophilus ampelinus* on the other hand a new species was incorporated to the genus as *Xanthomonas populi* earlier classified as *Aplanobacter populi* (Willems et al., 1987; Ride and Ride, 1992). Young et al. (1978) later proposed another reclassification guided by the former taxonomic system. Many studies were undertaken with the aim of accurately distinguishing the Xanthomonas species and bring clarity on the relationships between the pathovars using fatty acids (Young et al., 1993), DNA Restriction Fragment Length Polymorphism (RFLP) profiling and numerical taxonomy (Van Den Mooter and Swings, 1990).

1.6. *Xanthomonas euvesicatoria* pv. *allii*

Xanthomonas euvesicatoria pv. *allii* (Kadota et al. 2000; Constantin et al. 2016) is a gram-negative, obligate aerobic, rod-shaped, oxidative metabolism, catalase-positive, phytopathogenic bacterium with mucoid, smooth, circular, convex and yellow colonies (Kadota et al. 2000; Roumagnac et al. 2004). It belongs to the class Gammaproteobacteria and has as heterotypic synonyms *Xanthomonas campestris* pv. *allii* Kadota et al. 2000 and *Xanthomonas axonopodis* pv. *allii* (Kadota et al. 2000) Roumagnac et al. 2004.

The first description of this onion-pathogenic bacterium as belonging to the genus Xanthomonas was made by Alvarez et al. (1978) in Hawaii. *Xanthomonas euvesicatoria* pv. *allii* was initially classified as *X. campestris* pv. *allii* by Kadota et al. (2000), isolated from Welsh onion (*Allium fistulosum*) plants in Japan, based on their biochemical, physiological and host range tests. In 2004, Roumagnac et al., based on a polyphasic characterization using molecular techniques of DNA–DNA hybridization and FAFLP analysis (Fluorescent amplified fragment length polymorphism analysis), in addition to biochemical and phenotypic studies, reclassified the species as *X. axonopodis* pv. *allii*, defining as belonging to the rep-PCR 9.2 group of *X. axonopodis*. The last reclassification of the species was proposed by Constantin et al. (2016) who used molecular techniques of MLSA (Multilocus sequence analysis), DNA–DNA hybridization and genomic sequencing that allowed the reclassification of the species within group II of the genus Xanthomonas and in the species *X. euvesicatoria* pv *allii*.

Xanthomonas euvesicatoria pv. *allii* has been reported to infect different species of the genus Allium in addition to onions, such as garlic (*A. sativum*), Welsh onion (A. fistulosum) and shallot (*A. oschaninii*) under field conditions (Kadota et al. 2000; Roumagnac et al. 2004). Under greenhouse conditions, *X. euvesicatoria* pv. *allii* was able to induce symptoms in Citrus species similar to those caused by *X. axonopodis* pv. *citrumelo*, which causes citrus bacterial spot, however, under field conditions there is no evidence of occurrence in Citrus (Gente et al. 2005). There are already reports of epiphytic survival of *X. euvesicatoria* pv. *allii* on vegetables, beans, alfalfa, chickpeas, lentils and soybeans, under greenhouse conditions, but under field conditions isolated from *X. euvesicatoria* pv. *allii* were not recovered from soybeans and chickpeas (Gent et al. 2005). There are also reports of asymptomatic weed survival (Gente et al. 2005).

Currently *X. euvesicatoria* pv. *allii* contains only a complete genomic sequence (NCBI, 2021). The genome size of *X. euvesicatoria* pv. *allii* is 5,425,942 bp with a

G + C content of 64.4%, showing similarities with the gene content of other Xanthomonas species. It presents typical regions found in Xanthomonas, such as the type III secretion system (TTSS) and about 22 type III effector genes (Gagnevin et al. 2014). The isolates of *X. euvesicatoria* pv. *allii* have considerable genetic diversity and are less host-specific than is commonly expected of a pathovar of Xanthomonas (Gent et al. 2005; Gagnevin et al. 2014). Gent et al. (2003) identified a great genetic diversity in *X. euvesicatoria* pv. *allii* based on polyphasic characterization studies and using rep-PCR markers (BOX, ERIC and REP). Picard et al. (2008), also based on the same methodology, identified a great diversity among isolates from two islands of the Mascarenhas archipelago, reporting the occurrence of two groups from AFLP (Amplified fragment length polymorphism) and RFLP (restriction fragment length polymorphism) analyses. In a previous study, Gent et al. (2005), found by molecular markers rep-PCR, the genetic proximity between isolates of *X. euvesicatoria* pv. *allii* and *X. axonopodis* pv. *citrumelo*, speculating that they originated from a common ancestor.

Bacterial blight caused by *X. euvesicatoria* pv. *allii* is an expanding disease in the world, leading to outbreaks in several producing countries, a fact that raises concerns for vegetable production and further studies on this etiological agent are needed (Robène et al. 2015). Bacterial blight can cause losses of 10 to 50% in bulb yield, although it does not cause symptoms in the same, however, it interferes with the photosynthetic rate and nutrient assimilation for the plant due to foliar infection (Schwartz and Otto, 2000; Nunez et al. al. 2002). Due to the risk to onion production, since 2009 the bacterium considered as an absent quarantine pest (A1) by the European and Mediterranean Plant Protection Organization (EPPO) (EPPO, 2020).

1.7. Symptoms of bacterial blight of onion

The appearance of bacterial bight symptoms on onion varies with the types of varieties. In short-day varieties the symptoms generally appear at any stage of crop development. On the other hand, in long-day varieties the symptoms tend to emerge

during or after bulb initiation, (Pruvost et al., 2016). Initially lesions with water-soaked margins appear on the leaves. These lesions rapidly enlarge and their colour becomes tan or brown, characteristic of the heavy water-soaking (Picard et al., 2008; Nunez et al., 2002). In some cultivars chlorotic streaks may develop along the entire length of the leaves. When the temperature increases and relative humidity decreases (dry atmospheric conditions) the infected leaves become desiccated and brittle (EPPO, 2016).

As the disease further develops, coalescence of the lesions occurs causing tip dieback which leads to extensive blighting of the older, outer leaves (Gent and Schwartz, 2008). There is correlation between the loss of leaf area and stunted plants or undersized bulbs which are attained when the disease manifests. The dessication of the plant leaves accentuates senescence hence reducing photosynthetic area and dramatically shrinking the bulb size. A cocktail of factors such as weather conditions, timing of disease onset and cultivar susceptibility determine the extent of crop damage (Pruvost et al., 2016).

Onion bulb size is highly affected by the defoliation due to the disease during bulb initiation stage and less sensitive to defoliation towards maturation of the bulbs. Therefore, crop damage is most severe disease incidence occurs in the early growth stage (Bartolo et al., 1994). Roumagnac et al. (2004b) carried out temporal analysis on the development of onion bacterial blight in the tropical environment and discovered that bulb imitation is the most susceptible growth stage. When seedling infection occurs, severe defoliation follows leading to complete loss of the crop. Though Xanthomonas leaf blight appear on the members of the Allium genus such as chive, leek, garlic, Welsh onion and shallot they are more severe on onion (Gent and Schwartz, 2008).

In Barbados some strains of the bacterium have been identified as pathogenic to leguminous plants such as snap beans (*Phaseolus vulgaris*), soybean (*Glycine max*), lima bean (*Phaseolus lunatus*), pea (*Pisum sativa*), moth bean (*Vigna aconitifolia*)

and winged bean (*Psophocarpus tetragonolobus*). However, endeavours to reproduce disease symptoms on legumes in other places outside Barbados have been futile. Alvarez et al. (1978) reported that a hypersensitive response was in snap bean by isolates of the bacteria from Hawaii. Certain strains of *X. axonopodis* pv. *allii* symptoms of small, water-soaked lesions on the leguminous host, snap bean. However, these lesions remain small and develop necrosis within 7 days (Roumagnac et al., 2004a). In an experiment carried out by Gent et al. (2004) snap bean inoculated with *X. axonopodis* pv. *allii* did not produce symptoms despite the fact that the pathogen multiplied in the bean leaves and attained high population levels. The results of these studies reveal that the pathogen can persist as an epiphyte that is asymptomatic on some leguminous hosts (Gent et al., 2005).

Fig 1. Characteristic symptoms of *X. euvesicatoria* pv. *allii*. Photographs from Wiley Online Library and Invasive.Org

Geographical distribution of *Xanthomonas euvesicatoria* pv. *allii*

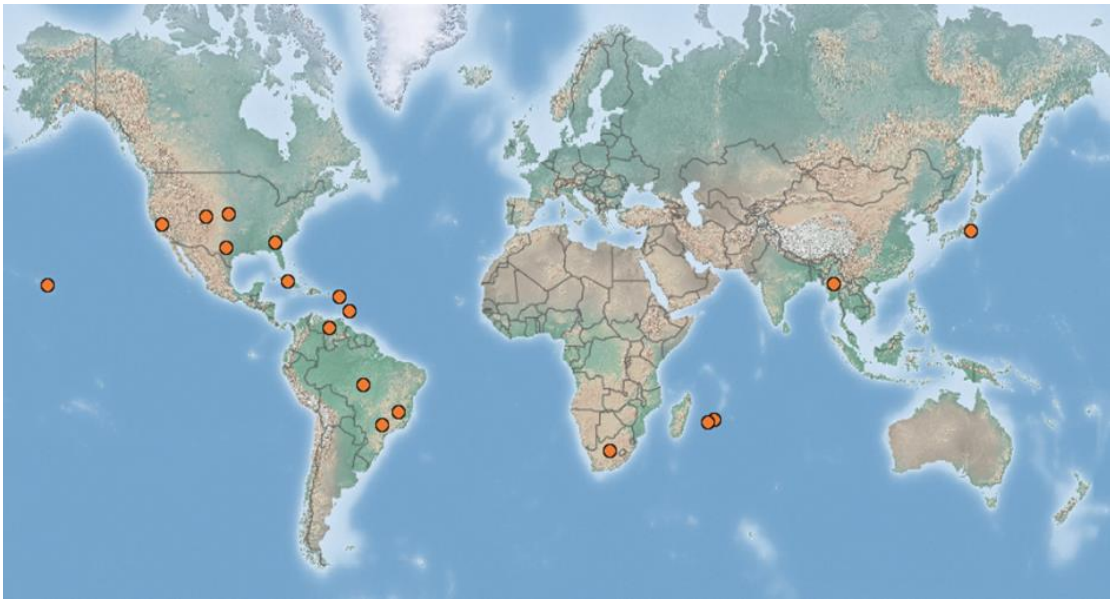


Fig 2. Map showing regions where the pathogens is present across the globe. Source: https://www.cabi.org/isc/

Xanthomonas euvesicatoria pv. *allii* is geographically distributed across 3 continents: America, Asia and Africa. On the American continent the pathogen is present across different parts i.e. North America (USA), Central America and Caribbean (Cuba, Barbados, St. Kitts Nevis) and South America (Venezuela, Brazil). In Asia the bacteria is established in Japan and Myanmar. On the African continent the pathogen's presence is in 3 countries, namely South Africa, Mauritius and Reunion (CABI, 2022).

Table 1. Distribution of *X. euvesicatoria* pv. *allii* across the globe

Geographical Region	Year of Report	Reference
Hawaii	1978	(Alvarez et al., 1978)
Brazil	1987	(Neto et al., 1987)

Barbados	1993	(Pauraj and O'Garro, 1993)
Cuba	1993	(Paulraj and O'Garro, 1993)
St Kitts and Nevis	1997	(O'Garro and Pauraj, 1997)
Venezuela	1999	(Trujillo and Hernandez, 1999)
Continental USA	2000	(Isakeit et al., 2000; Schwartz and Otto, 2000)
Japan	2000	(Kadota et al., 2000)
Mauritius	2000	(Roumagnac et al.,2000)
Reunion Island	2000	(Roumagnac et al., 2000)
South Africa	2001	(Serfontein, 2001)
Myanmar	2016	(IPPC, 2016)

1.8. Disease cycle and epidemiology

The ideal conditions for the development of the disease are regions of hot and humid climate with temperatures of 28 to 35 °C as well as high rainfall especially during bulb development (Schwartz et al. 2003; Humeau et al. 2006). Incessant rains especially accompanied with strong winds after bulb initiation create weather conditions conducive for the development of severe epidemics. Generally, in the initial stages of development temperature is the main driving factor whereas at harvesting stage disease severity is chiefly associated with rainfall amount and frequency (Schwartz et al., 2003).

The main reason for temperature playing an important role in disease development in the early growth stages is related to its vitality in the bulb initiation phase. After bulb development when disease incidence has taken place, disease spread and severity depend on rain (Schwartz et al., 2003; Gent et al., 2005b). Moreover, overhead irrigation and overcast humid conditions also favour disease development. Secondary infection takes place when splashing water or aerosols transmit *X. euvesicatoria* pv. *allii* onto leaves. Multiplication of the bacteria in turn occurs forming large epiphytic populations infecting the plants through natural openings i.e. stomata and wounds.

The main route of blight transmission is through onion seed contaminated by the pathogen, which may explain the fact of its rapid worldwide spread (Roumagnac et al. 2000). There are also reports of other sources of bacterial inoculum such as invasive plants, irrigation water, crop residues and other crops used in rotation with onion (Gent et al. 2005b). In a study by Roumagnac et al. (2004), a seed lot as a primary inoculum source with a contamination rate of 4/10,000 was shown to be efficient to develop an outbreak of bacterial blight under field conditions in the cold season of a tropical island. Disease pattern analyses revealed that the inoculum associated with the early stages of epidemics was most likely seedborne. Spatial analyses using several statistical methods revealed aggregated patterns of disease incidence data. Primary foci grew over time, and a few distinct secondary foci emerged after the occurrence of wind-driven rains (with gusts up to 15 m s–1). The distance range between primary and secondary foci was from less than 1 m (satellite foci) to 25 m. However, the authors acknowledge the possibility of long-distance dispersal of inoculum being partially involved in the later stages of the epidemics.

In a study by (Gent et al. 2005b) *X. euvesicatoria* pv. *allii* was recovered from irrigation tail water from fields where onion leaf blight symptoms were visible. Moreover, the bacterial populations increased with the increment in the number of days from the date of inoculum. This illustrates that irrigation tail water is an

efficient dispersal method of *X. euvesicatoria* pv. *allii* in onion fields. This is particularly rampant in scenarios where surface irrigation is used particularly furrow irrigation, as water is recollected in the canal to irrigate down-stream fields. The researchers identified crop debris as an important source of inoculum. Significant populations of *X. euvesicatoria* pv. *allii* were found overwintering in diseased onion leaves buried 25cm deep in the soil. However, the bacterial population found in debris on the soil surface was higher 10^6 CFU/ml as compared to that buried in the soil 10^4 CFU/ml, evaluated after a period of 8 months. Though they managed to recover *X. euvesicatoria* pv. *allii* in weeds, the study revealed that not all weed species support epiphytic growth of the bacteria. It was also observed that leguminous crop plants are also susceptible to the bacteria hence serving as alternative hosts and an inoculum source especially used in rotation with the onion crop. Additionally, the pathogen did not persist for more than a year on the hosts. This therefore reduces the likelihood of weeds being the primary medium for bacterial persistence in the absence of onion. Volunteer onion was consistently found to be an early season source of the pathogen.

1.9. Detection and identification

Since the realization that the spread of bacterial blight in onions was through seeds, studies based on PCR (Polymerase chain reaction) have been performed to make the detection of the pathogen in lots of contaminated seeds more efficient. Robene-Soustrade et al. (2010) using the Multiplex Nested PCR technique, performed the amplification of two specific genes present in the genome of *X. euvesicatoria* pv. *allii*, which resulted in the detection of the bacteria in seed lots with contamination rates 5×10^2 CFU/g, the Multiplex Nested PCR assay showed sensitivity in detecting 1 infected seed in 27,340 seeds. Robene et al. (2015) described a real-time PCR assay and was able to detect the presence of all isolates of *X. euvesicatoria* pv. *allii* tested, and the technique presented the detection limit of 1 infected seed in 32,790 seeds. These results are promising and of great importance to facilitate detection and

ensure that commercial seed lots are not contaminated, preventing the spread of this important onion crop pathogen.

1.10. Control

The main way to control the bacterial blight of onion caused by *X. euvesicatoria* pv. *allii* is the application of copper-based bactericides combined with ethylenebis dithiocarbamate fungicides. This is a method widely used in the United States, despite being a high-cost and environmentally harmful management (Gent and Schwartz, 2005). Integrated management can also be an option using crop rotation and biological control, but often not as effective as conventional control, since the range of hosts of the bacterium is not yet fully known and alternative controls such as biological can still be used not be so accessible (EPPO, 2016). Crop rotation is an option, but it should be further studied since the alternation between onions and some vegetables can favour the epiphytic survival of the bacteria or, in some cases, the bacteria can be pathogenic to these cultures (O'Garro and Paulraj, 1997; Gent et al. 2005). The application of a formulated product containing bacterial isolates of *Pantoea agglomerans* and *Pseudomonas fluorescens* for the biological control of bacterial blight in onions reduced the severity of symptoms, but the differences were not significant (Gent and Schwartz, 2005).

There are still few studies regarding other viable forms of control for bacterial blight in onions. Lang et al. (2007) using a plant defence activator, acibenzolar-S-methyl, and bacteriophages, showed a 50% reduction in disease severity in the field, which proves to be an alternative to massive applications of copper-based pesticides. A recent study by Nga et al. (2021) tested the efficacy of using specific lytic bacteriophages for *X. euvesicatoria* pv. *allii* in the control of bacterial blight in Welsh onion. The reduction in the incidence of symptoms in the leaf area in the presence of phages was about 70% in greenhouse. Under field conditions, one of the bacteriophages led to a reduction in the incidence of symptoms equal to the commercial bactericide Starner. Moreover, the phage strain and concentration play

a vital role in suppressing the bacteria. It was observed that bacterial phage Φ31 was the most effective in controlling the pathogen, reducing leaf infection to 26.6% as compared to 67.5% in the positive control. The optimal concentrations for satisfactory disease control were 10^7 and 10^8.

Yanti (2015) carried out a study involving the induction of resistance to bacterial blight caused by *X. euvesicatoria* pv. *allii*, evaluating the introduction of rhizobacteria in shallot (*Allium oschaninii*) plants, which contributed to the increase in the activity of the peroxidase enzyme that is involved in plant defence responses. However, six days after inoculation, this enzymatic activity began to decrease continuously. To date, there are no cultivars of the Allium genus that have any type of resistance to *X. euvesicatoria* pv. *allii*.

1.11. Pest significance and economic impact

The bacterium has been designated as a quarantine pest on EPPO's A1 list (2016). This is due to the horrendous damage the pathogen cause on onion and related *Allium species*. Cases have been reported where the pathogen caused 10-50% yield losses in the onion crop (Sanders et al., 2003; Nunez et al., 2002; Schwartz and Otto, 2000). In tropical regions the disease has higher impact for instance results of trials undertaken in Barbados revealed that in some cases there was destruction of the entire onion crop due to the bacteria (O'Garro and Paulraj, 1997). In South Africa Serfontein (2001) reported severe bulb size reduction when the disease occurred. Moreover *X. euvesicatoria* pv. *allii* increases the cost of onion production dramatically as farmers use various chemicals which are expensive. Lang et al. (2007) computed the cost of controlling the pathogen chemically using copper hydroxyl and mancozeb in infested fields and have disclosed that it costs approximately $250 per hectare.

Applying acibenzolar-S- methyl on average 3 times in a season as a preventive measure is 50% more expensive than the conventional copper-based control programs. Though bacteriophages e.g. (Agriphage) have demonstrated great

potential in controlling the disease the cost of applying them per hectare is twofold or threefold that of the conventional program (Lang et al., 2007). This means that until innovations have been made that can reduce the cost of bacteriophages onion producers will continue to heavily rely on unsustainable methods of control. Therefore, this pest poses a serious challenge to sustainable development.

1.13. Copper resistance in Xanthomonas

Copper-based products are widely used to control bacterial infections in agricultural crops, but long-term continuous use favours the selection of bacterial isolates insensitive to the chemical element, which makes effective control unfeasible (Sundin et al. 1989). There are several reports of Xanthomonas copper resistance in different crops. In citrus, isolates of *X. citri* subsp. *citri* were initially described by Canteros (1999) showing insensitivity to copper. Recently in Réunion and Martinique (Richard et al. 2016; Richard et al. 2017) isolates of *X. citri* subsp. insensitive citrus. In citrus nurseries in Florida (USA) the insensitivity to copper present in isolates of *X. alfalfae* subsp. *citrumelonis* (Behlau et al. 2011).

Copper resistance in phytopathogenic bacteria is linked to the presence of the copABCD operon. Initially described in *Pseudomonas syringae*, this operon encodes Cop proteins A, B, C and D, which confer insensitivity to cupric compounds, and generally is found on the plasmid (Mellano and Cooksey, 1988; Cha and Cooksey, 1991). Although most copper resistance genes in phytopathogenic bacteria are of plasmid origin, there are already reports of chromosomal resistance genes (Lee et al. 1994; Basim et al. 2005). According to Mellano and Cooksey (1988), the insensitivity to copper in bacteria is attributed to several genes usually organized in operons, therefore, it is unlikely that a spontaneous mutation that induces resistance to copper in bacterial populations will occur. Conjugation is the main mechanism of horizontal gene transfer that disseminates copper resistance in bacteria, demonstrated in a study by Behlau et al. (2013) in *X. citri* subsp. *citri* and *X. alfalfae* subsp. *citrumelonis*. In Xanthomonas,

in addition to the copABCD operon, the presence of the copLAB operon has already been reported,

Copper resistance genes have already been characterized and cloned in *X. arboricola* pv. *juglandis*, *X. vesicatoria* and *X. euvesicatoria* pv. *vesicatoria* (Lee et al. 1994; Basim et al. 2005; Voloudakis et al. 2005). In *X. arboricola* pv. *juglandis* copper insensitivity genes are present in the chromosome and have the same conformation as the copABCD operon of *Pseudomonas syringae*, differing only in gene size and in some regions of the DNA sequence (Lee et al. 1994). The copper resistance genes contained in the plasmid of X. blistert also resemble the cop operon of *P. syringae* (Vouloudakis et al. 1993).

The organization of copper resistance genes in *X. vesicatoria*, when it occurs in the chromosome, differs from when present in the plasmid, being rarer for the species (Basim et al. 2005). In the species *X. euvesicatoria* pv. *vesicatoria* it was verified that the transmission of copper resistance genes occurs via plasmids and the regulation of the expression of these genes is regulated by the copL operon (Voloudakis et al. 2005). In *X. euvesicatoria* pv. *vesicatoria* was genes homologous to the copRS regulatory operon of *P. syringae* were identified in the chromosome of a single isolate (Basim et al. 2005).

In addition to the genes that confer copper resistance in bacteria genes that induce levels of copper tolerance called cop genes have also been reported. These genes are involved in copper homeostasis, maintaining the balance of the amount of copper present inside the bacterial cell (Arguello et al. 2013). Isolates that present these genes are not considered sensitive, as they tolerate doses of copper and are not considered effectively resistant since they do not have the cop genes (Marin et al. 2019).

1.14. Genetic diversity of *Xanthomonas*

Over the years, taxonomic and diversity studies on Xanthomonas have been improving, incorporating molecular techniques based on genomics and phylogeny,

along with traditional methods such as phenotypic characterization (Vauterin et al. 1995; Vauterin and Swings 1997; Constantin et al. 2016). The genus Xanthomonas comprises one of the largest groups of bacteria described through DNA-DNA homology and genomic fingerprinting studies, and the internationally accepted DNA-DNA reassociation value to consider that two organisms belong to the same species must be equal to or greater than 70. %. However, DNA-DNA hybridization methods are more complex and expensive, which makes the analysis of a large number of isolates unfeasible and makes it difficult to study the diversity of bacterial isolates (Wayne et al. 1987; Rademaker et al. 2000).

With the use of different molecular markers that generate genomic fingerprinting (RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism), RAPD (random amplified polymorphic DNA), and ERIC (enterobacterial repetitive intergenic consensus), REP and BOX-PCR) to elucidate the relationships between isolates, was possible to improve the precision and agility of studies involving the diversity of Xanthomonas (Restrepo et al. 2000; Ogunjobi et al. 2010; Arshiya et al. 2014; Asgarani et al. 2015).

Restrepo et al. (2000) using analysis based on rep-PCR, AFLP and RFLP found a great diversity among 238 isolates of *X. phaseoli* pv. *manihotis* in Colombia and showed that the AFLP proved to be more robust for the analyzes compared to the RFLP. For *X. phaseoli* pv. *manihotis* from Nigeria, RAPD and AFLP techniques were applied to evaluate the genetic variability between isolates, both analyzes separate the isolates into four distinct clades, showing that they are reliable methods that complement each other in the study of diversity (Ogunjobi et al. 2010). A study demonstrated that the ERIC-PCR method is efficient to evaluate the diversity in Xanthomonas species according to the ability of isolates to produce xanthan gum or not, an exopolysaccharide produced by the genus and used mainly in the food industry (Asgarani et al. 2015). Mahuku et al. (2006) using the REP-PCR technique showed a high level of genetic distance between *X. citri* pv. *phaseoli* and *X. citri* pv.

phaseoli var. *fuscans* both pathogenic to common bean. Arshiya et al. (2014) evaluating isolates of *X citri* pv. *citri* using molecular markers such as REP, BOX and ERIC found a great genetic diversity among the isolates, BOX-PCR revealed more polymorphic bands than the other markers, and both REP and ERIC were less efficient for the analysis of diversity in *X. citri* pv. *citrus phaseoli* var. f*uscans* both pathogenic to common bean. Arshiya et al. (2014) evaluating isolates of *X citri* pv. *citri* using molecular markers such as REP, BOX and ERIC found a great genetic diversity among the isolates, BOX-PCR revealed more polymorphic bands than the other markers, and both REP and ERIC were less efficient for the analysis of diversity in *X. citri* pv. *citrus phaseoli* var. *fuscans* both pathogenic to common bean.

In *X. euvesicatoria* pv. *allii* studies using molecular markers were carried out, evidencing the diversity among isolates of the species, as Gent et al. (2003) using rep-PCR markers (BOX, ERIC and REP), Picard et al. (2008) who reported a great diversity among *X. euvesicatoria* pv. *allii* from two islands of the Mascarenhas archipelago, using AFLP (Amplified fragment length polymorphism) and RFLP (restriction fragment length polymorphism) markers and Gent et al. (2005) who showed by means of rep-PCR the genetic proximity between isolates of *X. euvesicatoria* pv. *allii* and *X. axonopodis* pv. *citrumelo*.

In general, the analysis of a single gene is not enough to assess the diversity in a bacterial population and elucidate phylogenetic issues at the species level, so, in order to improve the study of bacterial phylogeny, research based on the analysis of multiple genes emerged (Rodriguez et al. 2012). Young et al. (2008) using MLSA analysis (multilocus sequence analysis) and sequencing of four housekeeping genes (dnaK, fyuA, gyrB and rpoD), showed two different groups between species of the genus Xanthomonas. Young et al. (2010) using the same four housekeeping genes identified a great diversity in Xanthomonas isolates from New Zealand and proposed a new species *X. dyei*. Ntambo et al. (2019) used five genes atpD, glnA, gyrB, abc and rpoD to assess the phylogeny of *X. albilineans* evidencing the diversity among

isolates of the species. A study of MLSA revealed a high level of genetic diversity as a function of recombination and retention of point mutations in *X. campestris* (Fargier et al. 2011). Based on DNA-DNA hybridization, ANI (average nucleotide identity) and MLSA analysis, Constantin et al. (2016) reassessed the taxonomy of *X. axonopodis* and proposed some changes to the species, such as *X. axonopodis* pv. *allii* that belonged to the species *X. euvesicatoria*, since in the phylogeny it grouped in clade II of Xanthomonas. (2016) reassessed the taxonomy of *X. axonopodis* and proposed some changes to the species, such as *X. axonopodis* pv. *allii* that belonged to the species *X. euvesicatoria*, since in the phylogeny it grouped in clade II of Xanthomonas. (2016) reassessed the taxonomy of *X. axonopodis* and proposed some changes to the species, such as *X. axonopodis* pv. *allii* that belonged to the species *X. euvesicatoria*, since in the phylogeny it grouped in clade II of Xanthomonas.

With the increasing availability of fully sequenced genomes, studies based on comparative and evolutionary genomics have become facilitators for phylogenetic analyzes in Xanthomonas (Moreira et al. 2010, Rodriguez et al. 2012). Currently, there are more than 1,400 Xanthomonas genomes deposited in the database of the National Center for Biotechnology Information (NCBI), the GenBank (Timilsina et al. 2020). Genomic sequence analysis for species delimitation has been applied as a substitute for DNA-DNA hybridization and also for the ANI method (Konstantinidis and Tiedje, 2005). The ANI is a technique that makes use of sequences that can be retrieved from databases, the ANI value for an organism to be considered of the same species is equivalent to 95%-96% (Richter and Rosselló-Móra 2009). Young et al. (2008) performed a comparative study between DNA-DNA homology and genomic sequence similarity to evidence clades and species in Xanthomonas. Moreira et al. (2010) sequenced the genome of *X. fuscans* subsp. *aurantifolii* comparing with the genome of *X. citri* subsp. *citri* both causes citrus canker and showed several distinct genes between the species mainly related to pathogenicity. Rodriguez et al. (2012) based on phylogenomic analysis proposed that *X. citri* and

X. fuscans comprise the same species. Assis et al. (2021) in a comparative genomics study with *X. arboricola* pv.

Chapter 2
Materials and Methods

The *X. euvesicatoria pv. allii* strains (0378) and (0419) used in this study were accessed from the All-Russian Plant Quarantine Centre bacterial collection. Bacterial specimen stored at -80℃ were used to initiate the culturing process. Bacterial suspension was prepared by transferring a colony of the bacteria to 1ml of distilled water and mixing it on a high-speed vortex for 5 seconds. From this bacterial suspension, tenfold serial dilution was performed. Each bacterial dilution was plated in triplicate replication on Yeast Peptone Glucose Agar (YPGA) media by apportioning 50µl aliquots per petri dish. The Petri dishes were then incubated at 28℃ for 48 hours and the colonies were counted thereafter. The bacterial concentration in the series ranged from 3×10^1 to 3×10^7 CFU/ml.

2.1.1 Seed sample preparation

By virtue of BBO being seed-transmitted and seed samples being the object of interest in diagnostic assays for *X. euvesicatoria pv. allii*, plant extract was prepared using commercial onion seed. Thirty thousand onion seeds weighing 30 grams were used to make 6 samples of 5 g each for ease of carrying out the following procedure. Thereafter, 25ml of PBS buffer solution was added to each sample then left to soak at 4℃ for 24 hours before being put on a shaker at a rate of 200 rotations per minute for 2 hours. The samples were then transferred to filter bags. Each sample was pulverized and homogenized using a rubber mallet and the sap from the macerate was filtered into sterile tubes. The extract was centrifuged at 8000rpm/15 mins and the supernatant was blended in three 50ml tubes ready for use. Diagnostic assays using Real-Time PCR to ascertain the absence of the *X. euvesicatoria pv. allii* bacteria in the extract. Inoculation via sevenfold serial dilution with the bacteria from the prepared suspension was carried out on the samples.

2.1.2 DNA Extraction

The instructions provided in the manufacturers' protocols were strictly adhered to. To initiate the extraction process, 200ml aliquots in triplicate for each dilution stock were performed. For the Probe GC extraction method (Agrodiagnostica, Russia) the cell lysing agent was heated for 20 minutes at 50℃ prior to mixing with the silica sorbent and then added to the samples. The samples were then heated with a dry bath incubator (heat block) at 50℃ for 20 minutes. Subsequently, the tubes were centrifuged at 13 000g/min and the supernatant was discarded whilst the sorbent binding the DNA was retained. A series of washing and rinsing using the corresponding reagents for particular stages. The DNA was eluted from the silicon sorbent by adding 100µl of elution buffer that comes with the kit and incubating at 50℃ for 5 mins before centrifugation at 13000 g/min and transferring 70µl of the nucleic acids in sterile tubes.

The FitoSorb kit (Syntol, Russia) is a magnetic bead DNA purification method. Moreover, this method starts with the extraction phase, hence 500µl of extraction was added to each sample and centrifuged at 4000g/min. Thereafter, 700µl of the supernatant was transferred to new tubes for the lysing process whereby 500µl lysing buffer having been preheated at 60℃/20mins and 25 µl magnetic particle sorbent were added to each sample. The samples were then incubated at 65℃/10 minutes and the tubes were vortexed after every minute during the incubation period. The tubes were then centrifuged at 13000 g/5min and 600µl supernatant was put in tubes where 500µl of the precipitating solution and 60µl of sorbent were added. The tubes were held at room temperature (28℃) before centrifugation at 4000g/minute and put on a magnetic rack to retain the magnetic bead-bound DNA whilst the supernatant was discarded. A sequence of washing and rinsing stages using appropriate reagents provided in the kit followed. Finally, DNA was eluted by adding 100µl of elution buffer and 100µl of the nucleic acids was put in new tubes.

The Sorb-GMO kit (Syntol, Russia) is optimized for DNA extraction from food products, plant material and feeds. The method utilizes a silicon sorbent to capture the DNA. It constitutes an ionic detergent CTAB which provides maximum DNA release from plant components. To start off, 800µl of preheated (60°C/20mins) buffer and 15µl proteinase was added to every sample before being incubated on the heat block at 60°C for 60 minutes. The tubes were left to cool for 2 minutes and then centrifuged for 5 minutes at 13000 g/min. Subsequently, 600µl of non-precipitate supernatant was transferred to new tubes. After this 500µl of chloroform was added to remove the decomposed protein, hence releasing the DNA. Furthermore, 300µl of non-precipitate supernatant was transferred to new tubes in which 500µl of the precipitating agent and 25µl of silicon sorbent were added, to retain the DNA. Washing and rinsing procedures followed before the DNA was eluted by adding 100 µl of TE buffer to each sample.

2.1.3. Real-time PCR analysis

Simplex real-time PCR (Robene-Soustrade et al., 2010), specific for *X. euvesicatoria pv. allii* using the Xaa avr primers designed by Evrogen (Moscow, Russia) was utilized to compare the influence of the 3 extraction kits on the sensitivity of the assay. The reactions were carried out in 20µl volumes, containing 5µl 5x PCR master mix (Evrogen, Russia), 0.1µl MGB probe (AGENCY KHEMEXPERT LLC, Russia), 1µl for each primer, 12.9µl of distilled water and 2µl of sample DNA or distilled water in the negative controls. The thermal cycle program was as follows: 1 cycle at 50°C for 2 minutes, initial denaturation phase at 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Real-time PCR was carried out using the DNA Technology amplifier (DNA Technology, Russia).

2.2.1 Experimental design- evaluation of varying bacterial concentration on onion seed germination

The experiment was arranged in a complete randomized design (CRD) replicated thrice. Three bacterial concentrations were prepared, 10^8, 10^6 and 10^4. Distilled water was used as a negative control. Twelve commercial varieties of onion were procured. For each variety the concentrations were replicated 3 times. One hundred seeds were put in each petri dish.

Table 2. Commercial onion varieties that were used in the study

No.	Variety	Originator	Main features
1	Pierrot	Agrofirm Aelita	Welsh onion (*Allium fistulosum* L.)
2	Russian winter	FSBSI- Federal Research Center for Vegetable Production	Welsh onion (*Allium fistulosum* L.)
3	April	Far Eastern Experimental Station – VNIIR/CJSC LANS Company	Welsh onion (*Allium fistulosum* L.)
4	Karantansky	Agrofirm Aelita LLC/Heterosis Selection LLC	Leek (*Allium porrum* L.)
5	Summer breeze	Gavrish vegetable breeding company	Leek (*Allium porrum* L.)
6	Strigunovsky local	CJSC Research and Production Firm "RUSSIAN SEEDS";	Bulb onion (*Allium cepa* L.)

		Association for Seed Production of Vegetable Crops “SORTSEMOVOSCH”; Agrofirm Aelita LLC; FSBI "Federal Research Center of Vegetable Production"; Sativa LLC; Heterosis Selection LLC	
7	Carmen MS	Agrofirm Aelita LLC/Alt Semena	Bulb onion (*Allium cepa* L.)
8	Schtuttgarter Riesen	Samen Mauser Quedlinburg, Germany	Bulb onion (*Allium cepa* L.)
9	Myachkovsky 300	FSBI "Federal Research Center of Vegetable Production"; CJSC "LANS Company"; JSC "OZYORY"	Bulb onion (*Allium cepa* L.)
10	Danilovsky 301	Federal Research Center for Vegetable Production, Agrofirm Aelita LLC, LANS Company	Bulb onion (*Allium cepa* L.)
11	Chalcedony	Yuves 2000 LLC, Intersemya LLC, Agrofirm Poisk LLC, Heterosic Selection LLC	Bulb onion (*Allium cepa* L.)

12	Ellan	FSBEI HE "Kuban State Agrarian University Named after I.T. Trubilin"	Bulb onion (*Allium cepa* L.)

2.2.2. Inoculation

In each petri dish containing 100 seeds, 5ml of bacterial suspension was aliquoted. The petri dishes were then incubated at room temperature for 2 hours. Following the incubation period, the bacterial suspension was drained from the seeds. The seeds were then left to dry at room temperature.

2.2.3 Incubation

The dry inoculated seeds were then transferred to petri dishes lined at the bottom with 9cm filter paper. Distilled water was added to the petri dishes. The petri dishes were then placed in an incubator at 21℃ under dark conditions. The seeds were incubated for 14 days.

2.2.4. Data Recording

Daily routine checks were conducted and water was added when necessary. The first recording taking into account germination energy was done on the 5th day. The second and final recording was done on the 14th day.

2.3.1 Greenhouse Trial

Two seed samples were secured, one comprising of fungicide treated seed and the other with non-treated seed. One hundred seeds were put in each container, whereby bacterial suspension was added, immersing the seed. The containers were then put on a shaker at 100g/min for 4 hours. The bacterial suspension was drained from the seeds. The seeds were dried. Upon drying the seeds were taken to the greenhouse for sowing. Four 14-cell trays were filled with garden peat. Two seeds were sown per cell and watered. The plants were exposed to an 18-hour photoperiod whereby thegreenhouse lights were automatically switched on at 10 pm and automatically

switched off at 9 am when natural light would be available and able to penetrate. The dark period began from 4 pm to 10 pm, thus 6 hours of non-exposure to light. This was done so as to enhance photosynthesis, thus accelerating growth of the plants. Relative humidity was kept at 70% so as to allow for optimum transpiration whilst not dehydrating the plants. Moreover, regular watering was done every other day, hence keeping the plants lush.

Hence there were 2 sections of the experiment, one constituting inoculated treated seed and the other with inoculated untreated seed. The trays were routinely watered as per crop water requirement. The aim was to identify and evaluate the bacterial symptoms on the growing plants.

2.3.2. PCR Tests on inoculated seed

Three seed samples with weights of 1g, 5g and 10g respectively were used. Each seed sample was replicated 3 times. In each sample 1% of the seed was separated and inoculated via tenfold serial dilution with the bacteria from the prepared suspension. The containers with the inoculated seed were then put on a shaker at 100g/min for 4 hours. The bacterial suspension was drained from the seeds. The seeds were dried. The dry inoculated seeds were then mixed with the rest of the seed samples. Thereafter, 25ml of PBS buffer solution was added to each sample in the homogenizer bags. Each sample was pulverized and homogenized using a homogenizer machine and the sap of the extract was filtered into sterile 50ml tubes ready for use. The objective was to ascertain using PCR whether the seeds had been successfully inoculated with the bacteria.

2.3.3. DNA extraction

Firstly, 200µl of seed extract in triplicate for each sample was taken for DNA extraction. The cell lysing agent was heated for 20 minutes at 50°C prior to mixing with the silica sorbent and then added to the samples. The samples were then heated with a dry bath incubator (heat block) at 50°C for 20 minutes. Subsequently the tubes were centrifuged at 13 000g/min and the supernatant was discarded whilst the

sorbent binding the DNA was retained. A series of washing and rinsing using the corresponding reagents for particular stages followed. The DNA was eluted from the silicon sorbent by adding 100µl of elution buffer and incubating at 50°C for 5 mins prior to centrifugation at 13000 g/min and aliquoting 70µl of the nucleic acids in sterile tubes. Diagnostic assays using Real-Time PCR to ascertain the absence of the *X. euvesicatoria* pv. *allii* bacteria in the extract were performed afterwards.

2.4. Host range of two strains of *Xanthomonas euvesicatoria* pv. *allii* on five members of the genus Allium

Seeds of five Allium species namely onion (*Allium cepa*), chives (*Allium schoenoprasum*), Welsh onion (*Allium fistulosum*), fragrant onion (*Allium ramosum*) and leek (*Allium porrum*) were procured for host selectivity studies. The research was premised upon exploring the host selectivity of 2 strains of *Xanthomonas euvesicatoria* pv. *allii* specifically 0377 and 0419 which belong to the bacterial collection of the All-Russian Plan Quarantine Centre.

2.4.1. Plant extract preparation from the seed

A sample of 5 grams was weighed for each species. This was done for the purpose of simplifying the procedure. The seed samples were then put in homogenization bags and 25 ml of PBS buffer solution was added to each of them. Subsequently the seeds were pounded in a Bagmixer homogenizer (Interscience, France). The liquid was separated from the crushed seed particles by the bag filter and collected into centrifuge tubes. The extract was then centrifuged at 8000g for 15 minutes at temperature of 4°C to aggregate the plant cells. The supernatant was decanted and 1 ml sterile PBS buffer was added to the precipitate, hence resuspending it.

2.4.2. Inoculation of the extract (plant cells) with *X. euvesicatoria* pv. *allii*

Conserved colonies of the 2 bacterial strains of *X. euvesicatoria* pv. *allii* namely 0377 and 0419 were put in 1ml distilled water forming a concentrated bacterial solution which was used to make stock solutions. Ten-fold serial dilutions with the bacterial suspensions was carried out on all the samples, thus inoculating the various

plant extracts with the 2 bacterial strains. Moreover, tenfold serial dilutions with distilled water were also performed for the plate counting exercise. This was done in a bid to determine the amount of colony forming units (CFU) for each dilution factor.

2.4.3. DNA extraction

For each dilution stock per sample, 3 replicates of 200 ml inoculated extract were taken for DNA extraction. Furthermore, negative controls using distilled water were also taken for extraction together with the inoculated samples. The DNA kit Probe GC adapted for DNA extraction in plant material was used to isolate the DNA from the plant extract. The extracted DNA was put in new and labelled microtubes ready for PCR tests.

2.4.4. PCR Evaluation

Before the PCR tests were carried out, the plate counting results were taken into consideration. It was decided to take for PCR tests the dilution factors with CFU in the range 10^1, 10^2 and 10^3. The CFU range corresponded with the stock dilutions 6, 5 and 4 respectively. Therefore, for the 2 bacterial strains tested each seed specimen had 3 dilution factors replicated thrice, hence 9 treatments per sample and 90 entries for the whole experiment. Two *X. euvesicatoria* pv. *allii* primers (Xa.a. AVR and X.a.a. PIL) were used to detect the bacteria in the samples and the results were used to determine the bacteria's host selectivity among the 5 *Allium species*.

2.5. Testing of molecular genetic tests

Below is a list of oligonucleotides, composition of reaction mixtures and amplification modes for PCR (Tables 3, 4, 5).

The target in the genome are two sequences encoding proteins PilW/PilX (pil marker) and the avrRxv avirulence gene (avr marker).

2.5.1. Real-time PCR according to Robene et al. (2015)

List of oligonucleotides:

Please, give as a Table with basic properties of the primers (genome localization, length, Ta, expected product of PCR)

Xaa-pil (HEX) 5'-TGGTGGCCTCAGGAG-3' -MGB

Xaa-pilF 5' -CACGACCACTGCTGGAACA-3'

Xaa-pilR 5'-CATATCGACCGGCAAGGTTT-3'

Xaa-avr (FAM) 5' -TGCTGAGTCAGCCTC-3'-MGB

Xaa-avrF 5'-TCGAGCAGCAGTCGTTTTCA-3'

Xaa-avrR 5'-GGAGGCGTAGACGCCTTACT-3'

Table 3 - Composition of reaction mixtures and amplification program for real-time PCR according to Robene et al. (2015)

Components	**Volume (V) µl**	**Working concentration**
ultrapure water	12.9	-
5x qPCRmix-HS (Eurogen)	5.0	1x
X.a.a. avr- F	0.75	10 ppm
X.a.a. avr- R	0.75	10 ppm
X.a.a. avr- MGB dye FAM	0.5	1 ppm
Internal control- **CY5 dye (Syntol)**	0.1	-
Sample DNA	5.0	-
Total Volume	25.0	-
Components	**Volume (V) µl**	**Working concentration**
ultrapure water	12.9	-
5x qPCRmix-HS (Eurogen)	5.0	1x
X.a.a. pil-F	0.75	10 ppm
X.a.a. pil-R	0.75	10 ppm

X.a.a. pil-MGB dye HEX	0.5	1 ppm
Internal control- **CY5 dye (Syntol)**	0.1	-
Sample DNA	5.0	-
Total Volume	25.0	-
Amplification conditions		
Temperature °C	**Time**	**Number of cycles**
95	10 min	1
95	15 s	40
60	1 min	

Table 4 - Composition of reaction mixtures and amplification program for real-time PCR according to Robene et al. (2015) with modified probe

Components	**Volume (V) µl**	**Working concentration**
ultrapure water	11.9	-
5x PCR buffer*	5.0	1x
X.a.a. avr- F	1.0	10 ppm
X.a.a. avr- R	1.0	10 ppm
X.a.a. avr- BHQ1- FAM dye	1.0	5 ppm
Internal Control- **CY5 dye (Syntol)**	0.1	-
Sample DNA	5.0	-
Total Volume	25.0	-
Components	**Volume (V) µl**	**Working concentration**
ultrapure water	12.9	-
5x PCR buffer*	5.0	1x
X.a.a. pil-F	0.75	10 ppm
X.a.a. pil-R	0.75	10 ppm

X.a.a. pil- BHQ1- HEX dye	0.5	5 ppm
Internal control- **CY5 dye (Syntol)**	0.1	-
Sample DNA	5.0	-
Total Volume	25.0	-
Amplification conditions		
Temperature °C	**Time**	**Number of cylces**
95	10 min	1
95	15 s	40
60	1 min	

* In this experiment, two PCR buffers 5x MasCFE Mix-2025 (Dialat) and 5x qPCRmix-HS (Eurogen) were tested.

2.5.2. Nested PCR according to Robene-Soustrade et al. (2010)

List of oligonucleotides:

Please, give as a Table with basic properties of the primers (genome localization, length, Ta, expected product of PCR)

Primers for the first stage of PCR

Pxaa1U 5’-GGCTCTAATACGACGTTGACGAT-3'

Pxaa1L 5'-AAATTCATGCGCGTTTTCAATAG-3'

Pxaa2U 5' -CTCAAGCAGCAGTCGTTTTCA-3'

Pxaa2L 5'-ATGCTTCGATTGACATGCTGT-3’

Primers for the second stage of PCR

Nxaa1U 5’-TTACGTCGCAAACAATCCAGATA-3'

Nxaa1L 5’-GGGCACCATTGACATTATCAGTT-3'

Nxaa2U 5’-ATGCCTGGTTTCGTGAA-3'

Nxaa2L 5’-CTACGGCTCAGCGACTC-3'

The amplicons are 995 bp in size. (marker avr) and 697 b.p. (pil marker) for the first PCR step. Amplicon sizes for the second stage of PCR 401 bp. (marker avr) and 447 b.p. (marker pil).

Table 5 - Composition of nested PCR reaction mixtures according to Robene-Soustrade et al. (2010) and amplification program

Components	**Volume (V) µl**	**Working concentration**
marker *pil*		
Ultrapure water	14	-
5x ScreenMix-HS (Eurogen)	5.0	1x
Pxaa1U (I stage)	1.0	10ppm
Pxaa1L (I stage)	1.0	10ppm
Nxaa1U (II stage)	1.0	10ppm
Nxaa1L (II stage)	1.0	10ppm
Sample DNA	2.0	-
Total Volume	25.0	-
marker *avr*		
Ultrapure water	14	-
5xqPCRmix-HS	5.0	1x
Pxaa 2U (I stage)	1.0	10ppm
Pxaa 2L (I stage)	1.0	10ppm
Nxaa 2U (II stage)	1.0	10ppm
Nxaa 2L (II stage)	1.0	10ppm
Sample DNA	2.0	-
Total Volume	25.0	-
Amplification conditions		
Temperature °C	Time	Number of cycles

95	10 min	1
94	30 s	40
57	30 s	
72	40 s	
72	5 min	1

Note: After the first stage with the first pair of primers, amplicons diluted 1:40 with ultrapure water were used to set up the second stage.

2.6. Determining performance criteria for PCR tests

2.6.1. Determination of analytical sensitivity

In accordance with EPPO Standard PM 7/98 (4), 3 series (replicates) of extracts artificially contaminated with the target organism are used to determine analytical sensitivity.

Samples with different levels of infection were prepared according to the following scheme:

1. An extract of vegetative parts of onion was prepared in the amount necessary to evaluate the two methods of DNA extraction by mixing previously tested extracts stored at -20 °C.

2. Prepared 7 10-fold dilutions of the base suspension with concentration.

3. 900 µl of the plant extract was transferred into new tubes and 100 µl of the bacterial suspension was added, starting from the base, with a concentration of 10^7 CFU/ml, and then sequentially, ending with a concentration of 10^1 CFU/ml. A total of 3 series (replicates) of samples were prepared from each of the 10-fold dilutions. Next, each sample was divided into sub-samples, the volume of which was 200 µl.

4. Pure PBS buffer and target-free plant extract were used as negative controls.

Table 6 . - List of strains used to verify the exclusivity of tests

No.	No. of strain in the collection	Bacterium name
1.	0028	*Clavibacter michiganensis* subsp. *sepedonicus*
2.	0039	*Ralstonia solanacearum*
3.	0044	*Erwinia billingiae*
4.	0048	*Ochrobactrum anthrapi*
5.	0049	*Clavibacter michiganensis* subsp. *michiganensis*
6.	0050	*Xilophilus ampelinus*
7.	0078	*Erwinia tasmaniensis*
8.	0093	*Acidovorax citrulli*
9.	0092	*Acidovorax citrulli*
10.	0093	*Acidovorax citrulli*
11.	0113	*Erwinia piriflorinigrans*
12.	0120	*Pantoea stewartii* subsp. *stewartii*
13.	0137	*Clavibacter michiganensis* subsp. *sepedonicus*
14.	0141	*Pectobacterium carotovorum* subsp. *carotovorum*
15.	0142	*Pectobacterium atrosepticum*
16.	0144	*Dickeya* sp.
17.	0148	*Xanthomonas campestris* pv. *raphanin*
18.	0149	*Xanthomonas aboricola* pv. *pruni*
19.	0172	*Erwinia amylovora*
20.	0174	*Ralstonia solanacearum*
21.	0204	*Pantoea stewartii* subsp. *stewartii*
22.	0222	*Pseudomonas syringae* pv. *syringae*
23.	0226	*Xanthomonas campestris* pv. *campestris*
24.	0239	*Clavibacter michiganensis* subsp. *michiganensis*
25.	0267	*Erwinia amylovora*

26.	0298	*Pantoea agglomerans*
27.	0321	*Pectobacterium atrosepticum*
28.	0327	*Pectobacterium carotovorum* subsp. *odoriferum*
29.	0329	*Pectobacterium betavasculorum*
30.	0330	*Pectobacterium cacticida*
31.	0331	*Dickeya chrysanthemi*
32.	0332	*Dickeya dadantiii* subsp. *dadantii*
33.	0333	*Dickeya paradisiaca*
34.	0334	*Dickeya zeae*
35.	0335	*Pseudomonas fuscovaginae*
36.	0419	*Xanthomonas euvesicatoria* pv. *allii*
37.	0344	*Xanthomonas gardneri*
38.	0345	*Xanthomonas fragariae*
39.	0346	*Xanthomonas fragariae*
40.	0352	*Pectobacterium atrosepticum*
41.	0353	*Dickeya solani*
42.	0367	*Strenotrophomonas maltophilia*
43.	0373	*Xanthomonas* sp.
44.	0374	*Xanthomonas vesicatoria*
45.	0375	*Xanthomonas* sp.
46.	0376	*Xanthomonas oryzae pv. oryzicola*
47.	0378	*Rathayibacter tritici*
48.	0380	*Curtobacterium flaccumfaciens* pv. *oortiii*
49.	0381	*Curtobacterium flaccumfaciens* pv. *poinsettiae*
50.	0389	*Clavibacter michiganensis* subsp. *nebraskensis*
51.	0394	*Xanthomonas* sp.
52.	0398	*Pantoea ananatis*
53.	0401	*Pseudomonas corrugate*
54.	0403	*Pseudomonas syringae* pv. *tomato*

55.	0404	*Xanthomonas gardneri*
56.	0405	*Xanthomonas vesicatoria*
57.	0406	*Xanthomonas campestris*
58.	0417	*Agrobacterium tumafaciens*
59.	0441	*Pseudomonas syringae* pv. *maculicola*
60.	0442	*Acidovorax avenae*
61.	0443	*Pantoea stewartii* subsp. *Indologenes*
62.	0445	*Rathayibacter iranicus*
63.	0446	*Xanthomonas hyacinthi*
64.	0448	*Paraburkholderia andropogonis*
65.	0451	*Pseudomonas nitroreducens*
66.	0453	*Chryzeobacterium* sp.
67.	0457	*Pectobacterium betavasculorum*
68.	0462	*Pectobacterium atrosepticum*
69.	0465	*Xanthomonas paradisiaca*
70.	0466	*Paraburkholderia plantarii*
71.	0467	*Rhodococcus fascians*
72.	0468	*Rathayibacter tritici*
73.	0470	*Agrobacterium rubi*
74.	0471	*Pectobacterium cacticida*
75.	0472	*Xanthomonas nitroreducens*
76.	0473	*Xanthomonas citri* pv. *glycines*
77.	0474	*Pseudomonas syringae* pv. *tomato*
78.	0475	*Pseudomonas cannabina* pv. *alisalensis*
79.	0109	*Erwinia piriflorinigrans*
80.	0181	*Rahnella aquatilis*
81.	0306	*Xylella fastidiosa*
82.	0336	*Dickeya dadantii* subsp. *dieffenbachiae*
83.	0337	*Xanthomonas translucens*

84.	0338	*Xanthomonas euvesicatoria*
85.	0341	*Athrobacter* sp.
86.	0343	*Xanthomonas perforans*
87.	0350	*Ochrobactrum lupini*
88.	0382	*Pseudomonas savastanoi* pv. *phaseolicola*
89.	0385	*Pseudomonas savastanoi* pv. *glycinea*
90.	0386	*Xanthomonas axonopodis* pv. *phaseoli*
91.	0420	*Xanthomonas axonopodis* pv. *axonopodis*
92.	0427	*Xanthomonas phaseoli* pv. *soyrense*
93.	0458	*Pseudomonas viridiflava*
94.	0477	*Paraburkholderia cepacian*
95.	0480	*Paraburkholderia graminis*
96.	0482	*Ralstoniamannitolilytica*
97.	0483	*Xanthomonas* sp.
98.	0484	*Pseudomonas syringae* pv. *pisi*

2.6.2. Determination of repeatability

To establish the degree of conformity of the PCR test results, a series of 10-fold dilutions of artificially infected extracts was studied in 6-fold repetition at the level of the threshold concentrations of the phytopathogen established during the sensitivity determination, by one person and on the same device, under the same conditions and for a short period of time.

2.6.3. Determination of reproducibility

To determine the reproducibility, artificially inoculated extracts with a low and medium level of infection were prepared in 6-fold replication using a method similar to determining analytical sensitivity. At the same time, testing of the series was carried out by 3 operators in different labs with different equipment and at different times.

2.7. Pathogenicity test

To carry out the infection of plants and onion seeds, a bacterial suspension was prepared in distilled water at a concentration of 10^7 CFU/ml.

2.7.1 Method of onion seed inoculation.

Onion seeds in an amount sufficient for the experiment were placed in a container with a bacterial suspension and left on a shaker for 4 hours at 100 rpm. Next, the suspension was removed, and the thus treated seeds were dried at room temperature on filter paper.

2.7.2. Method of inoculation of plants

On the experimental plot, turnip onion, onion sets (Setton) and seeds (Stuttgarter Riesen) were planted. Onion plants were infected 14 days after planting in open ground by a stem prick. At the same time, 0.5-1 cm3 of a suspension of the pathogen of onion leaf burn was introduced.

Additionally, the negative control plants (onion bulbs, onion sets and seeds) were inoculated with distilled water.

2.7.3. Analysis of test results

The effectiveness of infection was checked by isolating DNA from the vegetative parts of the onion, as well as the bulbs (the bottom, roots and succulent scales were analyzed separately) using the Probe-GS reagent. Detection of infection results was carried out by real-time PCR.

Chapter 3

RESULTS AND DISCUSSION

3.1 Determination of conventional nested PCR and real-time PCR Sensitivity

The PCR sensitivity of both the conventional nested PCR and real-time (RT) PCR was determined. Bacterial dilutions 1 to 7 with concentrations ranging from 10^1 to 10^7 CFU/ml were tested with the PCR assays.

3.1.1 Sensitivity of nested PCR using AVR primers

As shown in table 7 and buttressed by figure 3 the conventional PCR using AVR primers could detect the bacteria from the highest concentration 10^7 to 10^2 CFU/ml corresponding with 1st bacterial dilution to the 6th dilution, having all 3 replicates at each concentration turning positive. However, on the 7th dilution with a bacterial concentration of 10^1 CFU/ml only 2 out of 3 replicates could be detected. Therefore, detection at this level was recorded as null.

Table 7 exhibiting sensitivity of conventional PCR assay for *X. euvesicatoria* pv. *allii* as a function of the AVR primers

Suspension dilution	Bacterial concentration	Replications		
		Rep 1	Rep 2	Rep 3
SD1	10^7	+	+	+
SD2	10^6	+	+	+
SD3	10^5	+	+	+
SD4	10^4	+	+	+
SD5	10^3	+	+	+
SD6	10^2	+	+	+
SD7	10^1	+	+	−
К- Negative Control	dH_2O	−	−	−
ЧК- Negative Control- PCR zone	dH_2O	−		
К+ Positive Control	10^8	+		

* + symbolizes that the sample turned positive whilst – signifies a negative sample
§dH_2O- distilled water was used in all the negative controls

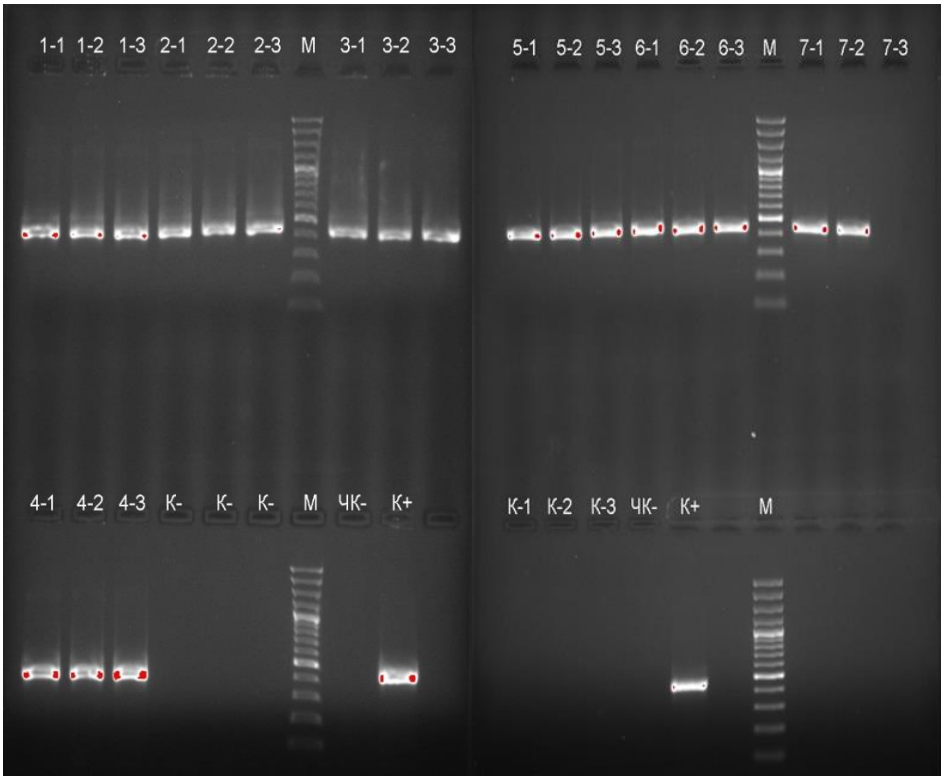


Fig. 3 Shows triplicate replications of different bacterial concentrations. For the concentration 10^1 only 2 replications of the sample could be detected by the assay. In the electrogram К- represents the negative control in all the replications, ЧК- symbolizes the negative for the PCR clean zone and К+ signifies the positive control (*X. euvesicatoria* pv. *allii*).

3.1.2 Determination of nested PCR assay sensitivity using PIL primers

When the PIL primers were used to detect the bacteria in different dilutions and concentrations, a similar trend as that observed for AVR primers was replicated as shown in table 8. As exhibited by the electrogram, (fig. 4) the highest concentration in which the bacteria could be detected was 10^7 CFU/ml cascading to concentration 10^2 CFU/ml corresponding with dilutions 1 to 6. At the lowest concentration evaluated 10^1 CFU/ml i.e. dilution 7 the bacterium was only detected in 1 out of 3 replicates, hence the result was recorded as null.

Table 8 showing sensitivity of conventional PCR for *X. euvesicatoria* pv. *allii* using PIL primers

Suspension dilution	Bacterial concentration	Replications		
		Rep 1	Rep 2	Rep 3
SD1	10^7	+	+	+
SD2	10^6	+	+	+
SD3	10^5	+	+	+
SD4	10^4	+	+	+
SD5	10^3	+	+	+
SD6	10^2	+	+	+
SD7	10^1	+	–	–
К- Negative Control	dH_2O	–	–	–
ЧК- Negative Control- PCR zone	dH_2O	–		
К+ Positive Control	10^8	+		

* + symbolizes that the sample turned positive whilst – signifies a negative sample
§dH_2O- distilled water was used in all the negative controls

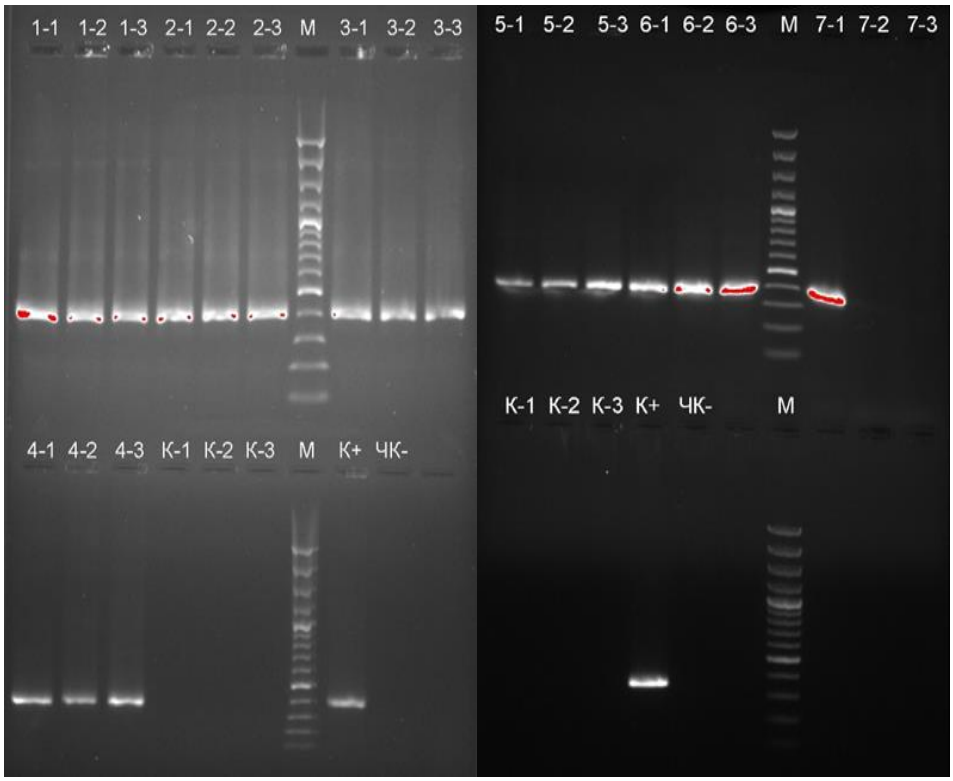


Fig. 4 The electrogram shows that the bacterial detection was stable at higher concentrations but when the lowest concentration was assessed irregularities were exhibited. In the electrogram К- represents the negative control in all the replications, ЧК- symbolizes the negative for the PCR clean zone and К+ signifies the positive control (*X. euvesicatoria* pv. *allii*).

3.1.3 Sensitivity of Duplex nested PCR as a function of AVR and PIL primers

The duplex nested PCR, combining both AVR and PIL primers had the same level of sensitivity as when the individual sets of primers were used. The PCR assay could detect the bacteria in the lowest dilution with a bacterial concentration of 10^7 CFU/ml up to the 6^{th} dilution with a concentration of 10^2 CFU/ml. Nonetheless at the highest dilution, 7^{th} with a concentration of 10^1 CFU/ml, only 1 out of 3 replicates turned to be positive. Consequently, the assay results at this dilution were designated as null.

Table 9 Highlighting the sensitivity of the duplex (AVR + PIL) nested PCR assay across the 7 concentrations in triplicate

Suspension Dilution	Bacterial Concentration	Replications		
		Rep1	Rep 2	Rep 3
SD1	10^7	+	+	+
SD2	10^6	+	+	+
SD3	10^5	+	+	+
SD4	10^4	+	+	+
SD5	10^3	+	+	+
SD6	10^2	+	+	+
SD7	10^1	+	–	–
К- Negative Control	dH_2O	–	–	–
ЧК- Negative Control- PCR zone	dH_2O	–		
К+ Positive Control	10^8	+		

* + symbolizes that the sample turned positive whilst – signifies a negative sample
§dH2O- distilled water was used in all the negative controls

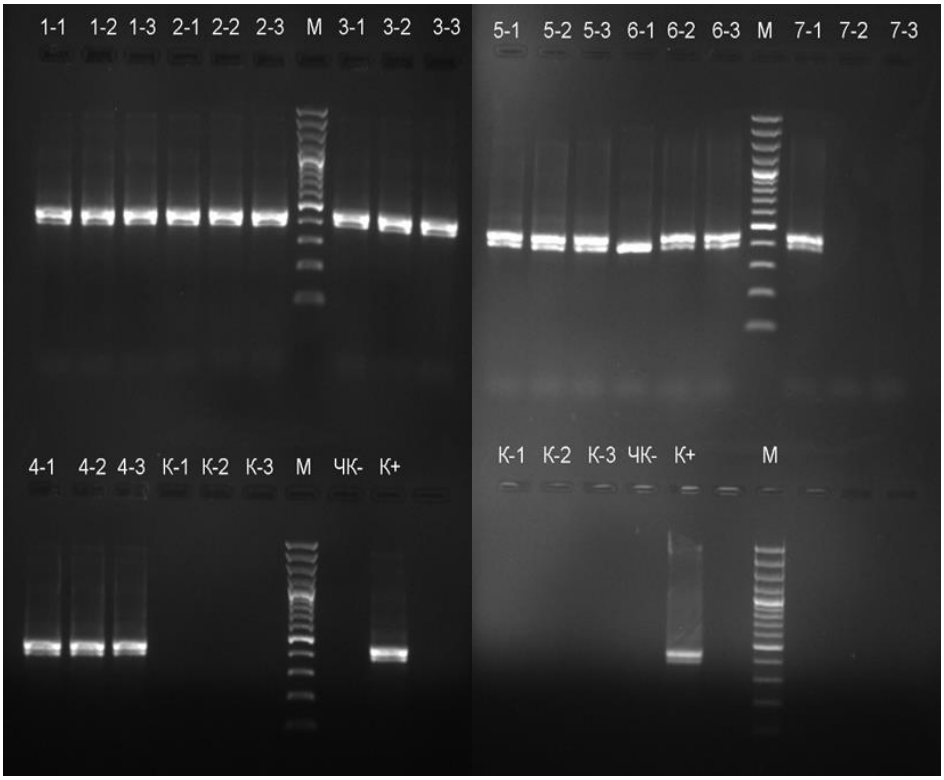


Fig. 5 Projecting the same trend as that noted when individual sets of primers were used, the assay could detect the bacteria in dilutions 1 to 6 corresponding with concentrations 10^7 cascading to 10^2 CFU/ml. However, in the highest dilution or lowest concetration only the first replication could be detected whilst the rest could not, rendering aggregate result null. In the electrogram К- represents the negative control in all the replications, ЧК- symbolizes the negative for the PCR clean zone and К+ signifies the positive control (*X. euvesicatoria* pv. *allii*).

3.2. Sensitivity of Real-Time PCR assay for *Xanthomonas euvesicatoria* pv. *allii*

The real-time PCR (qPCR) assay for *X. euvesicatoria* pv. *allii* was also evaluated for sensitivity. Tenfold serial dilution was performed yielding 7 dilutions with concentrations ranging from $6.8x10^1$ to $6.8x10^7$ CFU/ml, where the first dilution D1 corresponded with the highest concentration and the last dilution D7 tallied with the lowest concentration.

3.2.1 Sensitivity of the qPCR using AVR primers

To assess the sensitivity of the qPCR using AVR primers different dilutions 1 to 7 with corresponding concentrations $6.8x10^7$ CFU/ml cascading down to $6.8x10^1$ CFU/ml. The PCR assay could detect the bacteria at the lowest concentration 10^1 with a mean cycle threshold value (CT value) of 36.1 as shown table 7. At the highest concentration 10^7 CFU/ml bacterial detection was rapid and robust as indicated by a mean CT value of 19.9.

Table 10- The sensitivity of the assay using AVR primers tested with 7 bacterial dilutions having 7 corresponding concentrations

Bacterial Dilutions	Concentrations	Replications	CT Values	Averages
D1	10^7	1.1	20,1	19.91
		1.2	19,1	
		1.3	20,5	
D2	10^6	2.1	22,9	23.13
		2.2	22,2	
		2.3	24,3	
D3	10^5	3.1	24,8	25.23
		3.2	24,9	
		3.3	26,0	
D4	10^4	4.1	28,3	28.76
		4.2	28,6	
		4.3	29,4	
D5	10^3	5.1	32,0	31.90
		5.2	31,9	
		5.3	31,8	
D6	10^2	6.1	34,9	34.86
		6.2	34,8	
		6.3	34,9	
D7	10^1	7.1	36,2	36.06
		7.2	36,1	
		7.3	35,9	

3.2.2 Sensitivity of qPCR using PIL primers

The sensitivity of qPCR using PIL markers with bacterial dilutions 1 to 7 constituent of corresponding concentrations $6.8x10^7$ CFU/ml descending to $6.8x10^1$ CFU/ml respectively. The lowest concentration at which the PCR assay could detect bacteria

was 10^1 CFU/ml with a CT value of 36.1 as indicated in table 8. The highest concentration 10^7 CFU/ml had the lowest CT value 20.13 in the experiment. Generally, PIL primers had higher CT values than the AVR primers (tables 7 and 8) at each bacterial concentration or dilution though not significantly ($p>0.05$).

Table 11- Exhibiting the sensitivity of the assay at 7 different concentrations

Bacterial Dilution	Concentrations	Replications	CT Values	Averages
D1	10^7	1.1	18,8	20.13
		1.2	20,8	
		1.3	20,8	
D2	10^6	2.1	20,9	22.73
		2.2	23,8	
		2.3	23,5	
D3	10^5	3.1	25,6	25.47
		3.2	25,5	
		3.3	25,3	
D4	10^4	4.1	28,7	28.73
		4.2	28,7	
		4.3	28,8	
D5	10^3	5.1	32,1	31.63
		5.2	31,9	
		5.3	30,9	
D6	10^2	6.1	34,5	35.00
		6.2	35,4	
		6.3	35,1	
D7	10^1	7.1	35,8	36.10
		7.2	36,3	
		7.3	36,2	

3.3 Specificity of the PCR assay for *Xanthomonas euvesicatoria* pv. *allii*

To carry out the pathogen specificity studies a total of 99 bacterial species from the All-Russian Plant Quarantine Center were evaluated. Of the 99 bacteria 97 were assessed for exclusivity purposes whilst the only 2 strains of *X. euvesicatoria* pv. *allii* in the collection of the All-Russian Plant Quarantine Center were used for inclusivity tests. Investigations were conducted using both qPCR and conventional PCR.

Table 12. Shows the results of the 2 sets of primers AVR and PIL as well as duplex when tested for fidelity using 99 bacteria. The acronym qPCR represents real-time PCR whilst PCR denotes conventional PCR.

Species/strain number	Bacterial species	qPCR AVR Result	qPCR PIL Result	qPCR AVR+ PIL	PCR AVR	PCR PIL	PCR AVR+ PIL
1.	*Clavibacter michiganensis* subsp. *Sepedonicus*	–	–	–	–	–	–
2.	*Ralstonia solanacearum*	–	–	–	–	–	–
3.	*Erwinia billingiae*	–	–	–	–	–	–
4.	*Ochrobactrum anthrapi*	–	–	–	–	–	–
5.	*Clavibacter michiganensis* subsp. *michiganensis*	–	–	–	–	–	–
6.	*Xilophilus ampelinus*	–	–	–	–	–	–
7.	*Erwinia tasmaniensis*	–	–	–	–	–	–
8.	*Acidovorax citrulli*	–	–	–	–	–	–
9.	*Acidovorax citrulli*	–	–	–	–	–	–
10.	*Acidovorax citrulli*	–	–	–	–	–	–
11.	*Erwinia piriflorinigrans*	–	–	–	–	–	–
12.	*Pantoea stewartii* subsp. *stewartia*	–	–	–	–	–	–
13.	*Clavibacter michiganensis* subsp. *Sepedonicus*	–	–	–	–	–	–
14.	*Pectobacterium carotovorum* subsp. *Carotovorum*	–	–	–	–	–	–
15.	*Pectobacterium atrosepticum*	–	–	–	–	–	–
16.	*Dickeya* sp.	–	–	–	–	–	–
17.	*Xanthomonas campestris* pv. *raphanin*	–	–	–	–	–	–
18.	*Xanthomonas aboricola* pv. *pruni*	–	–	–	–	–	–
19.	*Erwinia amylovora*	–	–	–	–	–	–

20.	*Ralstonia solanacearum*	–	–	–	–	–	–
21.	*Pantoea stewartii* subsp. *Stewartii*	–	–	–	–	–	–
22.	*Pseudomonas syringae* pv. *syringae*	–	–	–	–	–	–
23.	*Xanthomonas campestris* pv. *campestris*	–	–	–	–	–	–
24.	*Clavibacter michiganensis* subsp. *michiganensis*	–	–	–	–	–	–
25.	*Erwinia amylovora*	–	–	–	–	–	–
26.	*Pantoea agglomerans*	–	–	–	–	–	–
27.	*Pectobacterium atrosepticum*	–	–	–	–	–	–
28.	*Pectobacterium carotovorum* subsp. *Odoriferum*	–	–	–	–	–	–
29.	*Pectobacterium betavasculorum*	–	–	–	–	–	–
30.	*Pectobacterium cacticida*	–	–	–	–	–	–
31.	*Dickeya chrysanthemi*	–	–	–	–	–	–
32.	*Dickeya dadantiii* subsp. *Dadantii*	–	–	–	–	–	–
33.	*Dickeya paradisiaca*	–	–	–	–	–	–
34.	*Dickeya zeae*	–	–	–	–	–	–
35.	*Pseudomonas fuscovaginae*	–	–	–	–	–	–
36.	*Xanthomonas gardneri*	–	–	–	–	–	–
37.	*Xanthomonas fragariae*	–	–	–	–	–	–
38.	*Xanthomonas fragariae*	–	–	–	–	–	–
39.	*Pectobacterium atrosepticum*	–	–	–	–	–	–
40.	*Xanthomonas euvesicatoria pv. allii*	+	+	+	+	+	+
41.	*Strenotrophomonas maltophilia*	–	–	–	–	–	–
42.	*Pectobacterium betavasculorum*	–	–	–	–	–	–

43.	*Xanthomonas vesicatoria*	–	–	–	–	–	–
44.	*Xanthomonas* sp.	–	–	–	–	–	–
45.	*Xanthomonas oryzae pv. oryzicola*	–	–	–	–	–	–
46.	*Dickeya solani*	–	–	–	–	–	–
47.	*Rathayibacter tritici*	–	–	–	–	–	–
48.	*Curtobacterium flaccumfaciens* pv. *oortiii*	–	–	–	–	–	–
49.	*Curtobacterium flaccumfaciens* pv. *poinsettiae*	–	–	–	–	–	–
50.	*Clavibacter michiganensis* subsp. *nebraskensis*	–	–	–	–	–	–
51.	*Xanthomonas* sp.	–	–	–	–	–	–
52.	*Pantoea ananatis*	–	–	–	–	–	–
53.	*Pseudomonas corrugate*	–	–	–	–	–	–
54.	*Pseudomonas syringae* pv. *tomato*	–	–	–	–	–	–
55.	*Xanthomonas gardneri*	–	–	–	–	–	–
56.	*Xanthomonas vesicatoria*	–	–	–	–	–	–
57.	*Xanthomonas campestris*	–	–	–	–	–	–
58.	*Agrobacterium tumafaciens*	–	–	–	–	–	–
59.	*Xanthomonas euvesicatoria pv. allii*	+	+	+	+	+	+
60.	*Pseudomonas syringae* pv. *maculicola*	–	–	–	–	–	–
61.	*Acidovorax avenae*	–	–	–	–	–	–
62.	*Pantoea stewartii* subsp. *Indologenes*	–	–	–	–	–	–
63.	*Rathayibacter iranicus*	–	–	–	–	–	–
64.	*Xanthomonas hyacinthi*	–	–	–	–	–	–
65.	*Paraburkholderia andropogonis*	–	–	–	–	–	–

66.	*Pseudomonas nitroreducens*	–	–	–	–	–	–
67.	*Chryzeobacterium* sp.	–	–	–	–	–	–
68.	*Rathayibacter tritici*	–	–	–	–	–	–
69.	*Pectobacterium atrosepticum*	–	–	–	–	–	–
70.	*Xanthomonas paradisiaca*	–	–	–	–	–	–
71.	*Paraburkholderia plantarii*	–	–	–	–	–	–
72.	*Rhodococcus fascians*	–	–	–	–	–	–
73.	*Xanthomonas sp.*	–	–	–	+	+	+
74.	*Agrobacterium rubi*	–	–	–	–	–	–
75.	*Pectobacterium cacticida*	–	–	–	–	–	–
76.	*Xanthomonas nitroreducens*	–	–	–	–	–	–
77.	*Xanthomonas citri* pv. *glycines*	–	–	–	–	–	–
78.	*Pseudomonas syringae* pv. *tomato*	–	–	–	–	–	–
79.	*Pseudomonas cannabina* pv. *alisalensis*	–	–	–	–	–	–
80.	*Erwinia piriflorinigrans*	–	–	–	–	–	–
81.	*Rahnella aquatilis*	–	–	–	–	–	–
82.	*Xylella fastidiosa*	–	–	–	–	–	–
83.	*Dickeya dadantii* subsp. *Dieffenbachiae*	–	–	–	–	–	–
84.	*Xanthomonas translucens*	–	–	–	–	–	–
85.	*Xanthomonas euvesicatoria*	–	–	–	–	–	–
86.	*Athrobacter* sp.	–	–	–	–	–	–
87.	*Xanthomonas perforans*	–	–	–	–	–	–
88.	*Ochrobactrum lupini*	–	–	–	–	–	–
89.	*Pseudomonas savastanoi* pv. *phaseolicola*	–	–	–	–	–	–
90.	*Pseudomonas savastanoi* pv. *glycinea*	–	–	–	–	–	–

91.	*Xanthomonas axonopodis* pv. *phaseoli*	–	–	–	–	–	–
92.	*Xanthomonas axonopodis* pv. *axonopodis*	–	–	–	–	–	–
93.	*Xanthomonas phaseoli* pv. *soyrense*	–	–	–	–	–	–
94.	*Pseudomonas viridiflava*	–	–	–	–	–	–
95.	*Paraburkholderia cepacia*	–	–	–	–	–	–
96.	*Paraburkholderia graminis*	–	–	–	–	–	–
97.	*Ralstoniamannitolilytica*	–	–	–	–	–	–
98.	*Xanthomonas* sp.	–	–	–	–	–	–
99.	*Pseudomonas syringae* pv. *pisi*	–	–	–	–	–	–

3.3.1 Specificity of the duplex nested PCR

The 99 bacterial strains evaluated for the specificity test included 2 *X. euvesicatoria* pv. *allii*, one of which was used for the inclusivity test. The other strain (0377) was used as the positive control for the whole PCR assay. The strain (0419) designated by the number 40 in table 9 tested positive to the assay, comparable to the control. For the exclusivity test the other 97 various bacterial strains were assessed and a *Xanthomonas sp.* (0373) designated by the number 73 in table 9 was wrongly identified as *X. euvesicatoria* pv. *allii*. The rest 96 bacteria tested negative to the assay as indicated in table 9. Therefore, the conventional duplex nested PCR assay had a fidelity or specificity of 99%.

3.3.2 Specificity of the Real-time PCR assay

To determine the specificity of the real-time PCR using two sets of primers AVR and PIL, 99 bacteria were used as indicated in table 12. The 99 bacteria included 2 *X. euvesicatoria* pv. *allii* strains denoted by strain identification numbers 0377 and 0419. Strain 0377 served as the standard control for the specificity assessments

whilst strain 0419 served to confirm inclusivity of the PCR the target isolates/strains of *X. euvesicatoria* pv. *allii*. Only these 2 *X. euvesicatoria* strains tested positive when evaluated using both AVR and PIL markers. The other 97 strains all tested negative to both AVR and PIL primers. Based on these findings the qPCR using either AVR or PIL markers had 100% specificity.

3.4 Determining the repeatability of the qPCR

The repeatability of the PCR assay using both AVR and PIL primers was determined. Thus 6 replications of 7 bacterial concentrations ranging from $6.6x10^1$ to $6.6x10^7$ were tested.

3.4.1 Repeatability using the AVR marker

The qPCR using AVR markers was assessed for repeatability. Thus 6 replications of 7 bacterial concentrations ranging from $6.6x10^1$ to $6.6x10^7$ CFU/ml were tested. As shown graphically in fig. 6 the CT values showed stable or regular patterns in the first 3 lowest concentrations 10^1 to 10^3. As the concentration increased, the patterns became irregular especially at concentration 10^4 and 10^5 CFU/ml. However, the CT value patterns were less irregular at concentrations 10^6 and 10^7 CFU/ml. Despite the differences in CT value patterns the positive responses were recorded for all concentrations hence 100% repeatability.

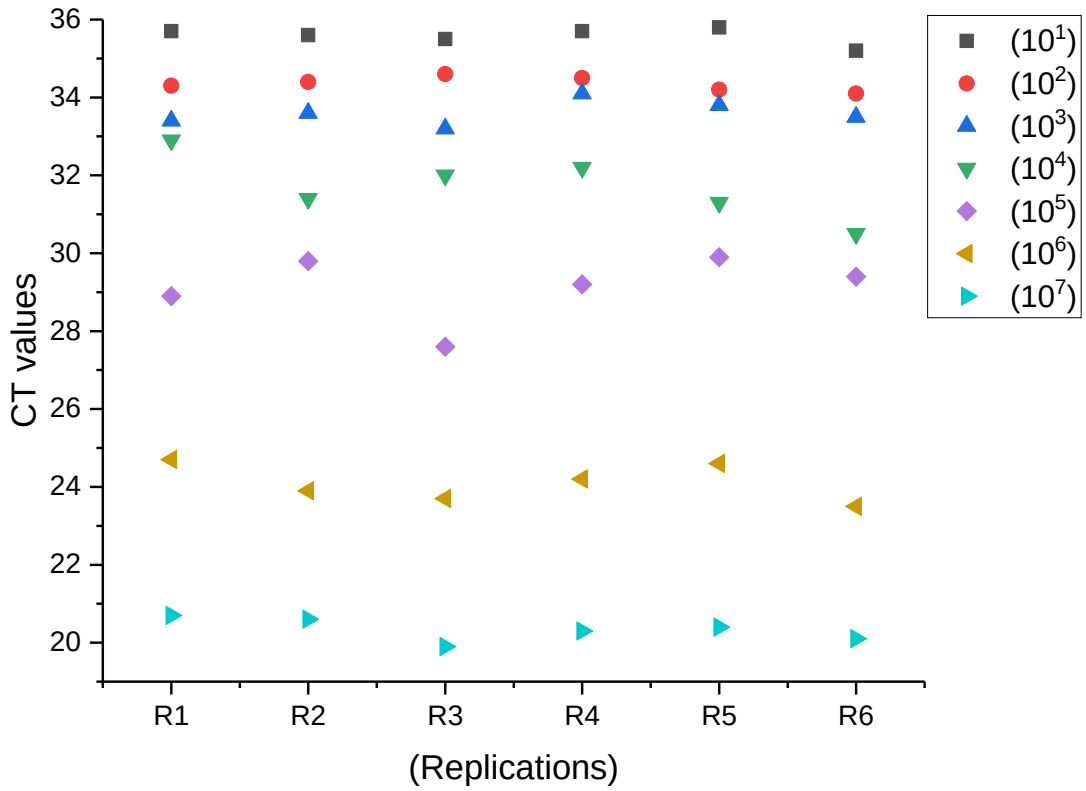


Fig. 6 Showing CT values for each replication of the bacterial concentrations

3.4.2 Standard-deviation- Repeatability of qPCR using AVR primers

The graphical-statistical presentation in fig 7 further unravels details of the PCR assay's repeatability using AVR primers. In the 3 lowest concentrations $6.6x10^1$ CFU/ml to $6.6x10^3$ CFU/ml there was positively skewed distributions of the mean CT values, hence stability of the replications. As the concentrations mean Ct values shifted especially at concentration $6.6x10^5$ CFU/ml where there was negatively skewed distribution of the mean, indicating non-homogenous patterns of replication at this concentration. Moreover, the range of CT values at concentrations 10^4 and 10^5 CFU/ml was wide. However, at higher concentrations 10^6 and 10^7 CFU/ml the range was reduced non-homogenous patterns at such concentrations. Nevertheless, detection of the pathogen was repeatable at all concentrations.

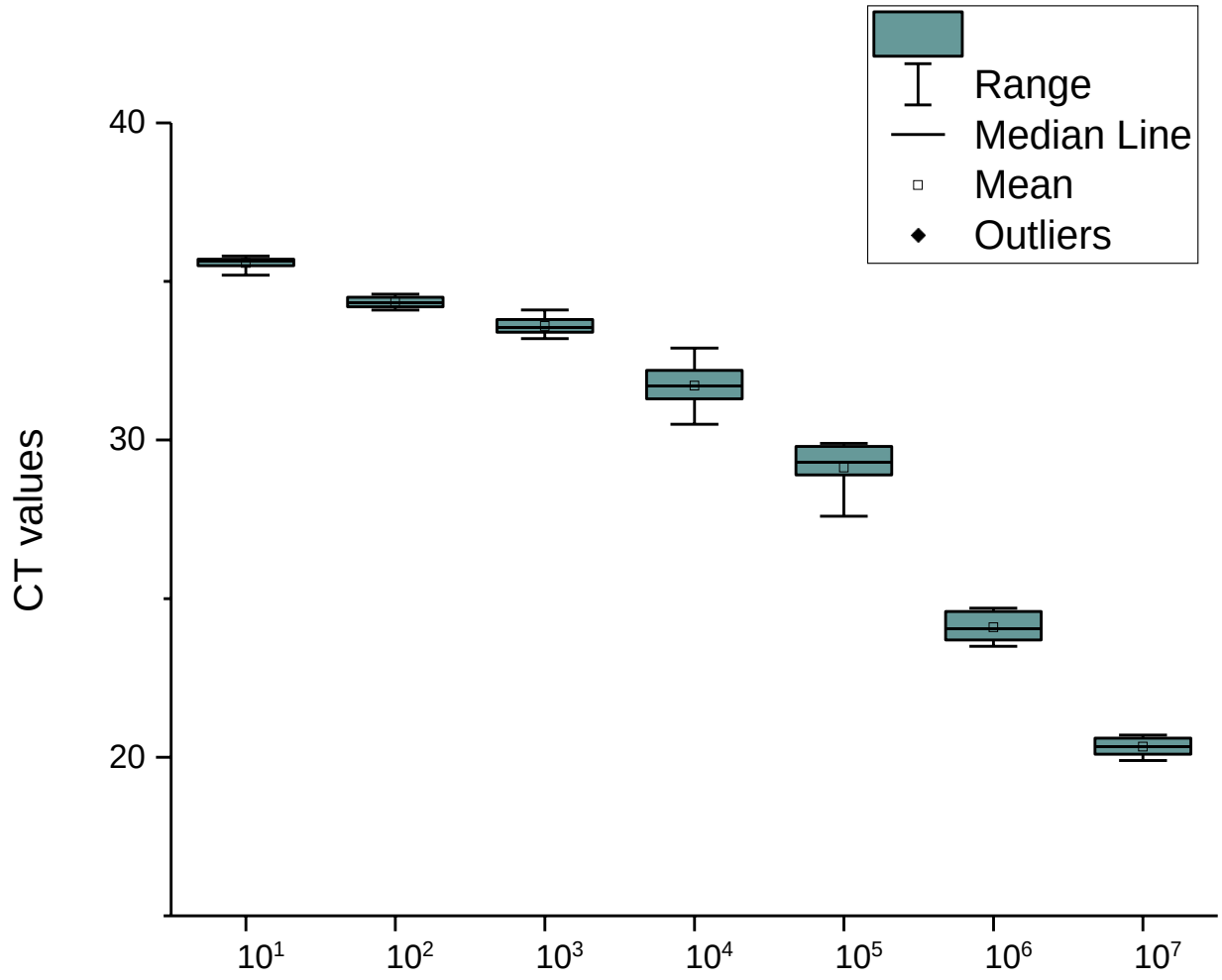


Fig. 7 The mean CT values per concentration

3.4.3 Repeatability of the qPCR using the PIL marker

When the PIL primers were evaluated for repeatability the same patterns noticed for the AVR markers were also observed as highlighted in fig 8. The CT values were evenly distributed across the replications of the 3 lowest concentrations $6.6x10^1$ to $6.6x10^3$ CFU/ml. On the fourth concentration $6.6x10^4$ CFU/ml dramatic changes were noticed as the CT values were unevenly distributed across the replications. However, the pattern changed in the subsequent concentrations $6.6x10^5$ to $6.6x10^7$ CFU/ml as CT became more evenly distributed. In spite of the uneven distribution of the CT values at some of the concentrations, the test managed to detect the bacteria at all 7 concentrations, registering high repeatability of 100%.

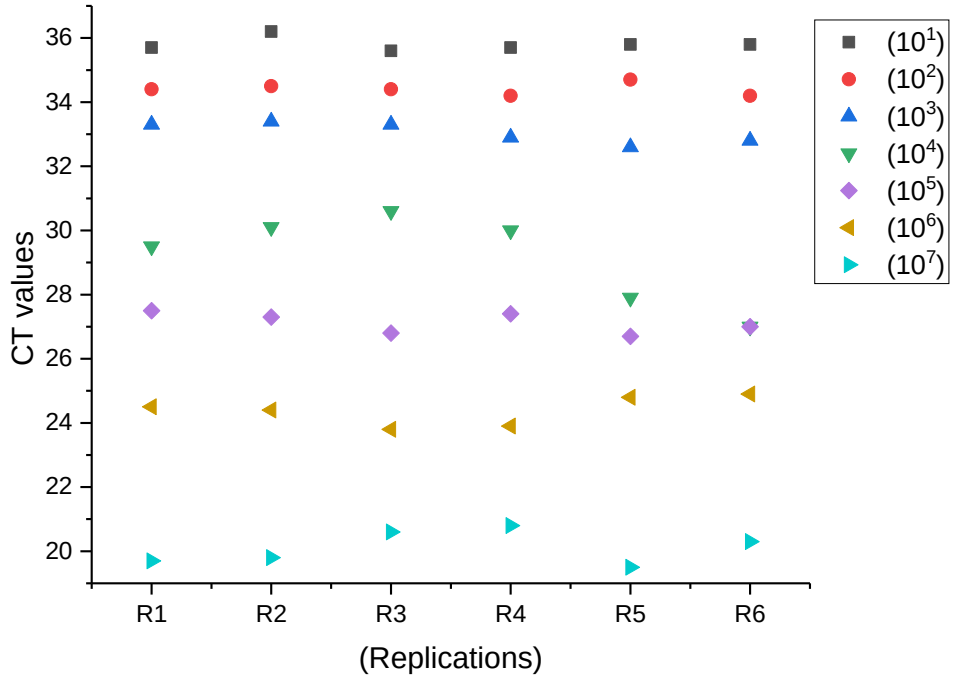


Fig. 8 Replications of the various bacterial concentrations

3.4.4 Standard deviation in assessing the repeatability of the qPCR assay using PIL primers

Upon computing the means and the mean deviations the statistics revealed that from $6.6X10^1$ CFU/ml up to the third concentration $6.6x10^3$ CFU/ml the mean CT values exhibited positively skewed distribution, showing stable replication of the results across those concentrations, figure 9. The trend radically shifted on fourth concentration $6.6x10^4$ CFU/ml when the mean fell below the mean, as well as a wide range signaling a negatively skewed distribution and consequently high fluctuations of CT values at that concentration. However, as the concentration increased from 10^5 to 10^7 CFU/ml the range between the CT values decreased showing more symmetric distribution of the means. Despite the non-homogenous distribution of CT values, the test had 100% repeatability.

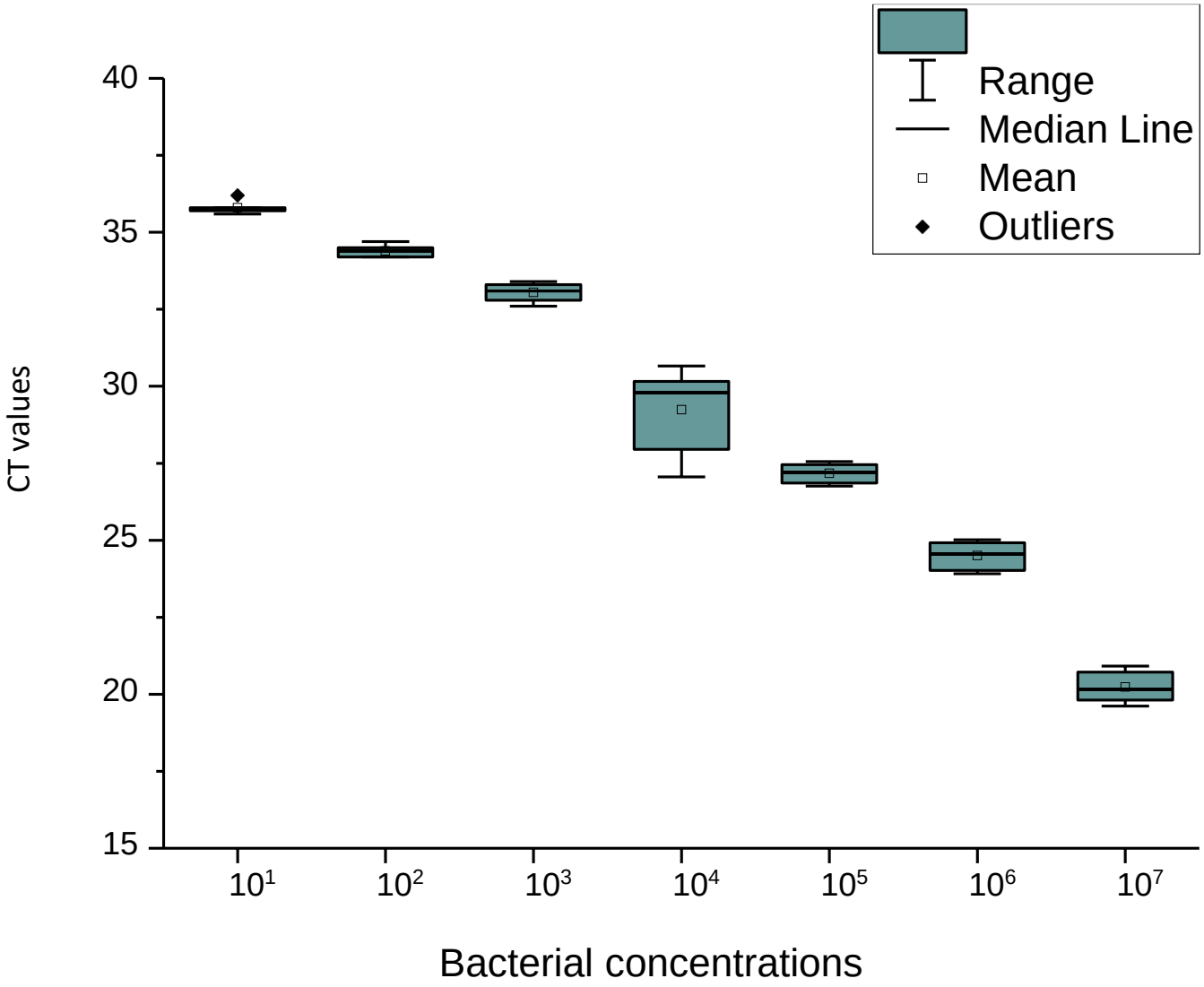


Fig. 9 Shows mean CT values of the bacterial concentrations

3.5. Determining reproducibility of the PCR assay

Reproducibility assessment was performed to determine the ability of the PCR assay to detect *X. euvesicatoria* pv. *allii* using 2 sets of primers AVR and PIL across 7 ($7{,}2x10^1$- $7{,}2x10^7$ CFU/ml) concentrations with 3 different operators and sets of equipment in triplicate.

3.5.1 Reproducibility of the PCR assay using the AVR marker

The data in table 13 illustrates that for the 7 concentrations evaluated with the AVR primers the reproducibility of the assay was high between the operators and equipment used. Variability was lowest at the highest bacterial concentration 10^7 CFU/ml as evidenced by relative standard deviation of 0,33%, whereas the

concentration 10^6 had the highest relative standard deviation of 1,62%. Moreover, with a standard deviation of 0,05 therefore high proximity to the mean CT cycle of 15,37 the test demonstrated high precision. Overall, with a relative standard deviation below 2% the assay exhibited good reproducibility.

Table 13- Shows the variance between the operators and equipment used across the 7 bacterial concentrations

Concentration	Operators & equipment	CT Values	Mean CT	Standard Deviation	Relative Std. Dev.
10^7	OE1	15,4	15,37	0,05	0,33%
	OE2	15,3			
	OE3	15,4			
10^6	OE1	19,4	19,13	0,31	1,62%
	OE2	18,7			
	OE3	19,3			
10^5	OE1	23,7	23,43	0,21	0,90%
	OE2	23,2			
	OE3	23,4			
10^4	OE1	28,2	27,77	0,33	1,19%
	OE2	27,7			
	OE3	27,4			
10^3	OE1	32,1	31,57	0,41	1,30%
	OE2	31,5			
	OE3	31,1			
10^2	OE1	34,9	34,53	0,39	1,13%
	OE2	34			
	OE3	34,7			
10^1	OE1	36,2	35,9	0,22	0,61%
	OE2	35,8			
	OE3	35,7			

3.5.3 Reproducibility evaluation of the PCR using PIL markers

Detection of the bacteria with the PIL primers at each of the 7 concentrations revealed exceptionally low variations in repeat tests between the operators and equipment used, hence good reproducibility. Reproducibility was most eminent at the concentrations 10^2 and 10^1 CFU/ml as indicated by relative standard deviation of

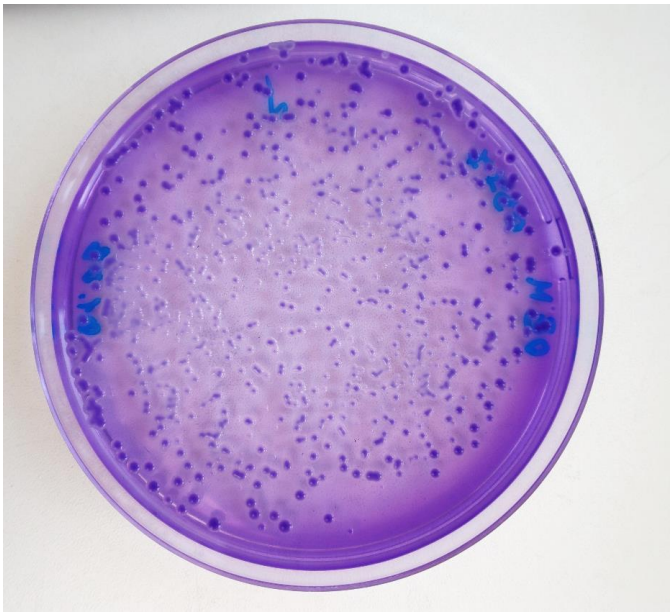
0,55% and 0,59% in table 14. With a peak relative standard deviation of 1.81% at concentration 10^7 CFU/ml the findings reflect that neither precision nor accuracy was significantly affected by the differences in operators and equipment used. Overall, for both sets of primers AVR and PIL the good reproducibility exhibited by the test between operators with their respective equipment demonstrates that the calculated relative standard deviation for the concentrations, especially the lower ones (10^1 and 10^2 CFU/ml) is indicative of the achievable accuracy by which the pathogen can be detected.

Table 14- Displays the reproducibility of the assay in detecting the bacteria at varied concentrations as quantified by the relative standard deviation

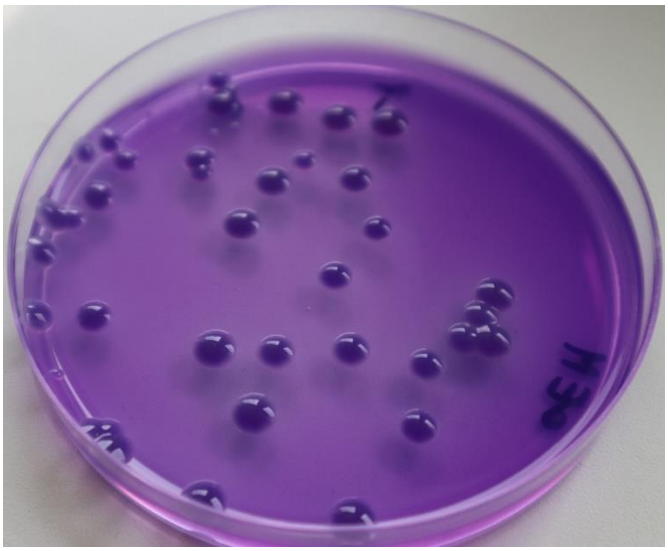
Concentration	Operators & equipment	CT Values	Mean CT	Standard Deviation	Relative Std. Dev.
10^7	OE1	19,9	20,33	0,37	1,81%
	OE2	20,3			
	OE3	20,8			
10^6	OE1	29,3	29.73	0,37	1,24%
	OE2	29,7			
	OE3	30,2			
10^5	OE1	31,8	31,5	0,24	0,76%
	OE2	31,2			
	OE3	31,5			
10^4	OE1	32,5	32,4	0,22	0,68%
	OE2	32,6			
	OE3	32,1			
10^3	OE1	34,2	33,76	0,31	0,92%
	OE2	33,6			
	OE3	33,5			
10^2	OE1	34,4	34,5	0,19	0,55%
	OE2	34,4			
	OE3	34,7			
10^1	OE1	35,9	35,86	0,21	0,59%
	OE2	35,6			
	OE3	36,1			

3.6 Comparing the cultural properties of *X. euvesicatoria* pv. *allii* on various nutrient media.

In the course of the research work, culturing was carried out on 4 nutrient media (Figs. 10-12).



(a) Strain Xaa 0377



(b) Strain Xaa 0419

Fig. 10 Growth of *X. euvesicatoria* pv. *allii* strains **0419 and 0377** on semi-selective OEM medium. Fifty microliters of bacterial suspension with a concentration of 6.6

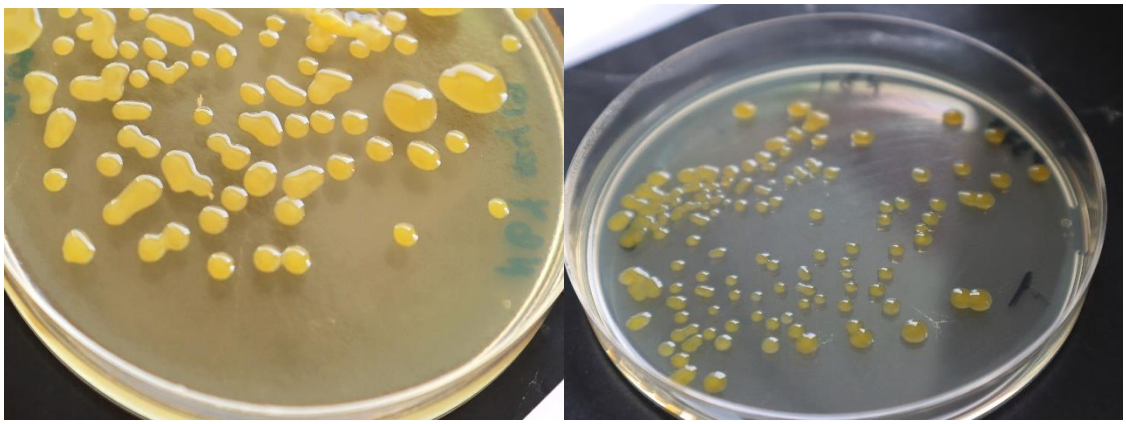
x 10^2 were plated on the media and incubated at 25 ℃ for 5 days since growth is typically slow on selective media.

The colonies of strain Xaa 0377 were obtained on onion extract media (OEM) medium at both 25℃ and 28℃ plate incubation. The strain formed small white colonies of white colour, mucous with a smooth edge and a slightly raised centre. On the 5^{th} day of culturing, the colonies acquired a dark-coloured centre, discolouring the medium.

The growth of strain 0419 was absent at 28℃ and was quite intense at 25℃. Colonies of strain 0419 did not differ in morphology from strain 0377.

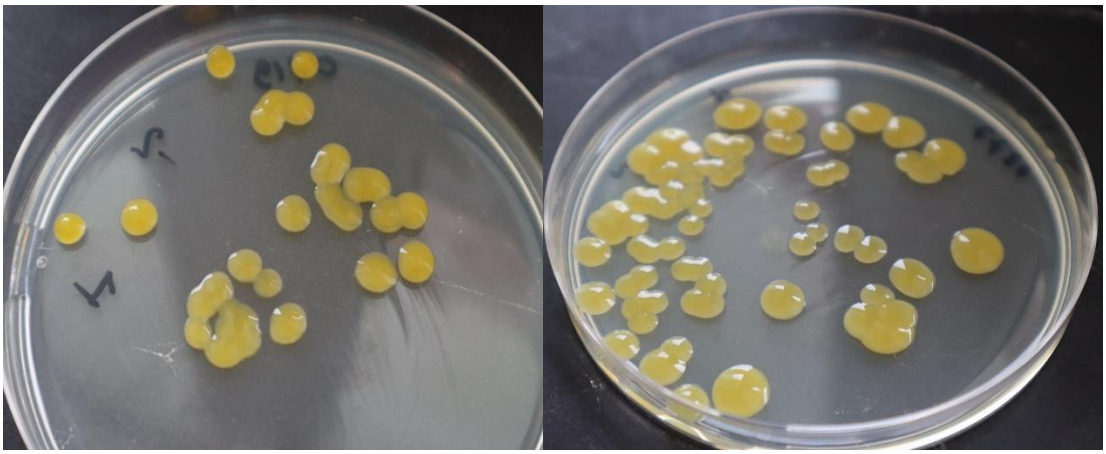
The OEM is prepared on the basis of a decoction obtained from the bulbs. Recommended by the developers for the isolation of onion phytopathogens and onion-related bacteria. According to the developer, the OEM environment is suitable for primary isolation of bacteria such as *Pantoea ananatis*, *P. agglomerans*, *Burkholderia 76epacian*, *Enterobacter cloacae*, *Pectobacterium carotovorum* subsp. *Carotovorum*, *Xanthomonas axonopodis* pv. *axonopodis* and several species of *Pseudomonas sp.*, from various onion tissues, including seeds, leaves, stems, bulbs, seedlings, and the soil in which the onion grew. Onion water is the only source of nutrients in the OEM environment. Onion tissue extract contains 9.34% carbohydrates, including 4.24% sugars, 1.1% proteins, trace elements and 89.11% water. These nutrients were sufficient to support the growth of pathogenic and onion-associated bacteria for 24 hours (Zaid et al., 2012).

When developing the medium, Zaid et al. (2012) did not take into account the onion leaf blight pathogen *X. euvesicatoria* pv. *allii*. In the course of studying and comparing the cultural properties of *X. euvesicatoria* pv. *allii* on different nutrient media, we found that the two main strains of this onion phytopathogen also grow quite well, although slowly (from 3 to 5 days) on OEM. At the same time, the temperature regime of 28°C, recommended by the developers of the medium, was not suitable for strain Xaa 0419.



(a) Strain 0419 (b) Strain 0377

Fig. 11 Growth of *X. euvesicatoria* pv. *allii* strains **0419 and 0377** on NBY medium. Fifty microliters of bacterial suspension with a concentration of 6.6 x 10^2 were plated on the media and incubated at 25 ℃ for 5 days so as to have homogenous conditions as those for OEM.



(a) Strain 0419 **(b) Strain 0377**

Fig. 12 Growth of *X. euvesicatoria* pv. *allii* strains **0419 and 0377** on YPGA medium. Fifty microliters of bacterial suspension with a concentration of 6.6 x 10^2

were plated on the media and incubated at 25 ℃ for 5 days so as to have homogenous conditions as those for OEM.

On nutrient broth yeast (NBY) and YPGA media, *X. euvesicatoria* pv. *allii* strains formed colonies of the same type: yellow mucous, with a raised top and a smooth edge. The colour of the colonies is yellow. On the 5^{th} day of incubation, the colonies reached a size of ≥ 5 mm and merged. The growth of the strains on both nutrient media was observed to be equally intense.

3.6.1 MXP nutrient medium results

The EPPO diagnostic protocol (EPPO, 2016) recommends inoculation on modified MXP medium. According to the results of inoculation of strains 0419 and 0377 on this medium and incubation at two temperature parameters, the bacterial cultures did not grow on this medium. As such it can be concluded that this medium contains a large number of selective components and requires additional modification and optimization of the composition. Thus, a modification of the composition will be carried out, which will preserve the selective properties of the medium and eliminate the inhibition of the growth of the target organism.

3.7 *Xanthomonas euvesicatoria* pv. *allii* host selectivity across different *Allium species*

To ascertain host selectivity of the *Xanthomonas euvesicatoria* pv. *allii* across various Allium species, two strains (0377) and (0419) were used to infect 5 *t.* Namely *A. cepa* (AC), *A. schoenoprasum* (AS), *A. fragrans* (AF), *A. ramosum* (AR) and *A. porrum* (AP). The infected plant cells were assessed using qPCR to determine host selectivity of the pathogen amongst the 5 species.

3.7.1 Host selectivity of strain 419 across 5 Allium species

Inoculum was prepared from *X. euvesicatoria* pv. *allii* strain 0419 with 3 concentrations ranging from 10^5 to 10^7 CFU/ml. These concentrations were then used to infect cells of the homogenised plant material of the 5 Allium species from

which DNA extraction was carried out. Subsequently level of pathogen proliferation in the different species was assessed by qPCR.

Table 15- Showing results of detecting the pathogen in 5 *Allium spp* inoculated with various concentrations of the bacteria in real-time PCR analysis.

Species	Bacterial Dilution	Concentration	Average CT cycle
A. cepa	D4	10^5	26,7
	D5	10^6	29,8
	D6	10^7	33,7
A. schoenoprasum	D4	10^5	26,4
	D5	10^6	29,1
	D6	10^7	32,8
A. fragrans	D4	10^5	25,8
	D5	10^6	28,9
	D6	10^7	31,7
A. ramosum	D4	10^5	27,0
	D5	10^6	30,8
	D6	10^7	33,3
A. porrum	D4	10^5	26,0
	D5	10^6	29,4
	D6	10^7	32,5

As shown in table 15 the strain 0419 was tested for host selectivity across the 5 Allium species the results showed non-distinct host specificity ($p>0.05$) at each bacterial concentration level which served as model of different degrees of virulence. This outcome is in tandem with results reported by Gagnevin et al. (2014) that *X. euvesicatoria* pv. *allii* are less host specific than a typical Xanthomonas pathovar.

3.7.2 Host selectivity of strain 0377 on 5 Allium species

Three concentrations of inoculum ranging from 10^5 to 10^7 CFU/ml were prepared from *X. euvesicatoria* pv. *allii*. These concentrations were then used to infect cells of the homogenised plant material of the 5 Allium species from which DNA

extraction was executed. Subsequently level of pathogen proliferation in the different species was assessed by qPCR.

Table 16- Detection levels of strain 0377 in the inoculated *Allium species* in real-time PCR analysis.

Species	Bacterial Dilution	Concentration	Average CT cycle
A. cepa	D4	10^5	27,1
	D5	10^6	31,1
	D6	10^7	33,2
A. schoenoprasum	D4	10^5	27,1
	D5	10^6	30,8
	D6	10^7	33,4
A. fragrans	D4	10^5	27,9
	D5	10^6	31,2
	D6	10^7	33,2
A. ramosum	D4	10^5	28,4
	D5	10^6	32,9
	D6	10^7	33,7
A. porrum	D4	10^5	28,7
	D5	10^6	32,0
	D6	10^7	33,8

Assessing the strain 0377 for host selectivity among the *Allium species* showed the same pattern of results as those obtained for strain 0419 whereby there was no distinct host specificity at each of the concentrations, as exhibited in table.

3.8 Artificial infection under field conditions of onion (*A. cepa*) plants grown from 3 different propagation material (seed, onion sets and onion bulbs) as well as evaluation of BHQ probe as alternative to MGB.

Field experiments were carried out in the summer season at the All-Russian Plan quarantine Centre, whereby 3 types of propagation material namely seed, onion sets and onion bulbs were (sown and planted) used. The rationale behind such an approach was to make use of the 3 most common methods used in the propagation of onions by farmers. A bacterial inoculum of *X. euvesicatoria* pv. *allii* at concentration $7.4x10^7$ CFU/ml was used to infect the plants. The leaves of the seedling plants were perforated by a pin and a cotton sab dipped in the bacteria

suspension was gently rubbed on the leaf perforations After a period of 4 months the plants were harvested and each of them was divided into 3 parts, in particular the leaves, the bulb apex and the roots. These 3 plant parts were assessed using qPCR to determine the presence of the bacteria and also unravel the plat part in which they were most abundant. Knowing the plant parts in which the bacteria is most abundant is key in identifying propagules that may serve as secondary sources of infection, thus preventing the occurrence of epidemics.

Table 17. Detection of *X. euvesicatoria* pv. *allii* in different plant parts of onions propagated from seed, using PIL markers and the MGB probe.

Plant ID according to propagation method	CT values of plant parts assessed		
	Stem Disc	Bulb apex	Leaves
Sd1	34.12	30.58	32.10
Sd2	34.00	31.30	32.62
Sd3	30.74	27.57	30.36
Sd4	28.16	29.00	31.60
Sd5	33.72	27.38	33.46
Sd6	27.24	32.36	33.40
Sd7	26.22	24.38	29.23
Sd8	33.89	30.80	32.90
Sd9	34.15	32.71	31.25
Sd10	33.70	30.45	30.48
Sd11	35.32	34.43	31.75

The 3 plant parts (leaves, bulb apex and stem disc) of the plant samples propagated from onion seed were assessed for pathogen presence and the results in table 17 were obtained. The bulb apex had low mean CT values in the majority of the samples except in sample Sd11 where the CT value 34.43 was higher than that of the leaf sample 31.75. The fact that the lowest CT value 24.38 recorded for all tested samples was for the bulb apex further consolidates the logic the concept that detection of the pathogen in this plant part was much more rapid in comparison with the other parts, for instance the stem disc which had the highet CT value 35.32. It can be reasoned that the other 2 plant parts namely the leaves and stem disc serve as habitats i.e. the phyllosphere (leaves) and rhizosphere to a lot more microorganisms that may have some inhibitory effects on the PCR assay as compared to the bulb apex which may have less inhibitory effect.

Table 18. *X. euvesicatoria* pv. *allii* detection using AVR primers with MGB probe in onion plants grown from inoculated seed

Plant ID according to propagation method	CT values of plant parts assessed		
	Stem disc	Bulb apex	Leaves
Sd1	31.14	28.25	30.16
Sd2	30.52	31.83	33.37
Sd3	33.15	24.39	29.39
Sd4	34.31	28.53	32.52
Sd5	32.23	29.01	31.48
Sd6	31.88	30.82	32.01
Sd7	28.24	27.73	32.62
Sd8	34.07	33.26	33.38

Sd9	32.49	17.88	30.83
Sd10	33.51	28.96	31.70
Sd11	33.85	31.03	32.24

When the AVR amrkers were used to the 3 plant parts of seed raised onions and employing the MGB probe as the reporter fluorophore, the mean CT values for all the samples were lower than those obtained using the PIL markers. For example, the lowest CT value recorded for the bulb apex and all samples included was 17.88 (table 18) which was markedly lower than the 24.38 obtained using the PIL markers (table 17). Nevertheless, the same trend where rapid detection was registered for the bulb apex was also observed in this case. The leaf samples generally had moderate detection as evidenced CT values ranging from 29.39 – 33.38, whereas the least level of detection was exhibited in the stem disc sample with the highest CT value of 34.31, though it was lower in comparison with the one obtained with the PIL markers 35.32 (table 17).

Table 19. Pathogen detection using AVR primers with BHQ probe in seed-raised inoculated plants

Plant ID according to propagation method	CT values of plant parts assessed		
	Stem disc	Bulb apex	Leaves
Sd1	34.53	15.36	18.60
Sd2	33.46	27.31	31.08
Sd3	28.19	16.56	25.36
Sd4	34.88	26.62	29.52

Sd5	22.85	15.63	19.31
Sd6	34.99	32.51	33.74
Sd7	33.57	30.69	32.60
Sd8	33.80	27.52	30.11
Sd9	33.89	28.47	30.83
Sd10	32.34	25.31	28.75
Sd11	21.00	18.43	22.56

As highlighted in table 19, when the BHQ probe was used as the reporter molecule coupled with the AVR marker in assessing plant parts of seed propagated onions, the mean CT values for the bulb apex and leaf samples significantly dropped. The lowest CT values recorded for the bulb apex and leaf samples were 15.36 and 18.60 in respective order. However, for the stem disc samples the CT values with a peak of 34.99 were slightly higher than when the MGB probe was used 34.31 in table 18. It can be explained that the stem disc by virtue of being in the rhizosphere generally has more remnant extraneous material that may interrup binding to the reporter molecule, in this case the BHQ probe. Moreover, the plant sample Sd6 where the highest value was recorded for stem disc samples also had higher values in other plant part samples, 32.51 for bulb apex and 33.74 for the leaves, thus further consolidating the notion that it may have had more extraneous material than the other plant samples.

Table 20. Pathogen detection using PIL primers with BHQ probe in seed-raised inoculated plants

	CT values of plant parts assessed

Plant ID according to propagation method	Stem disc	Bulb apex	Leaves
Sd1	30.34	28.51	33.62
Sd2	33.42	31.65	32.75
Sd3	33.57	28.42	30.14
Sd4	29.69	25.37	30.58
Sd5	32.37	27.78	31.46
Sd6	31.66	24.89	29.25
Sd7	33.28	25.66	28.71
Sd8	32.36	21.25	27.36
Sd9	34.31	30.17	33.86
Sd10	33.53	29.46	31.28
Sd11	33.54	26.24	31.61

The use of the BHQ probe in combination with the PIL markers in assessing seed raised plant samples saw the CT values dropping across all the plant part samples as indicated in table 20, comparing with the use of the MGB probe in table 17. For instance, in the bulb apex samples with the majority of low CT values, the lowest value recorded was 21.25 which was lower than when the MGB probe ws used with the PIL markers in table 17. Moreover, unlike the AVR markers where the CT values increased in the stem disc with use of the BHQ probe coupled with PIL primers, the highest CT value was 34.31 which was lower than when MGB probe was used 35.32

(table 17). Higher detection efficiency was therefore demonstrated for this plant part (stem disc) when the PIL markers were used in combination with the BHQ probe.

Table 21. Assessing 3 plant parts using PIL markers and the MGB probe for pathogen presence in inoculated onions propagated from onion bulbs.

Plant ID according to propagation method	CT values of plant parts assessed		
	Stem disc	Bulb apex	Leaves
Bb1	33.12	28.62	30.15
Bb2	32.56	29.07	31.43
Bb3	33.28	25.38	29.27
Bb4	30.64	24.86	28.75
Bb5	33.72	27.39	30.31
Bb6	29.63	21.28	24.56
Bb7	29.86	23.84	27.95
Bb8	33.85	26.77	30.62
Bb9	34.19	29.46	31.34
Bb10	31.27	28.58	31.61
Bb11	30.73	27.49	29.84

When the plant parts of onions propagated from onion bulbs were assessed for pathogen presence using PIL markers and the MGB probe it was observed as highlighted in table 21 that the mean CT values were lower than when seed was used as propagation material in table 17. The bulb apex had the lowest CT values than the other 2 plant parts, with the least value of 21.28 it was lower than that of the seed

propagated samples 24.38 (table 17). The same trajectory was witnessed for the stem disc whereby the highest CT value 34.19 was lower than that of seed raised samples. It is also important to note that the CT range for the leaf samples was 27.95 – 31.61 as compared to 29.23 – 33.46 for the seed samples (table 17). It can be postulated that the rapid vegetative phase of the bulb propagated plants may have stimulated accelerated reproduction of the bacteria in-planta hence aggravating proliferation of the pathogen the plant tissues. The quicker detection in the samples of bulb propagated plants as compared to the seed raised plants bolsters the rationale the quicker development of the leaves provided abundant nourishment to the initial population of the bacterial hence accelerated reproduction rate and subsequent abundance in the plant tissues.

Table 22. Assessing 3 plant parts using AVR markers and the MGB probe for pathogen presence in inoculated onions propagated from onion bulbs.

Plant ID according to propagation method	CT values of plant parts assessed		
	Stem Disc	Bulb Apex	Leaves
Bb1	33.57	30.24	32.36
Bb2	31.73	29.69	30.75
Bb3	32.62	27.41	31.53
Bb4	30.25	26.42	28.14
Bb5	33.81	29.71	30.62
Bb6	33.75	31.26	32.38
Bb7	32.36	26.57	29.12
Bb8	30.64	28.82	30.29

Bb9	29.31	25.15	27.51
Bb10	32.79	27.04	29.46
Bb11	33.52	27.92	30.81

As shown in table 22 the assessment of plant parts of bulb propagated plants using AVR markers and the MGB probe also exhibited a trend where the bulb apex had the lowest mean CT values whilst the highest were recorded for the stem disc samples. However, in comparison with the seed propagated samples assessed using AVR markers and the MGB probe, the lowest CT value 25.15 was higher than than recorded for seed raised samples 17.88 (table 18). This fits seamlessly with the trend observed for the PIL markers.

Table 23. Assessing 3 plant parts using PIL markers and the BHQ probe for pathogen presence in inoculated onions propagated from onion bulbs.

Plant ID according to propagation method	CT values of plant parts assessed		
	Stem Disc	Bulb Apex	Leaves
Bb1	29.96	27.47	28.11
Bb2	31.53	25.82	28.64
Bb3	32.46	27.61	30.31
Bb4	33.75	28.34	31.26
Bb5	34.34	30.58	33.53
Bb6	35.21	29.89	32.86
Bb7	30.62	24.29	27.35

Bb8	34.59	29.33	32.95
Bb9	29.48	25.07	28.34
Bb10	32.52	28.64	30.18
Bb11	31.38	28.75	30.37

The use of the PIL markers and the BHQ probe effected the quickest detection of 24.29, recorded for the bulb apex and the highest CT value 35.21 was recorded for the stem disc as illustrated in table 23. In comparison with the results obtained for the seed-raised plant tese CT values were higher, for instance the lowest CT value recorded for the bulb apex in seed propagate plants was 21.25 whilst the stem disc had the highest CT value of 34.31 (table 20). Additionally, the plant sample Bb7 with the least CT value 24.29 recorded for the bulb apex also had the lowest value 27.35 for leaf samples and the third lowest value for the stem disc, which indicates susceptibility in comparison with the other pant samples.

Table 24. Pathogen detection using AVR primers with BHQ probe in inoculated onions grown from bulbs

Plant ID according to propagation method	CT values for plant parts assessed		
	Stem disc	Bulb apex	Leaves
Bb1	33.01	28.38	29.94
Bb2	33.46	26.52	28.29
Bb3	31.61	28.13	30.38
Bb4	35.16	27.37	31.62
Bb5	28.17	20.06	25.31

Bb6	25.36	18.83	23.47
Bb7	32.52	29.16	30.36
Bb8	33.4	28.31	30.03
Bb9	32.15	29.41	30.26
Bb10	31.43	28.72	30.24
Bb11	32.51	27.58	29.64

As illustrated in table 24, assessment of the bulb propagated plants using the AVR markers and BHQ probe as the reporter fluorophore had yielded the lowest CT value in the bulb apex sample 18.83 whereas the highest 35.16 was noted for the stem disc. The leaf samples with a CT range of 25.31 – 31.62 exhibited moderate level of detection. Though the seed propagated samples had the lowest CT values for the 3 plant part samples, there was a wide range in the CT values of the samples indicating higher deviation from the mean. For instance, for the bulb apex samples the seed raised plants exhibited a CT range of 15.36 – 32.51 (table 19) whereas the bulb propagated samples had a range of 18.33 – 29.41 indicating lower deviation from the mean.

Table 25. Pathogen detection using PIL primerswith MGB probe in onion plants raised from onion sets inoculated with *Xanthomonas euvesicatoria* pv. *allii*

Plant ID according to propagation method	CT values for plant parts assessed		
	Stem Disc	Bulb Apex	Leaves
St1	32.85	28.91	29.47
St2	35.26	28.07	30.14

St3	34.24	29.62	31.86
St4	33.23	25.92	29.31
St5	30.70	23.64	27.38
St6	31.46	26.41	29.53
St7	31.63	28.58	30.45
St8	30.55	24.25	28.68
St9	33.62	28.92	30.89
St10	34.36	27.38	30.85
St11	33.81	28.56	31.72

The assessment of plant parts from onions propagated from onion sets using the PIL markers and the MGB probe showed the most efficient detection in the bulb apex whereby the lowest CT value was 23.64, whereas the least detection was noted for the stem disc 35.26 as demonstrated in table 25. The lowest recorded CT value for the leaf samples was 27.38 which was intermediate. In comparison with other methds of propagation the detection was quicker than in the seed raised samples 24.38 (table 17) whist the bulb raised onions 21.28 (table 21) showed better results over the other 2 methods. It can be ascribed to the fact that the onion bulbs provide more nutrient resources which promote accelerated growth of the bacteria as compared to the other 2 propagation material.

Table 26. Pathogen detection using AVR primers with MGB probe in plants raised from onion sets inoculated with *Xanthomonas euvesicatoria* pv. *allii*

	CT values for plant parts assessed

Plant ID according to propagation method	Stem Disc	Bulb Apex	Leaves
St1	32.69	27.31	30.71
St2	31.35	28.63	30.62
St3	32.65	28.71	31.18
St4	30.74	26.42	29.54
St5	34.82	25.26	30.36
St6	30.41	23.57	28.89
St7	30.15	28.39	30.82
St8	32.26	27.45	29.93
St9	33.89	28.38	31.26
St10	33.57	26.76	29.43
St11	32.39	29.21	30.14

Detecting *X. euvesicatoria* pv. *allii* using the AVR markers and the MGB probe in 3 plant parts of onions raised from onion sets revealed that the lowest CT value 23.57 was obtained in the bulb apex whilst the highest was recorded for the stem disc 34.82 as shown in table 26. Moreover, the plant sample St6 in which the lowest CT value was recorded for the bulb apex also had the lowest CT value 28.89 for the leaf sample as well as the second lowest CT value for the stem disc 30.41. This shows that the pathogen had extensively permeated the tissues of this plant sample. Comparing onion sets raised plants with other propagation methods showed that, for the bulb apex samples detection of the pathogen in onion sets samples (table 26) was

faster than in the bulb propagated plants (table 22) but second to that of seed raised samples. However, for the stem disc the onion set samples performed second to the bulb raised samples but better than the seed propagated samples.

Table 27. Detection of *X. euvesicatoria* pv. *allii* using AVR primers with BHQ probe in plants propagated from inoculated onion sets

Plant ID according to propagation method	Ct values of plant part assessed		
	Stem disc	Bulb apex	Leaves
St1	26.38	20.47	24.46
St2	27.43	25.02	26.12
St3	28.61	25.11	27.44
St4	28.87	26.01	27.40
St5	28.50	23.31	26.62
St6	29.36	25.75	27.83
St7	29.53	26.37	28.19
St8	29.65	25.14	27.83
St9	30.17	27.62	29.58
St10	30.38	27.47	29.24
St11	29.89	26.28	28.72

The detection of the bacteria using AVR primers and BHQ probe in inoculated onion plants propagated from onion sets exhibited higher efficiency levels for the bulb apex as indicated by the lower CT values in comparison with the stem disc and leaf samples, table 27. The lowest CT value was recorded for the bulb apex 20.47. In

contrast the highest CT value 30.38 was recorded for the stem disc. Worthy of note is the plant sample St1 in which the lowest CT values (20.47; 24.46 and 26.38) for all 3 plant part samples i.e. bulb apex, leaves and the stem disc respectively were obtained. In contrast the plant sample St9 had the highest CT values (27.62 and 29.58) for the bulb apex and the leaves, as well as the second highest value 30.17 for the stem disc. This shows that the bacteria's proliferation in the tissues of this plant was less than in the other plant samples. Comparing with other plant propagation methods, the bulb apex and leaves of the onion set samples were the least rapid in terms of detection. However, for the stem disc the onion set samples had the least range of CT values 26.38 - 30.38 (table 27), thus lower deviation than the other two propagation methods.

Table 28. Detection of *X. euvesicatoria* pv. *allii* using PIL primers with BHQ probe in plants propagated from inoculated onion sets

Plant ID according to propagation method	CT values for plant parts assessed		
	Stem Disc	Bulb Apex	Leaves
St1	28.22	21.12	24.58
St2	29.31	23.86	26.51
St3	28.35	25.16	27.47
St4	29.37	26.64	27.83
St5	30.05	26.53	28.36
St6	28.89	23.21	25.68
St7	30.41	24.47	28.92
St8	29.63	23.75	27.74

St9	31.38	28.18	30.26
St10	27.24	23.86	25.70
St11	31.73	27.14	29.31

Assessing the detection of the bacteria using PIL primers and BHQ probe in plants propagated from onion sets revealed significant differences amongst plant samples and plant parts assessed. The bulb apex was more adapted to the assay as indicated by the lowest CT value 21.12 whilst the highest CT value 31.73 was recorded for the stem disc, table 28. Just like the pattern witnessed when the AVR primers and the BHQ probe were used in assessing plant from onions propagated from onion sets, the plant sample St1 had all the lowest CT values (21.12; 24.58 and 28.22) viz. bulb apex, leaves and stem disc in respective order. In contrast the plant sample St9 had the highest values (28.18; 30.26) for the bulb apex and respectively as well as the second highest value for the stem disc 31.38. In comparison with the other methods of propagation the onion set samples had the quickest rate of detection for all plant parts, though slightly quicker (21.12, table 28) than the seed propagated samples (21.25, table 20).

3.9 The efficacy of DNA extraction methods on enhancement of the *Xanthomonas euvesicatoria* pv. *allii* PCR assay

Xanthomonas euvesicatoria pv. *allii* is a gram-negative, motile phytopathogenic bacterium, (EPPO, 2016). This bacterium causes bacterial blight of onion (BBO), a devastating foliar disease which seriously threatens onion production and the profitability of the enterprise, (Gent et al., 2004). The disease adversely affects the crop's leaf area index (LAI), hence reducing photosynthetic activity and retarding growth. The members of the *Allium* genus such as *A. sativum* (garlic), *A. fistulosum* (Welsh onion), *A. porrum* (leeks) and *A. schoenoprasum* (chives) are also susceptible to attack by this pathogen. Though the host range for *X. euvesicatoria*

pv. *allii* is broad, the incidence of the disease is frequently reported in the onion crop, (Pruvost et al., 2016). Furthermore, the bacteria is seed-borne and can be transmitted vertically, (Humeau et al., 2006). The pathogen is existent on three continents namely, Asia, America and Africa. *Xanthomonas euvesicatoria* pv. *allii* is classified as an A1 quarantine pathogen and measures are constantly being sought to curb its transmission to other regions, (Robène et al., 2015).

Rapidity and high accuracy of pathogen detection and identification are crucial in phytosanitary laboratories. A PCR protocol has been developed to competently detect the pathogen and reduce the risk of transmission especially through seed trade, (EPPO, 2016). Previous research has established that DNA extraction methods can influence the sensitivity of real-time PCR assays, (Dauphin et al., 2010; Queipo-Ortuño et al., 2008) therefore selection of the best method for *X. euvesicatoria* pv. *allii* is vital for diagnostic and identification purposes. PCR performance is dependent upon effective DNA extraction and purification, (Berensmeier, 2006). Though there are many commercial DNA extraction kits for plant samples it is necessary to identify the most optimum for recovering the nucleic acids of a particular pathogen, hence enhancing the throughput and reliability of the method, (Mahmoudi et al., 2011). Inefficient extraction of nucleic acids potentially leads to inaccurate diagnostic results, (Mölsä et al., 2016).

The thrust of this experiment was to evaluate commercially available DNA extraction kits optimized for plant material and identify the most suitable for isolating *X. euvesicatoria* pv. *allii*. The three commercial extraction kits, Probe GS, Sorb GMO and FitoSorb used in the study have different mechanisms of DNA recovery i.e. magnetic beads, silica-based sorbent as well as glass and diatomaceous earth.

3.9.1

Table 29. Shows mean Ct values and standard deviation at each bacterial concentration for the three kits

Extraction Method	Avg. CT (mean±SD) with following dilutions of *X. euvesicatoria pv. allii*						
	10^7	10^6	10^5	10^4	10^3	10^2	10^1
Probe GS	17.9[a] (±0.21)	20[a] (±0.47)	24.5[a] (±0.06)	27.3[a] (±0.06)	30.8[a] (±0.64)	34.2 (±0.45)	35.8 (±0.44)
Sorb-GMO	20.9[b] (±0.80)	23.7[c] (±0.26)	27.8[b] (±1.33)	31.4[b] (±1.59)	34.2[b] (±0.78)	ND	ND
FitoSorb	19.6[b] (±0.64)	22.6[b] (±0.15)	25.9[ab] (±0.69)	29.6[ab] (±1.08)	32.9[b] (±0.95)	ND	ND

§Different letters in the same column denote statistically significant differences

*ND, not determined, the average CT cycles were only calculated for samples which had three positive results out of three replicates

The different extraction methods effected a wide range of DNA detectability on the PCR assay ($p < 0.05$), table 29. The Probe GS extraction kit effected the highest degree of sensitivity on the PCR assay and had the most consistent detection ability at all seven concentrations used in the study. The method had all 3 replications of each sample being detectable at every concentration. At lower concentrations 10^2 CFU ml^{-1} and 10^1 CFU ml^{-1}, the other two methods Sorb GMO and FitoSorb could not attain 3 positive results per sample, hence the cut of thresholds at those concentrations were not determined, (ND). Moreover, the lowest cycle thresholds were achieved by Probe GS in comparison with the other two methods. At the five concentrations where all the kits produced positive results for *X. euvesicatoria* pv. *allii*, Sorb GMO had the highest Ct values ranging from 20.9-34.2 Ct. However, it

has to be noted that at concentrations 10^5 CFU ml^{-1} and 10^4 ml^{-1} FitoSorb had comparable ($p < 0.05$) cycle thresholds with Probe GS having values of 25.9; 29.6 and 24.5; 27.3 respectively.

3.9.2 The distribution of sample Ct values with respect to concentration on the scatter column and box-plot

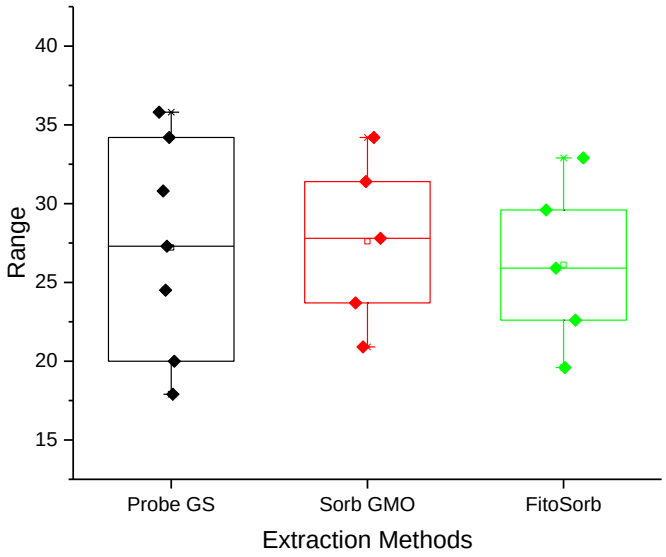


Fig 13. The box-plots showing the distribution of Ct values in the real-time PCR analysis as influenced by three different DNA extraction methods (Probe GS, Sorb GMO, FitoSorb).

The scatter columns and box-plots in fig 13 show the distribution of the mean Ct values obtained for the DNA recovered at each concentration of *X. euvesicatoria* pv. *allii* using the three extraction methods. The values for Probe GS formed a perfect column as compared to Sorb GMO and FitoSorb. This pattern corresponds with the low margins of standard deviation obtained for the method, whereas Sorb GMO and FitoSorb with wider margins of standard deviation had unevenly distributed scatter columns. At the concentrations that the kits were tested the Ct values were significantly different ($p < 0.05$; n=21). At the highest concentration of 10^7, Probe GS had the lowest Ct value of 17.9 and the lowest concentration 10^1 x 3 CFU/ml it

was the only kit whose Ct value could be determined. FitoSorb had at least one positive replication but not all three at the lowest concentrations 10^2 and 10^1, hence the Ct value could not be determined just as in the case of Sorb GMO.

3.9.3

Table 30. The limit of detection (LOD) for the DNA from the three extraction kits

Extraction Method	Limit of detection (CFU/ml^{-1})	Cycle threshold (CT)
Probe GC	3x10^1	35.8b
Sorb-GMO	3x10^3	34.2ab
FitoSorb	3x10^3	32.9a

*Different letters in the same column denote statistically significant differences

The limit of detection was determined as the lowest concentration at which all three replications per sample were positive as exhibited by the cycle threshold values < 40. Table 30 shows the limit of detection for all three extraction methods. The Probe GS method had the best limit of detection as it yielded DNA detectable at the lowest concentration used in the experiment, 10^1 x 3 CFU/ml. The DNA extracted by both Sorb GMO and FitoSorb had similar limit of detection 10^3 x 3 CFU/ml. Moreover, pairwise comparison revealed that the cycle threshold achieved by Probe GS 35.8 at its limit of detection 10^1 CFU/ml was statistically comparable ($p > 0.05$) to that of Sorb GMO 34.2 with a detection limit of 10^3, though significantly ($p < 0.05$) different from FitoSorb.

The objective for carrying out this study was to identify the most optimal commercially available DNA extraction kit for efficient detection of *X. euvesicatoria* pv. *allii*. The findings of the study revealed that the Probe GS

extraction kit which had DNA yield that achieved the lowest limit of detection is the best for recovering the DNA of *X. euvesicatoria* pv. *allii*.

Sorbent characteristics such as surface chemistry and porosity aspects play a major role in controlling reversible adsorption and ultimately DNA yield quality, (Günal et al., 2018). Surface chemistry is a function of the base material and synthesis process used in producing the sorbent. This explains why the three extraction methods had different DNA yield quality resulting in variable cross thresholds and limits of detection.

The results obtained from evaluating the extraction kits contradict the assertion that the methods which involve the use of magnetic sorbents are more robust and counter the difficulties associated with DNA extraction as compared to other techniques, (Berensmeier, 2006; Scobeyeva et al., 2018). To the contrary the glass and diatomaceous earth sorbent-based method Probe GS proved quicker in obtaining the DNA and had superior quality in comparison with FitoSorb which is magnetic based.

Moreover, Dauphin et al., (2009) observed that at lower concentrations purity has greater effect on detection than yield (quantity). Though magnetic particles are generally larger in size with diameter ranging from 0.5-10μm approximately, (Berensmeier, 2006), hence greater surface area for nucleic acid adsorption, the same space is also available for the retention of extraneous material such as proteins and carbohydrates from the lysed cells. These extraneous materials interfere with detection during PCR. Ip et al., (2015) observed lower peak heights for the vWA locus on the electropherogram with DNA isolated by two magnetic particle-based extraction kits, (QIAsymphony and IQ) and suspected either DNA purity or presence of inhibitors as possible reasons for the peak drop. This is consistent with the non-detection of FitoSorb isolated DNA at the lowest concentrations of 10^1 and 10^0 CFU/ml. However, at moderate concentrations of 10^3 and 10^4 CFU/ml the cycle thresholds were comparable to those of Probe GS prepared DNA. This shows that

the concentration reduces beyond a certain threshold point DNA purity becomes a crucial factor determining detectability.

In exploring the reasons why Sorb GMO and FitoSorb yielded DNA of lower quality than Probe GS as exhibited by the results from the PCR assay, it is important to consider three factors that lead to such results namely, cell lysis, adsorption of DNA to particulate material and degrading or shearing of DNA. Cell lysis mechanisms involve chemicals, enzymatic activity, mechanical action and heat, (Vesty et al., 2017). In gram-negative bacteria cell lysis is achieved with relative ease and this can be incompatible with methods that highly depend on mechanical action, (Coyne et al., 2004; Li et al., 2020; Martzy et al., 2019). The mechanical action of FitoSorb with magnetic particles adapted for bead beating may have sheared the DNA thus compromising quality. Moreover, the results for both Sorb GMO and FitoSorb are in tandem with findings from previous research which revealed that such methods with multiple changes of micro-centrifuge tubes throughout the extraction process result in poor DNA quality due to DNA manipulation during the transfers, (Cheng and Jiang, 2006).

This also explains the inconsistency of Sorb GMO results with stated findings, that the enzymatic approach produces DNA of higher yield and quality, (Vesty et al., 2017). Though Sorb GMO constitutes proteinase K+ which digests cell proteins therefore reducing their interference with the amplification process, DNA manipulations that occur during the transfer of the nucleic acids to new tubes at many stages reduces the quality. It also has to be noted that DNA purity is highly influenced by the ionic strength and pH of the elution buffer, (Vandeventer et al., 2012). The purity is compromised when the chaotropic salts that enhance the adsorption of DNA to the sorbent are carried over to the amplification reaction. A low ionic strength, high pH elution buffer is used to remove the DNA from the sorbent. Some buffers are highly compatible with the subsequent PCR amplification stage hence high detection levels. The results indicate that the reagents and elution

buffer used in the Probe GS method yielded DNA of better purity than Sorb GMO and FitoSorb.

3.10. Germination response of commercial onion varieties to inoculation with *Xanthomonas euvesicatoria* pv. *allii*

The object of carrying out this experiment was to investigate the effect of *Xanthomonas euvesicatoria* pv. *allii* on the germination of onion seeds. We sought to find the extent of influence the pathogen has on the germination parameters of onion. The study was executed in vitro giving a glimpse of the dynamics of what may take place in the soil. The research unravels the potential effects that may be caused on onion seed in soils that are infested with the bacteria.

3.10.14. Effect of inoculum concentration on onion seed germination

At day 5 of recording all 3 bacterial concentrations significantly inhibited onion seed germination. For most of the onion varieties the level of germination inhibition increased with an increase in bacterial concentration, as illustrated in table 31.

Table 31. Germination percentage at day 5 of recording

Variety	**Germination percentage per bacterial concentration- %**			
	10^8	**10^6**	**10^4**	**Control**
Pierrot	62c	71b	77b	91a
Russian winter	56b	58b	69a	85a
Karantansky	9b	14a	16a	17a
Carmen MS	2b	3b	4b	17a
Summer breeze	17c	23b	26ab	27a
Strigunovsky local	24c	26bc	30ab	35a
April	21b	30ab	35a	37a

Schtuttgarter Riesen	14c	20b	25b	30a
Myachkovsky 300	3c	14b	16b	29a
Danilovsky 301	42c	73b	79a	80a
Chalcedony	0b	0b	0b	2a
Ellan	7c	16b	22a	25a

*Different letters in the same row denote statistically significant differences. The Tukey's test was used to separate the means.

Though the highest concentration in Karantansky was significantly different 9%, the concentrations 10^6 and 10^4 CFU/ml with germination percentages of 14% and 16% respectively were statistically similar to the control, 17%. For varieties such as Carmen MS and Chalcedony there were no significant differences amongst the concentrations though all treatments were significantly different ($p < 0.05$) from the control. For these 2 varieties the level of inhibition was more pronounced. In Carmen MS the germination percentage dropped from 17% in the control to 2-4% in the treatments. In Chalcedony no germination was recorded in all 3 concentrations at day 5 of recording.

Table 32. Germination percentage at day 14 of recording

Variety	**Germination percentage per bacterial concentration- %**			
	10^8	**10^6**	**10^4**	**Control**
Pierrot	80c	84bc	86b	96a
Russian winter	59d	66c	75b	92a
Karantansky	54b	56b	57b	83a
Carmen MS	4c	6bc	8b	45a

Summer breeze	55c	61b	68a	73a
Strigunovsky local	45b	48b	51b	87a
April	45c	51bc	55b	82a
Schtuttgartter Riesen	21c	52b	67a	69a
Myachkovsky 300	4d	22c	35b	85a
Danilovsky 301	50d	76c	83b	94a
Chalcedony	0b	0b	0b	8a
Ellan	23c	26c	46b	53a

*Different letters in the same row denote statistically significant differences. The Tukey's test was used to separate the means.

At day 14 of recording the different inoculum concentrations had significant inhibition ($p < 0.05$) on the germination of various onion varieties, as shown in table 3. Moreover, for most of the onion varieties there were variations in the level of germination inhibition across different concentrations. Therefore, the highest concentration 10^8 CFU/ml effected the highest level of inhibition, whilst 10^6 CFU/ml had moderate levels and the least inhibition observed in the least concentration 10^4 CFU/ml. In the variety Carmen MS the pattern of germination inhibition shifted at day 14 of recording where the lowest germination percentage (4%) was observed in the highest bacterial concertation, though concentrations 10^6 and 10^4 CFU/ml were statistically similar. In Chalcedony, the similar pattern observed at day 5 of recording persisted at day 14 of recording, no seed germinated in any of the bacterial concentrations against a germination percentage of 8% in the control.

3.10.2 Effect of bacterial concentration on germination energy

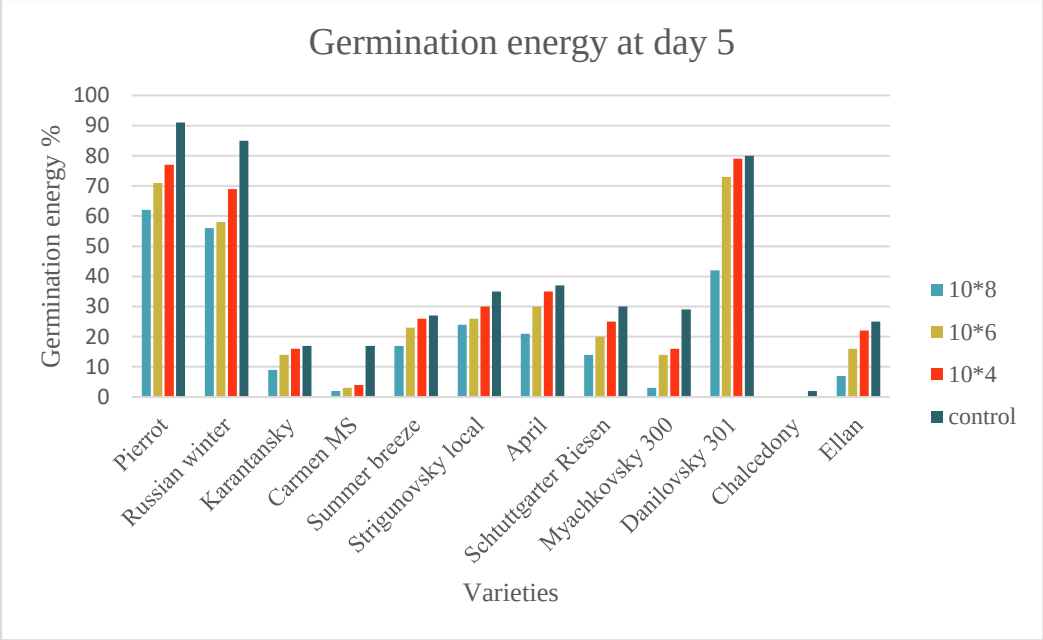


Fig 14. The chart shows germination energy at different concentrations on day 5 of recording

As shown in fig 14, on day 5 of recording the different inoculum concentrations had an influence ($p < 0.05$) on germination energy of the 12 onion varieties. The inoculum concentration and germination energy were inversely proportional i.e. the increase in bacterial concentration corresponded with low germination in the onion seeds. The highest inoculum concentration had the highest impact on 3 varieties namely, Myachkovsky 300, Carmen MS and Chalcedony with germination energy of 3, 2 and 0 respectively.

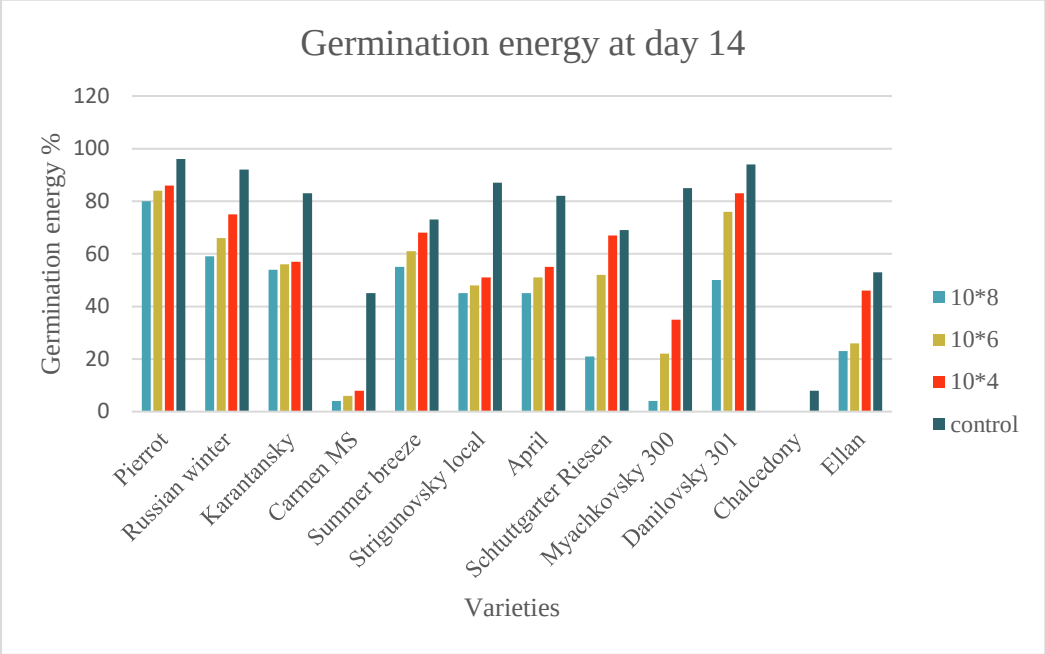


Fig 15. Shows germination level at different bacterial concentrations on day 14 of recording

At day 14 of recording all inoculum concentrations significantly reduced germination energy in the onion varieties as exhibited by fig 15. In the majority of the varieties, the highest bacterial concentration 10^8 CFU/ml effected the lowest germination energy followed by 10^6 CFU/ml and the least impactful being 10^4 CFU/ml. However, for the varieties Karantansky and Chalcedony there were no variations amongst the concentrations though all treatments significantly differed from the control ($p < 0.05$). In Chalcedony, though germination energy increased in the control from 2 to 8, in all treatments it remained constant at 0.

The research findings proved that the three bacterial concentrations significantly suppressed the germination of the 12 onion varieties. Bashan (1986) inoculated pepper and tomato seeds with *Xanthomonas campestris vesicatoria* and noted that germination reduced with increase in bacterial concentration. This aspect was observed for most of the varieties that were tested and the results were in tandem with that of (Bashan, 1986).

Some varietal differences can be attributed to the variations amongst the treatments (Bashan and Okon, 1981). There are varieties which succumbed uniformly to every bacterial concentration. At day 5 of recording the varieties Carmen MS and Chalcedony had their germination uniformly supressed at different concentrations. Nevertheless, at day 14 the highest concentration seemed to have effect on Carmen MS since it was significantly different from the other 2 concentrations 10^6 CFU/ml and 10^4 CFU/ml. However, in Chalcedony the germination pattern did no change as it remained homogenous in the 3 concentrations at day 14. It can be concluded that the variety Chalcedony is the most susceptible to *X. euvesicatoria* pv. *allii*.

The germination energy of the 12 varieties was affected by the bacterial concentrations. Dadon et al. (2004) mentioned of a factor in *Azospirillum brasilense* that significantly affects germination parameters such as germination energy in *Orobanche aegyptiaca*. Some low molecular peptide is produced by the bacteria which binds to the germination site on the seed thus affecting germination over a certain period of time, thus lowering germination energy (Miche et al., 2000). The higher the inoculum concentration the lower the germination energy exhibited by the varieties. A high inoculum concentration coincides with a protracted germination inhibition, hence low germination energy.

Though germination energy generally increased in most varieties it did not change in Chalcedony which remained constant at zero. Moreover, the fact that the different concentrations had homogenous effect on germination energy of Karantansky and Chalcedony may reveal susceptibility of the 2 varieties even at low concentrations. This is consistent with the findings of Ahonsi et al. (2002) whereby *Striga hermonthica* was proved to be highly susceptible to the fluorescent *Pseudomonas spp*. used in the study. Futhermore, Chalcedony had the lowest germination energy (0) showing high susceptibility of this variety to the bacteria *Xanthomonas euvesicatoria* pv. *allii*.

Conclusion

Based on the results of the research on the collection of data on the systematics, pathogenicity, and biology of bacteria, it was observed that the *X. euvesicatoria* pv. *allii* strains do not have a distinct host specificity among the various *Allium* species. Additionally, when disease effects in-planta on various parts of the onion plants grown from different propagation material under field conditions it was discovered that the bacteria multiplied vigorously and had prolonged persistence in the bulb apex as compared to the leaves and the stem-disc.

The study on the cultural properties and growth characteristics of bacteria on different growth media demonstrated that on the OEM incubation temperature was an influential factor especially for *X. euvesicatoria* pv. *allii* strain 0419 which did not grow on the media at 28°C but produced colonies at 25°C. On the NBY and YPGA media, both strains were able to produce colonies, but strain 0419 showed somewhat restricted growth on YPGA compared to strain 0377.

All technical aspects i.e. sensitivity, specificity, repeatability and reproducibility of the qPCR as well as the conventional nested PCR were evaluated and validated. Sensitivity of the qPCR was determined and the lowest detection concentration was $6.8x10^1$ CFU/ml whereas for the conventional nested PCR the least detectable concentration was $1.0x10^2$ CFU/ml. The test had a specificity of 99% when the conventional PCR was assessed whilst the qPCR exhibited 100% specificity. The test had high accuracy hence good repeatabiity, however at concentration 10^4 using both AVR and PIL primers was lower i.e. small variations between the concentration replications. For both AVR and PIL primers the good reproducibility, below 2% relative standard deviation exhibited by the test between operators with their respective equipment is indicative of the achievable accuracy by which the pathogen can be detected.

The BHQ probe was evaluated in testing the presence of the bacteria in different parts of inoculated onion plants and was found to be effective when using both AVR and PIL primers. The BHQ probe is therefore recommended as an alternative to the MGB probe which is not available on the Russian market.

The study confirmed that DNA isolation method influences the performance of the assay. The DNA extraction kit Probe GS enhanced PCR sensitivity such that the assay could detect the bacteria at the lowest concentration of 10^1 CFU/ml whilst the other 2 methods Sorb GMO and FitoSorb could only detect the bacteria at the lowest concentration of 10^3 CFU/ml. Therefore, Probe GS is recommended as the standard method of isolating DNA for *X. euvesicatoria* pv. *allii*.

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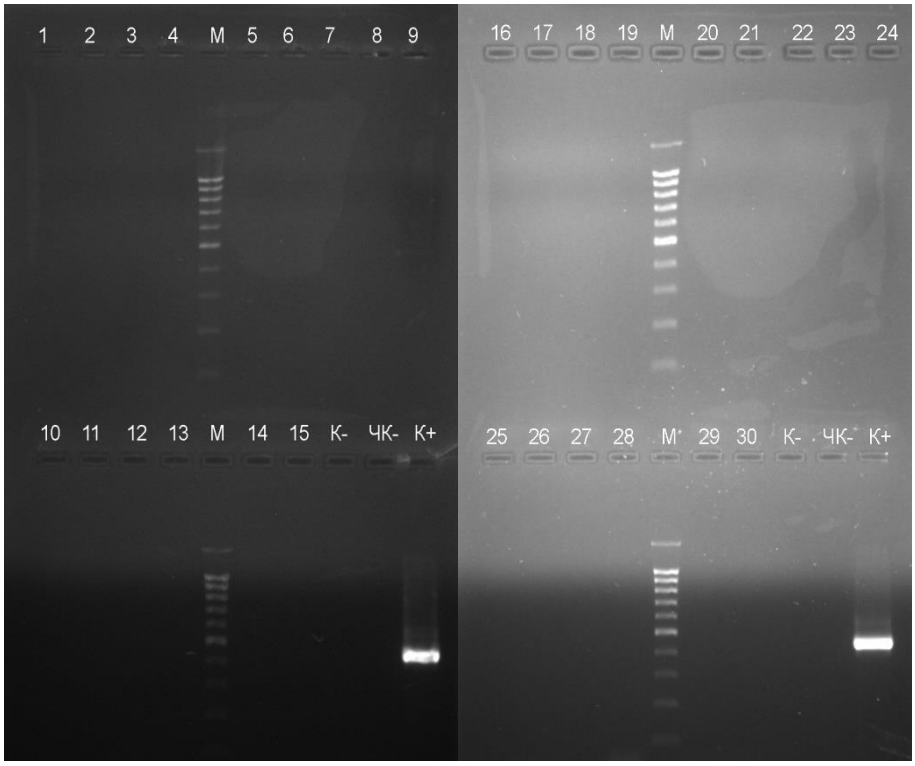
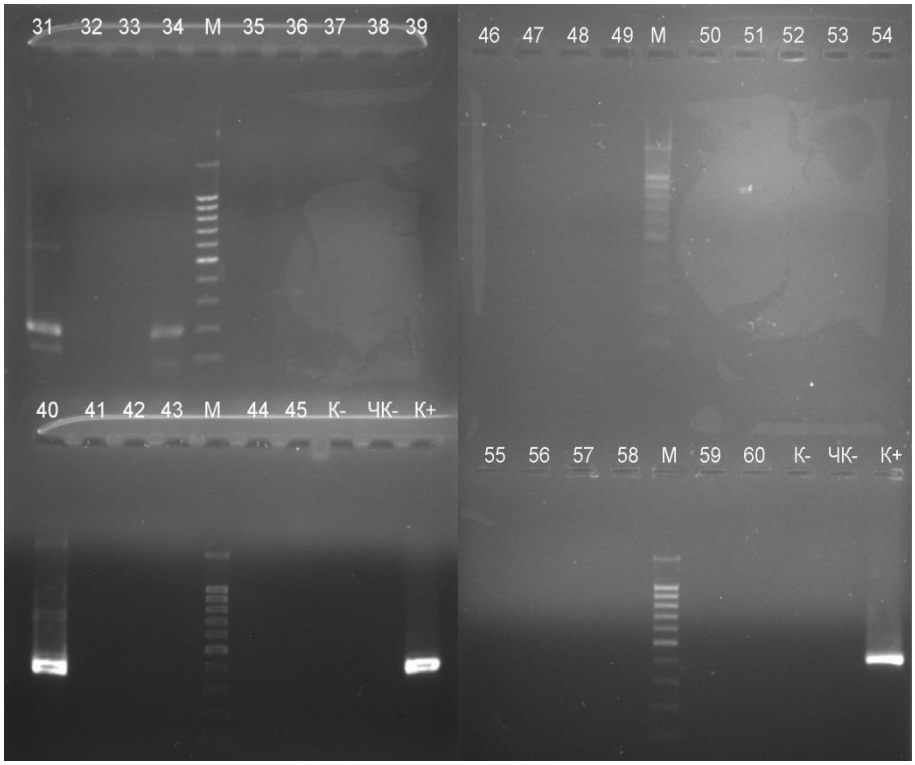
Appendix

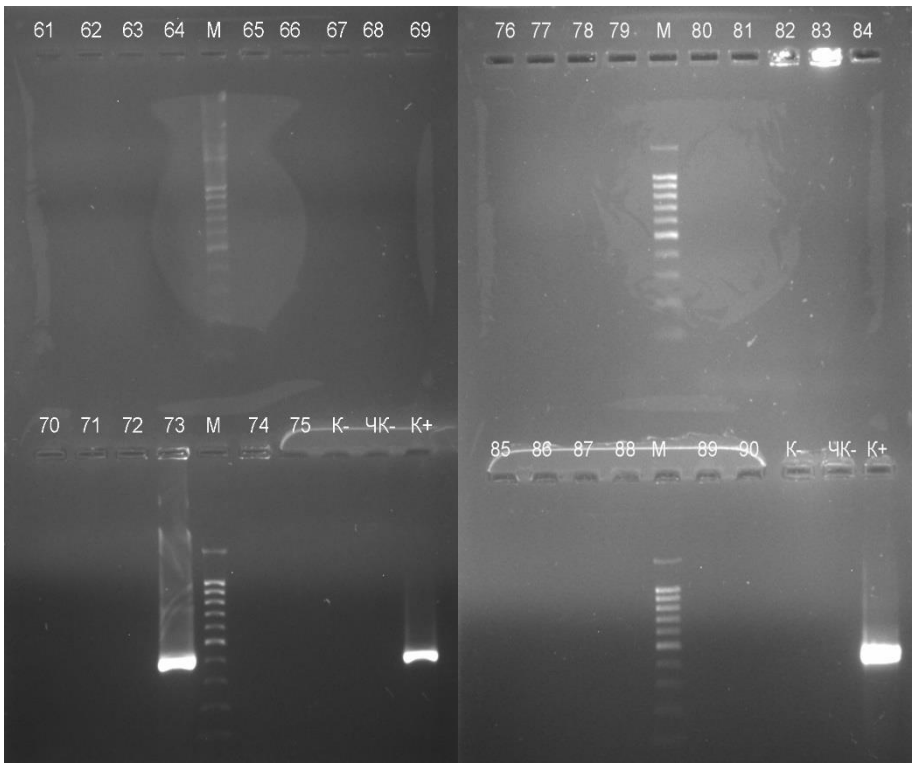
Electrograms for conventional PCR specifity evaluations



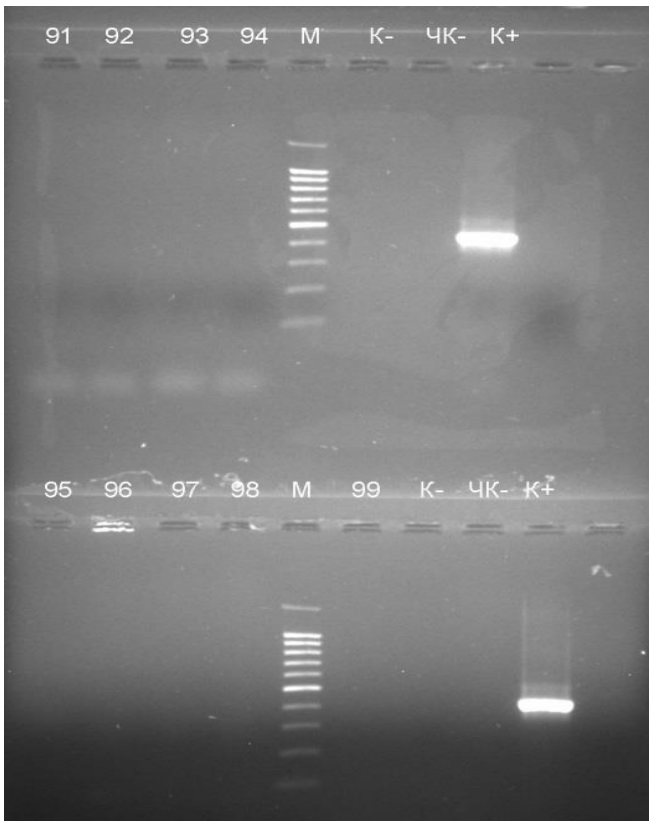
Appendix 1. Shows 30 bacterial species evaluated in the exclusivity test that tested negative to the assay, К- is the negative control, ЧК- is the second negative control and К+ is the positive control



Appendix 2. Number 40 in the electrogram represents a strain of *Xanthomonas euvesicatoria* pv. *allii* that tested positive to the assay.



Appendix 3. Shows a Xanthomonas species (73) which was wrongly identified as *Xanthomonas euvesicatoria* pv. *allii*



Appendix 4. Exhibiting the last 9 bacterial strains which tested negative to the PCR assay

Data on PCR performance as influenced by DNA extraction method

Номер лунки	Идентификатор пробирки	Ср, Fam	Ср, Hex	Результат
B1	Проба_ГС_1-1 (Xaa (X.alii))	17,8	19,0	+
B2	Проба_ГС_1-2 (Xaa (X.alii))	17,7	18,5	+
B3	Проба_ГС_1-3 (Xaa (X.alii))	18,1	18,7	+
B4	Проба_ГС_2-1 (Xaa (X.alii))	21,4	22,3	+
B5	Проба_ГС_2-2 (Xaa (X.alii))	20,5	21,5	+
B6	Проба_ГС_2-3 (Xaa (X.alii))	21,2	22,1	+
B7	Проба_ГС_3-1 (Xaa (X.alii))	24,5	25,1	+
B8	Проба_ГС_3-2 (Xaa (X.alii))	24,5	25,2	+
B9	Проба_ГС_3-3 (Xaa (X.alii))	24,6	25,3	+
B10	Проба_ГС_4-1 (Xaa (X.alii))	27,3	27,3	+
B11	Проба_ГС_4-2 (Xaa (X.alii))	27,2	27,5	+
B12	Проба_ГС_4-3 (Xaa (X.alii))	27,3	28,4	+
C1	Проба_ГС_5-1 (Xaa (X.alii))	30,1	32,3	+
C2	Проба_ГС_5-2 (Xaa (X.alii))	31,3	32,3	+
C3	Проба_ГС_5-3 (Xaa (X.alii))	31,1	32,6	+
C4	Проба_ГС_6-1 (Xaa (X.alii))	33,7	33,8	+

	C5	Проба_ГС_6-2 (Xaa (X.alii))	34,6	34,4	+
	C6	Проба_ГС_6-3 (Xaa (X.alii))	34,2	34,4	+
	C7	Проба_ГС_7-1 (Xaa (X.alii))	35,6	34,2	+
	C8	Проба_ГС_7-2 (Xaa (X.alii))	35,5	34,2	+
	C9	Проба_ГС_7-3 (Xaa (X.alii))	36,3	34,4	+
	C10	К-1 (Xaa (X.alii))		34,7	-
	C11	К-2 (Xaa (X.alii))		34,8	-
	C12	К-2 (Xaa (X.alii))		35,0	-
	D1	Сорб_ГМО_1-1 (Xaa (X.alii))	21,7	22,5	+
	D2	Сорб_ГМО_1-2 (Xaa (X.alii))	20,1	21,3	+
	D3	Сорб_ГМО_1-3 (Xaa (X.alii))	20,8	21,9	+
	D4	Сорб_ГМО_2-1 (Xaa (X.alii))	23,6	24,5	+
	D5	Сорб_ГМО_2-2 (Xaa (X.alii))	24,0	24,7	+
	D6	Сорб_ГМО_2-3 (Xaa (X.alii))	23,5	24,4	+
	D7	Сорб_ГМО_3-1 (Xaa (X.alii))	27,1	28,1	+
	D8	Сорб_ГМО_3-2 (Xaa (X.alii))	29,3	29,8	+
	D9	Сорб_ГМО_3-3 (Xaa (X.alii))	26,9	27,5	+
	D10	Сорб_ГМО_4-1 (Xaa (X.alii))	30,4	31,3	+
	D11	Сорб_ГМО_4-2 (Xaa (X.alii))	33,2	33,4	+
	D12	Сорб_ГМО_4-3 (Xaa (X.alii))	30,5	31,4	+
	E1	Сорб_ГМО_5-1 (Xaa (X.alii))	35,1	33,1	+
	E2	Сорб_ГМО_5-2 (Xaa (X.alii))	33,8	33,4	+
	E3	Сорб_ГМО_5-3 (Xaa (X.alii))	33,7	33,3	+
	E4	Сорб_ГМО_6-1 (Xaa (X.alii))	36,3	32,9	+
	E5	Сорб_ГМО_6-2 (Xaa (X.alii))	35,7	33,2	+
	E6	Сорб_ГМО_6-3 (Xaa (X.alii))		28,8	-
	E7	Сорб_ГМО_7-1 (Xaa (X.alii))		28,8	-

12.11.2020

	E8	Сорб_ГМО_7-2 (Xaa (X.alii))		32,4	-
	E9	Сорб_ГМО_7-3 (Xaa (X.alii))		32,3	-
	E10	К-1 (Xaa (X.alii))		24,8	-
	E11	К-2 (Xaa (X.alii))		28,3	-
	E12	К-3 (Xaa (X.alii))		33,7	-
	F1	ФитоСорб_1-1 (Xaa (X.alii))	20,3	21,1	+
	F2	ФитоСорьб_1-2 (Xaa (X.alii))	19,2	20,0	+
	F3	ФитоСорб_1-3 (Xaa (X.alii))	19,2	20,1	+
	F4	ФитоСорб_2-1 (Xaa (X.alii))	22,6	23,4	+
	F5	ФитоСорб_2-2 (Xaa (X.alii))	22,5	23,4	+
	F6	ФитоСорб_2-3 (Xaa (X.alii))	22,8	23,7	+
	F7	ФитоСорб_3-1 (Xaa (X.alii))	26,7	27,2	+
	F8	ФитоСорб_3-2 (Xaa (X.alii))	25,5	26,2	+
	F9	ФитоСорб_3-3 (Xaa (X.alii))	25,5	26,3	+
	F10	ФитоСорб_4-1 (Xaa (X.alii))	30,8	31,8	+
	F11	ФитоСорб_4-2 (Xaa (X.alii))	29,3	29,4	+
	F12	ФитоСорб_4-3 (Xaa (X.alii))	28,7	29,6	+
	G1	ФитоСорб_5-1 (Xaa (X.alii))	33,6	33,5	+
	G2	ФитоСорб_5-2 (Xaa (X.alii))	33,2	33,5	+
	G3	ФитоСорб_5-3 (Xaa (X.alii))	31,8	32,9	+
	G4	ФитоСорб_6-1 (Xaa (X.alii))		32,4	-
	G5	ФитоСорб_6-2 (Xaa (X.alii))	35,6	34,2	+
	G6	ФитоСорб_6-3 (Xaa (X.alii))	35,1	34,1	+
	G7	ФитоСорб_7-1 (Xaa (X.alii))	22,8	23,7	+
	G8	ФитоСорб_7-2 (Xaa (X.alii))	36,5	26,4	+
	G9	ФитоСорб_7-3 (Xaa (X.alii))		33,8	-
	G10	К-1 (Xaa (X.alii))		33,3	-
	G11	К-2 (Xaa (X.alii))		33,3	-
	G12	К-3 (Xaa (X.alii))		33,2	-
	H1	К+ (Xaa (X.alii))	17,8	17,8	+
	H2	К+ (Xaa (X.alii))	17,7	18,1	+
	H3	К+ (Xaa (X.alii))	18,1	18,3	+

H4	K- (Xaa (X.alii))		34,6	-

Appendix 5. Data on DNA isolation methods

Data for host selectivity trials

Номер лунки	Идентификатор пробирки	Ср, Hex	Ср, Hex	Результат
B2	1-4.1-0419 (X.a.a.)	26,9	26,9	+
B3	1-4.2-0419 (X.a.a.)	26,7	26,7	+
B4	1-4-3-0419 (X.a.a.)	26,6	26,6	+
B5	1-5-1-0419 (X.a.a.)	29,7	29,7	+
B6	1-5.2-0419 (X.a.a.)	30,0	30,0	+
B7	1-5.3-0419 (X.a.a.)	29,8	29,8	+
B8	1-6.1-0419 (X.a.a.)	35,7	35,7	+
B9	1-6.2-0419 (X.a.a.)	33,1	33,1	+
B10	1-6.3-0419 (X.a.a.)	32,4	32,4	+
C2	2-4.1-0419 (X.a.a.)	26,3	26,3	+
C3	2-4.2-0419 (X.a.a.)	26,3	26,3	+
C4	2-4.3-0419 (X.a.a.)	26,5	26,5	+
C5	2-5.1-0419 (X.a.a.)	29,1	29,1	+
C6	2-5.2-0419 (X.a.a.)	28,8	28,8	+
C7	2-5.3-0419 (X.a.a.)	29,3	29,3	+
C8	2-6.1-0419 (X.a.a.)	32,5	32,5	+
C9	2-6.2-0419 (X.a.a.)	33,0	33,0	+
C10	2-6.3-0419 (X.a.a.)	32,9	32,9	+
D2	3-4.1-0419 (X.a.a.)	26,0	26,0	+
D3	3-4.2-0419 (X.a.a.)	25,5	25,5	+
D4	3-4.3-0419 (X.a.a.)	25,8	25,8	+
D5	3-5.1-0419 (X.a.a.)	28,8	28,8	+
D6	3-5.2-0419 (X.a.a.)	29,2	29,2	+
D7	3-5.3-0419 (X.a.a.)	28,7	28,7	+
D8	3-6.1-0419 (X.a.a.)	31,8	31,8	+
D9	3-6.2-0419 (X.a.a.)	31,7	31,7	+
D10	3-6.3-0419 (X.a.a.)	31,6	31,6	+
E2	4-4.1-0419 (X.a.a.)	26,9	26,9	+
E3	4-4.2-0419 (X.a.a.)	27,0	27,0	+
E4	4-4.3-0419 (X.a.a.)	27,0	27,0	+
E5	4-5.1-0419 (X.a.a.)	31,2	31,2	+
E6	4-5.2-0419 (X.a.a.)	30,4	30,4	+
E7	4-5.3-0419 (X.a.a.)	30,7	30,7	+
E8	4-6.1-0419 (X.a.a.)	32,9	32,9	+
E9	4-6.2-0419 (X.a.a.)	33,4	33,4	+
E10	4-6.3-0419 (X.a.a.)	33,6	33,6	+
F2	5-4.1-0419 (X.a.a.)	26,0	26,0	+
F3	5-4.2-0419 (X.a.a.)	25,7	25,7	+
F4	5-4.3-0419 (X.a.a.)	26,4	26,4	+
F5	5-5.1-0419 (X.a.a.)	29,4	29,4	+
F6	5-5.2-0419 (X.a.a.)	29,4	29,4	+
F7	5-5.3-0419 (X.a.a.)	29,3	29,3	+
F8	5-6.1-0419 (X.a.a.)	32,5	32,5	+
F9	5-6.2-0419 (X.a.a.)	32,8	32,8	+

Appendix 6. Data-sheet on selectivity trials

Reproducibility data

Protocol

1: 95,0°C for 10:00
2: 95,0°C for 0:15
3: 60,0°C for 1:00
Plate Read
4: GOTO 2, 39 more times

Quantification Data

Well	Fluor	Target	Content	Sample	Cq	Cq Mean	Cq Std. Dev
B02	FAM		Unkn	1(1)	16,04	16,04	0,000
B03	FAM		Unkn	1(2)	16,56	16,56	0,000
B04	FAM		Unkn	1(3)	16,72	16,72	0,000
B05	FAM		Unkn	2(1)	22,84	22,84	0,000
B06	FAM		Unkn	2(2)	23,17	23,17	0,000
B07	FAM		Unkn	2(3)	22,80	22,80	0,000
B08	FAM		Unkn	3(1)	27,47	27,47	0,000

Quantification Data

Well	Fluor	Target	Content	Sample	Cq	Cq Mean	Cq Std. Dev
B09	FAM		Unkn	3(2)	27,05	27,05	0,000
B10	FAM		Unkn	3(3)	26,53	26,53	0,000
C02	FAM		Unkn	4(1)	30,87	30,87	0,000
C03	FAM		Unkn	4(2)	31,22	31,22	0,000
C04	FAM		Unkn	4(3)	30,95	30,95	0,000
C05	FAM		Unkn	5(1)	33,33	33,33	0,000
C06	FAM		Unkn	5(2)	33,5	35,5	0,000
C07	FAM		Unkn	5(3)	32,7	32,7	0,000
C08	FAM		Unkn	6(1)	34,5	34,5	0,000
C09	FAM		Unkn	6(2)	34,6	34,6	0,000
C10	FAM		Unkn	6(3)	34,2	34,2	0,000
D02	FAM		Unkn	7(1)	35,3	35,3	0,000
D03	FAM		Unkn	7(2)	35,6	35,6	0,000
D04	FAM		Unkn	7(3)	35,8	35,8	0,000
D07	FAM		Neg Ctrl	-	39,35	39,35	0,000
D08	FAM		Neg Ctrl	-	38,08	38,08	0,000
D09	FAM		Neg Ctrl	-	N/A	0,00	0,000
D10	FAM		Neg Ctrl	-	N/A	0,00	0,000
B02	HEX		Unkn	1(1)	19,85	19,85	0,000
B03	HEX		Unkn	1(2)	20,3	20,3	0,000
B04	HEX		Unkn	1(3)	20,83	20,83	0,000
B05	HEX		Unkn	2(1)	29,29	29,29	0,000
B06	HEX		Unkn	2(2)	29,7	29,7	0,000
B07	HEX		Unkn	2(3)	30,18	30,18	0,000
B08	HEX		Unkn	3(1)	31,8	31,8	0,000
B09	HEX		Unkn	3(2)	31,15	31,15	0,000
B10	HEX		Unkn	3(3)	31,5	31,5	0,000
C02	HEX		Unkn	4(1)	32,5	32,5	0,000
C03	HEX		Unkn	4(2)	32,6	32,6	0,000
C04	HEX		Unkn	4(3)	32,1	32,1	0,000
C05	HEX		Unkn	5(1)	34,2	34,2	0,000
C06	HEX		Unkn	5(2)	33,6	33,6	0,000
C07	HEX		Unkn	5(3)	33,5	33,5	0,000
C08	HEX		Unkn	6(1)	34,4	34,4	0,000
C09	HEX		Unkn	6(2)	34,36	34,36	0,000
C10	HEX		Unkn	6(3)	34,7	34,7	0,000
D02	HEX		Unkn	7(1)	35,34	35,34	0,000
D03	HEX		Unkn	7(2)	35,5	35,5	0,000
D04	HEX		Unkn	7(3)	35,7	35,7	0,000
D07	HEX		Neg Ctrl	-	25,15	25,15	0,000
D08	HEX		Neg Ctrl	-	31,30	31,30	0,000
D09	HEX		Neg Ctrl	-	27,47	27,47	0,000
D10	HEX		Neg Ctrl	-	2,95	2,95	0,000

Appendix 7. Reproducibility trials findings

Data on reproducibility assessments

Номер лунки	Идентификатор пробирки	Ср, Fam	Ср, Hex
B2	1(1)_pil (X.a.a avr)		14,5
B3	2(1)_pil (X.a.a avr)		17,5
B4	3(1)_pil (X.a.a avr)		21,2
B5	4(1)_pil (X.a.a avr)		24,8
B6	5(1)_pil (X.a.a avr)		28,1
B7	6(1)_pil (X.a.a avr)		30,9
B8	7(1)_pil (X.a.a avr)		34,5
B9	1(2)_pil (X.a.a avr)		13,4
B10	2(2)_pil (X.a.a avr)		17,2
B11	3(2)_pil (X.a.a avr)		21,3
C2	4(2)_pil (X.a.a avr)		24,6
C3	5(2)_pil (X.a.a avr)		27,7
C4	6(2)_pil (X.a.a avr)		31,3
C5	7(2)_pil (X.a.a avr)		34,5
C6	1(3)_pil (X.a.a avr)		14,1
C7	2(3)_pil (X.a.a avr)		17,2
C8	3(3)_pil (X.a.a avr)		20,8
C9	4(3)_pil (X.a.a avr)		24,4
C10	5(3)_pil (X.a.a avr)		27,6
C11	6(3)_pil (X.a.a avr)		30,6
D2	7(3)_pil (X.a.a avr)		34,3
D3	1(1)_avr (X.a.a avr)	15,4	
D4	2(1)_avr (X.a.a avr)	19,4	
D5	3(1)_avr (X.a.a avr)	23,7	
D6	4(1)_avr (X.a.a avr)	28,2	
D7	5(1)_avr (X.a.a avr)	32,1	
D8	6(1)_avr (X.a.a avr)	34,9	
D9	7(1)_avr (X.a.a avr)	35.8	
D10	1(2)_avr (X.a.a avr)	15,3	
D11	2(2)_avr (X.a.a avr)	18,7	
E2	3(2)_avr (X.a.a avr)	23,2	
E3	4(2)_avr (X.a.a avr)	27,7	
E4	5(2)_avr (X.a.a avr)	31,5	
E5	6(2)_avr (X.a.a avr)	34,0	
E6	7(2)_avr (X.a.a avr)	36.1	
E7	1(3)_avr (X.a.a avr)	15,4	
E8	2(3)_avr (X.a.a avr)	19,3	
E9	3(3)_avr (X.a.a avr)	23,4	
E10	4(3)_avr (X.a.a avr)	27,4	
E11	5(3)_avr (X.a.a avr)	31,1	
F2	6(3)_avr (X.a.a avr)	35,1	
F3	7(3)_avr (X.a.a avr)	36.2	
F4	K+ (X.a.a avr)	12,8	
F5	K+ (X.a.a avr)		11,5

	F6	K- (X.a.a avr)		
	F7	K- (X.a.a avr)		
	F8	K- (X.a.a avr)		
	F9	K- (X.a.a avr)		
	F10	K- (X.a.a avr)		
	F11	K- (X.a.a avr)		
	G2	K- (X.a.a avr)		

Appendix 8. Another data-set for Reproducibilty trials.

Data for qPCR specificity evaluations

Номер лунки	Идентификатор пробирки	Ср, Fam	Ср, Hex	Результат
A1	0445 (Xaa (X.alii))		33,9	-
A2	0446 (Xaa (X.alii))		33,6	-
A3	0419 (Xaa (X.alii))	15,8	27,7	+
A4	0394 (Xaa (X.alii))		33,1	-
A5	0398 (Xaa (X.alii))		33,0	-
A6	0399 (Xaa (X.alii))		33,3	-
A7	0401 (Xaa (X.alii))		33,3	-
A8	0403 (Xaa (X.alii))		33,7	-
A9	0404 (Xaa (X.alii))		34,3	-
A10	0405 (Xaa (X.alii))		33,9	-
A11	0406 (Xaa (X.alii))		34,1	-
A12	0417 (Xaa (X.alii))		33,9	-
B1	0389 (Xaa (X.alii))		33,3	-
B2	0329 (Xaa (X.alii))		34,4	-
B3	0321 (Xaa (X.alii))		36,0	-
B4	0298 (Xaa (X.alii))		33,5	-
B5	0267 (Xaa (X.alii))		33,3	-
B6	0239 (Xaa (X.alii))		33,6	-
B7	03227 (Xaa (X.alii))		34,2	-
B8	0226 (Xaa (X.alii))		31,0	-
B9	K- (Xaa (X.alii))		33,1	-
B10	0222 (Xaa (X.alii))		26,7	-
B11	0204 (Xaa (X.alii))		30,0	-
B12	0174 (Xaa (X.alii))		30,2	-
C1	0181 (Xaa (X.alii))		33,0	-
C2	0044 (Xaa (X.alii))		32,5	-
C3	0028 (Xaa (X.alii))		29,1	-
C4	0039 (Xaa (X.alii))		33,2	-
C5	0048 (Xaa (X.alii))		29,3	-
C6	0049 (Xaa (X.alii))		30,9	-
C7	0050 (Xaa (X.alii))		32,1	-
C8	0092 (Xaa (X.alii))		31,5	-
C9	0093 (Xaa (X.alii))		31,6	-
C10	0078 (Xaa (X.alii))		31,7	-
C11	0137 (Xaa (X.alii))		30,3	-
C12	0142 (Xaa (X.alii))		31,3	-
D1	0144 (Xaa (X.alii))		31,3	-
D2	0149 (Xaa (X.alii))		32,1	-
D3	0172 (Xaa (X.alii))		32,6	-

	D4	0141 (Xaa (X.alii))		32,2	-
	D5	0148 (Xaa (X.alii))		32,2	-
	D6	K- (Xaa (X.alii))		33,9	-
	D7	0109 (Xaa (X.alii))		33,6	-

	D8	0113 (Xaa (X.alii))		33,7	-
	D9	0120 (Xaa (X.alii))		33,0	-
	D10	0327 (Xaa (X.alii))		33,3	-
	D11	0330 (Xaa (X.alii))		33,3	-
	D12	0331 (Xaa (X.alii))		33,7	-
	E1	0332 (Xaa (X.alii))		34,3	-
	E2	0333 (Xaa (X.alii))		33,9	-
	E3	0334 (Xaa (X.alii))		34,1	-
	E4	0335 (Xaa (X.alii))		33,9	-
	E5	0344 (Xaa (X.alii))		33,3	-
	E6	0328 (Xaa (X.alii))		34,4	-
	E7	345p (Xaa (X.alii))		36,0	-
	E8	0353 (Xaa (X.alii))		33,5	-
	E9	0367 (Xaa (X.alii))		33,3	-
	E10	0373 (Xaa (X.alii))		33,6	-
	E11	0374 (Xaa (X.alii))		34,2	-
	E12	0375 (Xaa (X.alii))		31,0	-
	F1	0376 (Xaa (X.alii))		33,1	-
	F2	0380 (Xaa (X.alii))		26,7	-
	F3	K- (Xaa (X.alii))		30,0	-
	F4	0378 (Xaa (X.alii))		30,2	-
	F5	0381 (Xaa (X.alii))		33,0	-
	F6	0352 (Xaa (X.alii))		32,5	-
	F7	0462 (Xaa (X.alii))		29,1	-
	F8	0465 (Xaa (X.alii))		33,2	-
	F9	0466 (Xaa (X.alii))		29,3	-
	F10	0467 (Xaa (X.alii))		30,9	-
	F11	0468 (Xaa (X.alii))		32,1	-
	F12	0470 (Xaa (X.alii))		31,5	-
	G1	0441 (Xaa (X.alii))		31,6	-
	G2	0442 (Xaa (X.alii))		31,7	-
	G3	0473 (Xaa (X.alii))		30,3	-
	G4	0472 (Xaa (X.alii))		31,3	-
	G5	0474 (Xaa (X.alii))		31,3	-
	G6	0475 (Xaa (X.alii))		32,1	-
	G7	0471 (Xaa (X.alii))		32,6	-
	G8	0453 (Xaa (X.alii))		32,2	-
	G9	0451 (Xaa (X.alii))		32,2	-
	G10	0457 (Xaa (X.alii))		32,4	-
	G11	0448 (Xaa (X.alii))		32,0	-
	G12	0443 (Xaa (X.alii))		31,8	-
	H1	K+ (Xaa (X.alii))	16,6	27,6	+
	H2	K+ (Xaa (X.alii))	15,5	28,8	+

Appendix 9. Specificity- data on exclusivity as well as inclusivity trials