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Research Article

Isolation, identification, and functional analysis of quorum sensing producing strain *Pectobacterium atrosepticum* associated with blackleg disease in potatoes

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ABSTRACT

Potato is an important food and cash crop that production losses have a double effect on society, causing both a food security and financial crisis. The significant problem in the potato fields is blackleg of potato caused by *Pectobacterium atrosepticum (Pa)*. The research concentrates on the isolation and characterization of *P. atrosepticum,* as one of the major causal organisms of the potato blackleg disease, in particular its prevalence in the Pakistani regions. Differential diagnosis by using biochemical methods and Koch's Postulates, the etiological agent was identified. The involvement of N-Acyl Homoserine Lactones (NAHLs)-based quorum sensing (QS) in modulating Pa behaviors was clarified by the investigations. Novel QS inhibitors showing non-lethal quorum quenching capacity surfaced, however the long-term possibilities could be further improved. Initial data reflect a strong drop of virulence markers up to Day 16, which demonstrates the importance of increasing the knowledge of limiting influence arising from characteristics of bioactive substances or intrinsic ecological dynamics. Overall, this research contributes significantly to comprehending *P. atrosepticum*-associated threats and lays foundation for devising eco-friendly intervention tactics centered on quorum quenching paradigms.

Keywords: *Pectobacterium atrosepticum*, Quorum sensing, Quorum quenching, Potato agriculture, Biopesticides.

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INTRODUCTION

Potato (*Solanum Tuberosum L*.) belonging family *Solanaceae*, having a rich source of starch and protein. In terms of human consumption, it comes in third place, behind rice and wheat (FAO, 2014). With an average yield of 21.77 t/ha in 2020–21, it is the first non-cereal crop in the world, producing 359.1 million tons yearly above 16.5 million per ha (FAO, 2022). More than 10 million people are fed by potatoes, which are a staple meal in many countries (Majeed & Muhammad 2018). The production and yield potentials of the potato crop are also threatened by a number of factors, including an increase in bacterial and fungal pathogen infections as well as abiotic factors like temperature fluctuations, soil conditions, and water availability (Majeed et al. 2014; Majeed & Muhammad 2018). Potato producers suffer a significant loss in potato production due to the severe potato disease known as "Blackleg disease of Potato" caused by the bacterial pathogen *Pectobacterium atrocepticum* (Rivedal et al. 2021). Blackleg causes 0.3-5.0% of infections in parts of Pakistan that cultivate potatoes, including Okara, Sahiwal, Faisalabad, and Gujranwala. In India, losses of 45% have been reported (Rashid *et al.*, 2012).

Potato blackleg appears shortly after the plant first appears.

The underground portion of the stem deteriorated badly and turned dark brown to black. On the other hand, if the pith portion is attacked, it might rot upward and become susceptible to blackleg. The development of the potato crop affected by blackleg is stunted (Perombelon et al., 2000). Potato blackleg has two negative effects on the economy: it lowers yield when it exceeds 5–10% of the crop, and it also deteriorates the crop during seed certification (Farran *et al.*, 2006). Rotten tubers and infected crop residues are important sources of inoculum; in potato production, latent infection in seed tubers is the main source of disease. Bacteria spread from mother tubers to the lenticels of offspring tubers in the field (Parrot, 2010). Proteases, pectin methylesterases, polygalacturonases, pectate lyases, and cellulases are only a few of the numerous exoenzymes that Pba produces during an infection. These enzymes destroy plant tissues and release nutrients that speed up bacterial development. Now that Pba has produced and released the enzymes that break down PCW under the QS process, all is noticeable.

Numerous quorum quenching techniques work against the virulence components that are part of the *Pectobacterium* quorum sensing system. These include the development of transgenic plants that express lactonases, which are enzymes that break down bacterial AHLs (Dong et al., 2001); the detection and stimulation of cells of soil bacteria, such as *Bacillus thuringiensis* and *Rhodococcus erythropolis* (Dong et al., 2005, Cirou et al., 2007), that break down bacterial AHLs; and the synthesis and identification of artificial and natural compounds that function as quorum sensing inhibitors (QSIs).

MATERIALS AND METHODS

Collection of diseased samples

In three distinct potato-growing regions of Punjab includes Chiniot, Faisalabad, and Okara, thirty diseased black leg of potato samples were collected and brought to lab for identification.

Isolation and Purification

The samples were isolated using standard isolation techniques (Riker and Riker, 1936). After surface sterilization pieces were plated on Nutrient Agar (NA) medium-filled Petri plates. The Petri plate was kept at 27°C±2°C for 24h. Following that, the bacterium were purified by streak plate method on the fresh NA media plates.

Biochemical and physiological tests

All the isolates that grew on the NA media culture plate was identified morphologically by observing its color, type of colony and conducting biochemical tests viz., Gram staining and 3% KOH test (Holt et al. 2000; Mubeen et al. 2015).

Pathogenicity test

Preparation of Inoculum

Bacterial inoculum was prepared by overnight shaking method following the protocol of Hoque, M.E. and J.W. Mansfield (2005), and adjusted to a final concentration of 10^6cfu/ml using spectrophotometer (Hoque and Mansfield, 2005).

Pathogenicity test with Potato tuber assay

To provide a humid atmosphere conducive to the growth of disease, potatoes were placed in a container that was transparent. The 12 tubers were injected with 3 replicas of 0.5 ml of 103 cfu/ml of 12 bacterial solutions using sterile yellow tips. The control group provided simply sterile water treatment. Three, six, and nine days following inoculation, maceration symptoms on potato tubers were observed.

NAHL Studies

NAHL studies were carried out with the help of biosensors. An overnight culture of *P. atrosepticum* was subjected to solvent extraction using ethyl acetate. Supernatant obtained after centrifugation contained putative NAHLs. Samples underwent separation according to hydrophobicity differences between distinct types of NAHLs. Free supernatant fractions were applied onto silica gel coated TLC plates. Soft agar overlays infused with appropriate indicator biosensors were used to determine the existence of active NAHLs. Reporter strain *Chromobacterium violaceum* CV026 sensitive to Long Chain Acrylamide type NAHLs, resulting in purple pigment formation called violacein indicating positive responses. The presence of violet spot shows Quorum sensing, NAHL presence and absence of spot shows, there is no QS.

Agar plate method to check Test Molecule

Test molecules were tested to see whether they have any impact on reactivity and growth of Biosensor and *Pba*. BS and *Pba* were streaked in agar plates independently on individual plates. Then test molecule and NAHLs were placed at a distance of 2.5 cm from the middle of plate. In same way BS was plated with test molecule.

MIC determination

MIC is the minimum inhibitory concentration of a molecule that will inhibit the visible growth of microorganism in overnight incubation. Using a spectrophotometer, the concentration of several molecules—12.5 ug/ml, 25 ug/ml, 50 ug/ml, 100 ug/ml, and 150 ug/ml—was added to a bacterial culture. OD was then measured at 600 nm at intervals of 30 minutes to 6 hours.

QSI studies

QSI studies were carried out by quenching assay or NAHL interference assay. LB medium was produced, and test molecule cultured at a concentration of 12 ug/ml overnight. An equivalent volume of ethyl acetate was added to remove NAHLs from the culture and after shaking the 10 ul free supernatant and soft agar seeded biosensors was deposited in LB plates at opposite sides. In the middle 12ug/ml was placed. In the control plate, sterile water was poured instead of molecule. Absence or presence of spots showed that either there was QS or QQ. And this all was done to select the test molecules as Quorum Quenchers.

Pathogenicity quenching tuber assay

Inoculum containing 12 ug/ml of quencher molecule, 25 ul and 103 cfu/ml of Pbainoculum and quencher plus Pba, 100 ug/mL of Tetracycline plus Pca, and sterile water. To maintain the desired temperature and humidity, the tubers were kept within a box. By visually inspecting the maceration zone that the pathogen pectobacterium created after inoculating the potato tuber, pectinolytic activities were evaluated.

Statistical Analysis

The statistical analysis program Statistix 10 was used to record and examine the data. The LSD test was used to compare means at a 5% significance level after the ANOVA test was used to assess all potential interactions and variables (Steel *et al.,* 1997).

RESULTS AND DISCUSSION

Isolation and purification of bacterium

The samples were isolated using typical isolation techniques. There were 7, 9, and 8 bacteria found on each of the ten plates from chiniot, Faisalabad, Okara, respectively.

Biochemical tests

Gram staining

Isolates were examined under a microscope after undergoing the gram staining process. The findings showed that all 24 isolates were gram negative, rod-shaped, and that there were differences in the isolates' sizes.

KOH test (3%)

Each of the isolate produced thread-like string using a toothpick, demonstrating +ve results in the KOH test. According to these +ve findings, the bacteria is gram negative.

Pathogenicity test by Tuber Assay

The pathogenicity of *P. atrosepticum* was also confirmed using the potato tuber test. After inoculation, maceration symptoms on potato tubers were seen three, six, and nine days later.

Extraction and Quantification of NAHLs

After adding the same quantity of ethyl acetate to an overnight culture of 12 Pba, NAHLs were extracted. The free supernatant was then placed on TLC plates, covered with soft agar that had been seeded with biosensors, and shaken.

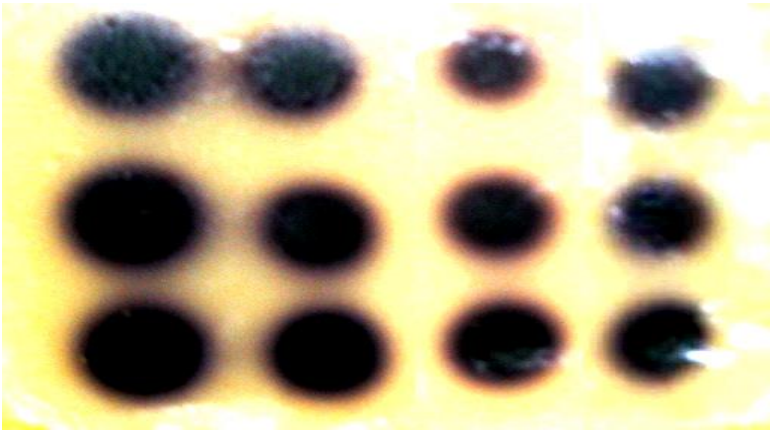

Figure 1. NAHL detection with CVO26

Detection of N-AHSL

The biosensors *Chromobacterium violaceum* CV026 were used to detect N-AHSL. All 12 Pba displayed violet spots based on quorum sensing.

Test Molecule bt Agar plate method

The agar plate technique was used to test twelve molecules. Six test molecules eliminated Pba, two activated Pba, three did nothing, and three eliminated BS. those chosen to serve as Quorum Quenchers.

Table 1. Showing the reaction of test molecules.

Test Molecule	Kill BS	Kill *Pba*	Activate BS	Do nothing
TM1			+	
TM2				+
TM3		+		
TM4				+
TM5		+		
TM6	+			
TM7	+			
TM8				+
TM9		+		
TM10		+		
TM11	+			
TM12		+		

MIC determination

In three bacterial cultures with three replications, different concentrations of molecules (12.5 ug/ml, 25 ug/ml, 50 ug/ml, 100 ug/ml, and 150 ug/ml) were added. Using a spectrophotometer, the OD was measured at 600 nm at intervals of 30 minutes to 1 day.

OD of at different concentrations of first bacteria

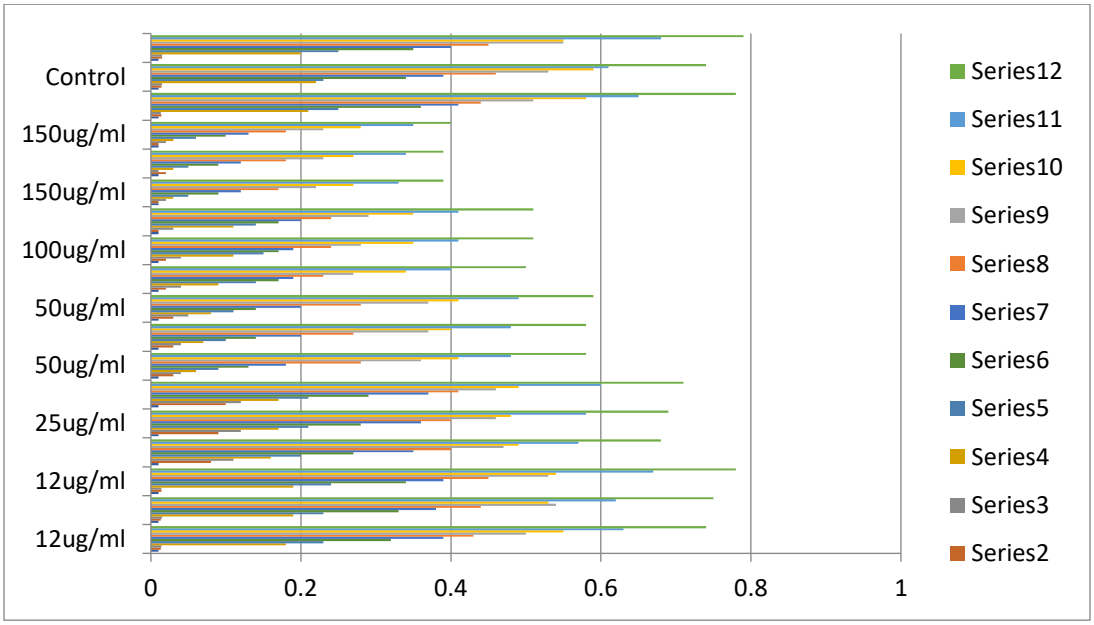

Figure 2. OD of First bacteria.

By increasing chemical concentration, the Maceration zone also decreased. 12 ug/ml is minimum inhibitory concentration for *Pba* at which bacterial OD is continuing to increase.

OD of second bacteria at different concentration

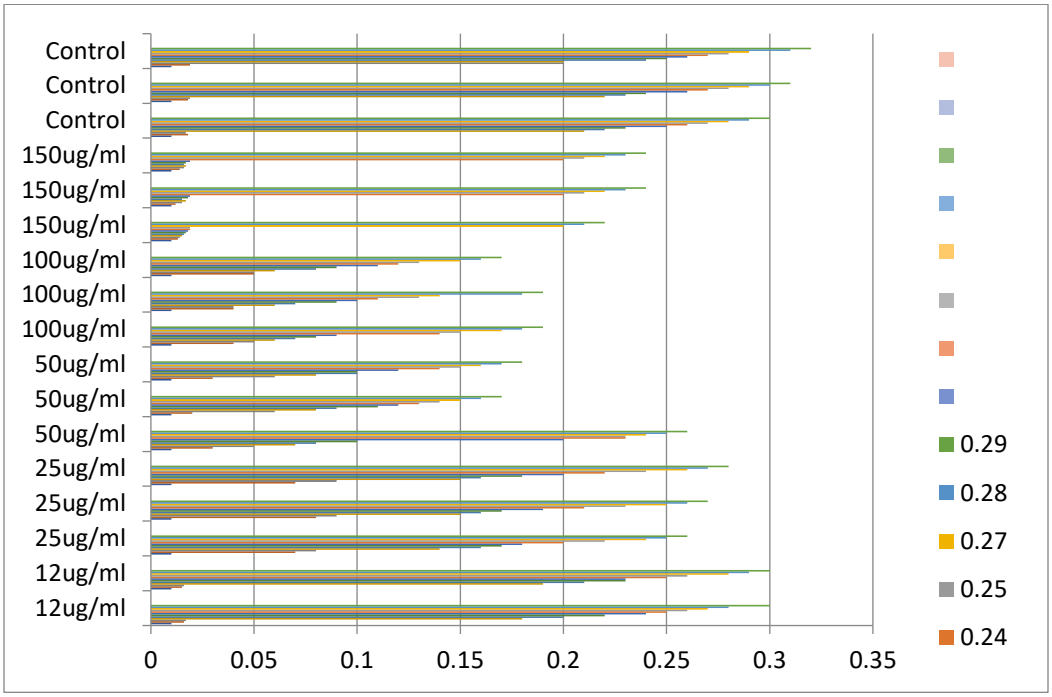

Figure 3. OD of second bacteria.

In this graph it is shown that by increasing chemical concentration the Maceration zone also decreased. 12 ug/ml is minimum inhibitory concentration for *Pba* at which bacterial OD is continuing to increase.

OD of third bacteria at different concentrations

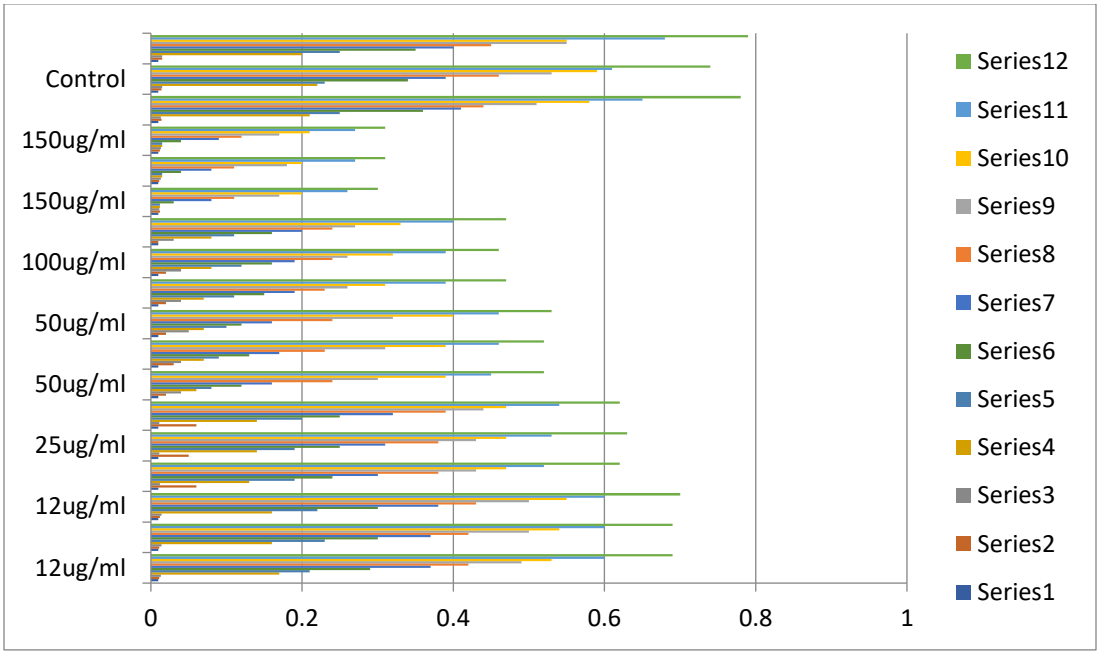

Figure 4. OD of third bacteria.

In this graph it is shown that by increasing chemical concentration the Maceration zone also decreased. 12de/ml is minimum inhibitory concentration for *Pba* at which bacterial OD is continuing to increase.

Test molecules result

Using a spectrophotometer, the OD of the test molecule (12.5 ug/ml of molecule MIC) and the control molecule (1/4 of Tetracycline MIC) were measured at 600 nm at 30-minute intervals to substantiate the assertion above. As time went

by, OD continued to rise. This demonstrated that although the number of bacteria was rising, the test molecule did not destroy the biosensor or Pca. Instead of being dead, bacteria were living.

Detection of Quorum Sensing Inhibitors

The results demonstrated that no color was produced in the plates where test molecules were applied, and that the lack of molecules in the control plate resulted in the creation of violet spots. The plates where was no chemical applied, there was spot production as shown in results.

Pathogenicity quenching tuber assay

By visually inspecting the maceration zone that the pathogen pectobacterium produced after inoculating the potato tuber, pectinolytic activities were evaluated.

Figure 5. Quenching assay.

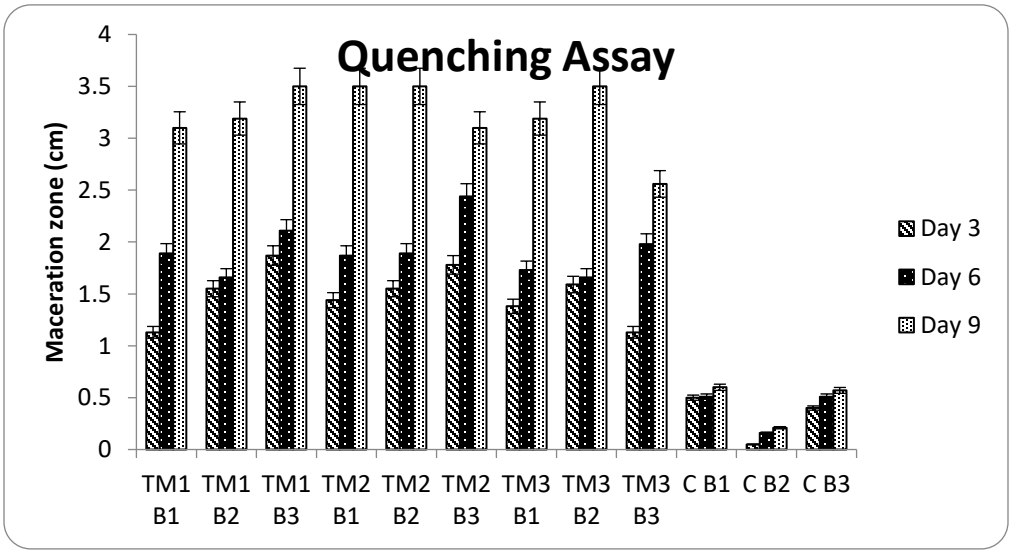

Figure 6. Maceration zone graph.

In this quenching assay graph, it is shown that TM1B3, TM2B1,TM2B2 and TM3B2 show max maceration zone at day 9.

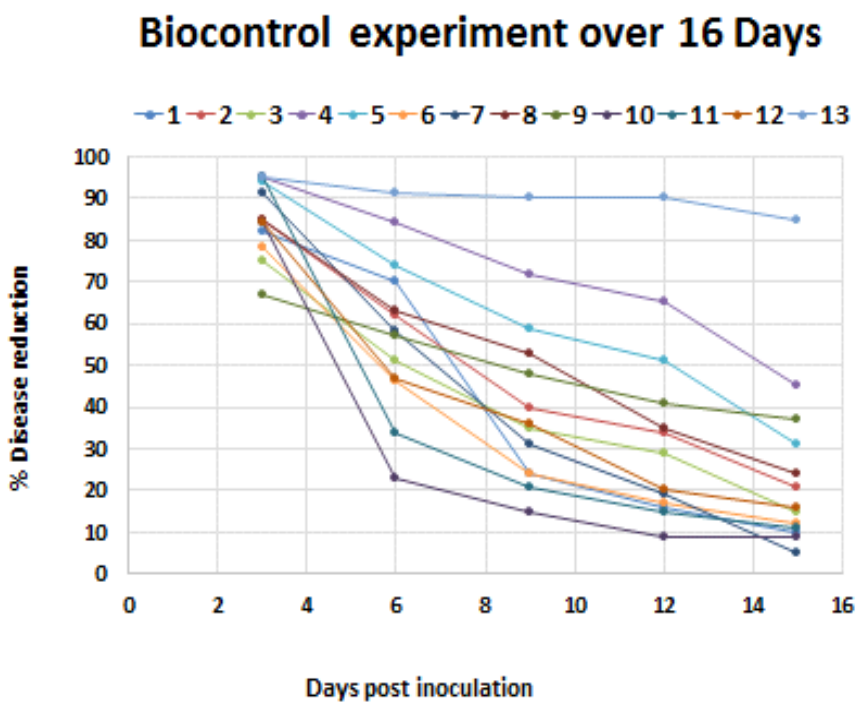

Figure 7**.** Percentage of disease reduction graph.

This graph illustrates how the chemicals' ability to manage the illness was declining relative to the control when data was collected after 16 days. Only control was able to keep the illness under control.

The presented study involving the Confirmation of Pathogenicity through a Tuber Assay aligns with similar findings reported in various sources related to testing the pathogenicity of Pectobacterium spp. on potato tubers. Specifically, Study conducted by Lukianova et al., (2021) describes experiments conducted on artificially inoculated potato tubers, including symptoms observed during infection, such as those seen in your own study's observation of maceration on potato tubers within specific timeframes post-inoculation. Additionally, Study reports experimental evidence demonstrating varying levels of maceration abilities across several strains of Pectobacterium, further supporting the validity of conducting tuber assays for confirming pathogenicity (Han et al., 2023).

Han et al., (2023) also describes a similar experimental setup involving a potato tube maceration assay to confirm the pathogenicity of various strains of *Pectobacterium*. It reports symptoms observed within 72 hours post-inoculation, while in this study observing them up to 9 days after inoculation. Regarding extraction and quantification of N-acyl homoserine lactones (NAHLs), there is alignment between your protocol and techniques mentioned in the study Girard et al., (2017), they describe the use of genetic engineering or natural systems, specifically employing biosensors like *Chromobacterium violaceum* CV026, to identify and analyze NAHL signals produced by microbes—similar to how you utilized it for detecting N-AHSL via bioactivity on TLC plates. In particular, study conducted by Fan et al., (2020) used varying volumes of mixtures consisting of QQ strain D-2 + pathogen vs. other controls (including E. coli DH5α, B23, and Streptomycin) to demonstrate the ability of QQ strain D-2 to suppress disease symptoms when co-injected with the pathogen into potato tuber slices. Similarly, study conducted by Zhang et al., (2020) performed bioassays on potato tubers comparing the effect of mixing Acinetobacter sp. XN-10 with the pathogen versus controls without it, showing reductions in maceration zones after treating infected samples with the QQ agent compared to untreated ones.

CONCLUSION

The research aimed to isolate and characterize *P. atrosepticum,* a causative agent of blackleg disease in potatoes, specifically focusing on identifying potential quorum sensing (QS) signal-producing strains and exploring methods to disrupt QS mechanisms. Research has effectively isolated and verified *P. atrosepticum,* responsible for potato blackleg disease, demonstrating its use of N-Acyl Homoserine Lactones (NAHLs) for quorum sensing. Promising quorum quenching compounds were identified for controlling blackleg disease progression through interference with QS systems, additional studies are necessary to optimize application strategies and address challenges related to long-term stability and compatibility with natural environments. Further investigation into the underlying mechanism(s)

behind reduced activity past day 16 will provide valuable insights towards developing effective anti-virulence approaches based upon quorum quenching principles.

COMPETING OF INTEREST

The authors declare no competing interests.

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