ADVANCES IN THE QUANTIFICATION OF *Pectobacterium spp* IN THE POTATO SEED AS A METHOD TO DETERMINE SEED QUALITY UNDER AN INTEGRATED MANAGEMENT APPROACH IN CHILE.

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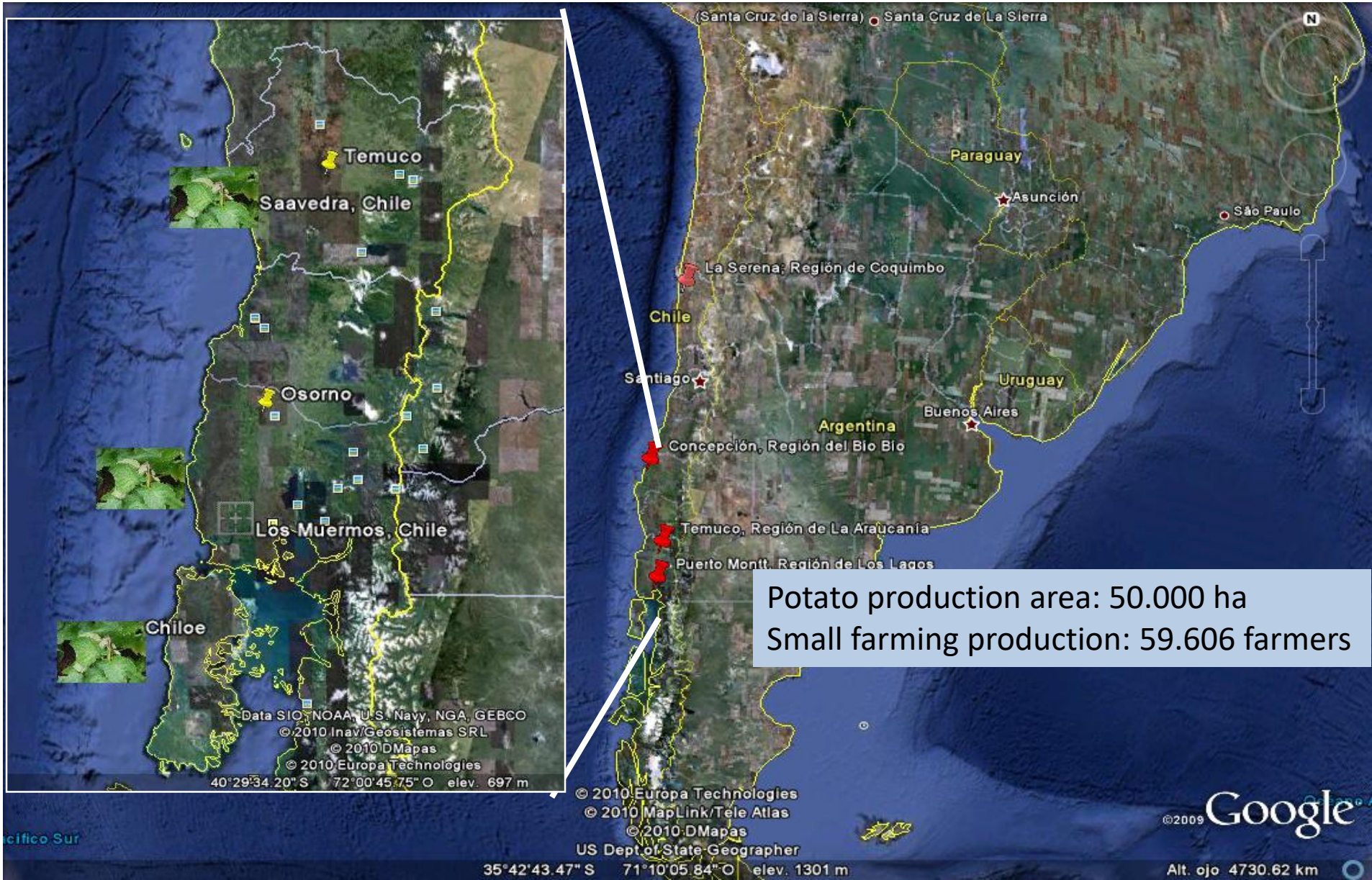
EAPR Pathology and Pest Section Meeting, 2019, Neuchâtel, Switzerland

Ministerio de Agricultura

Gobierno de Chile

CHILE LO HACEMOS TODOS

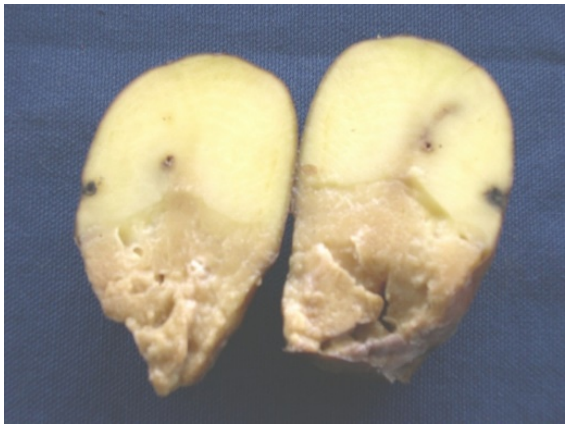
Potato crop in Chile



Potato production area: 50.000 ha
Small farming production: 59.606 farmers

Seed decay, Soft rot and Black leg in Chile

- **A survey done by INIA shows that 13.5% and 64.8% of the strains causing the disease are *P. atrosepticum* and *P.c. carotovorum*, respectively.**
- **Also, three seasons ago, one isolate was described as *P.c. brasiliense.***
- **In addition, SAG in 2015, reported *Dickeya* spp. in potato crops in the VI Region and Metropolitan Regions. They described *D. dadantii*, *D. solani* and *D. dianthicola.* The fields are under official control and quarantine resolution (SAG, 2016).**



Today…new production system…

Tuber seed (in vitro), new varieties

Irrigation systems

Mechanical harvesting

Deficient storage facilities

Seed decay, soft rot and black leg

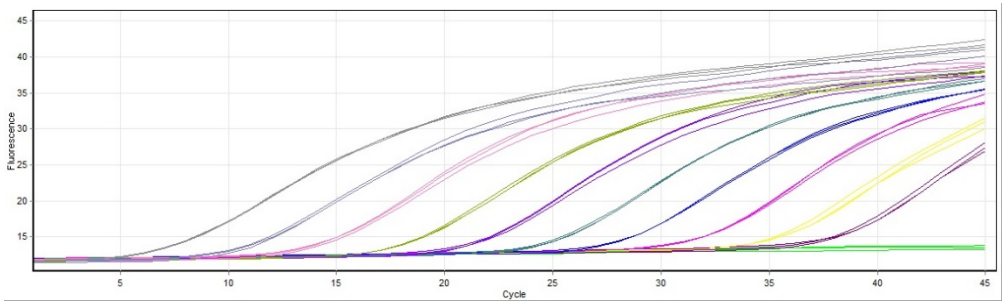
Losses of up to 30% on susceptible cultivars and rejection of seed lots up to 24%.

Actual situation

- **These results indicate that new bacteria are associated with potato causing black leg and soft rot, therefore, new studies need to be performed to have conclusive results.**
- **A proposal was granted by the Foundation for Agricultural Innovation of Chile (FIA) in 2017.**

Main Objective:

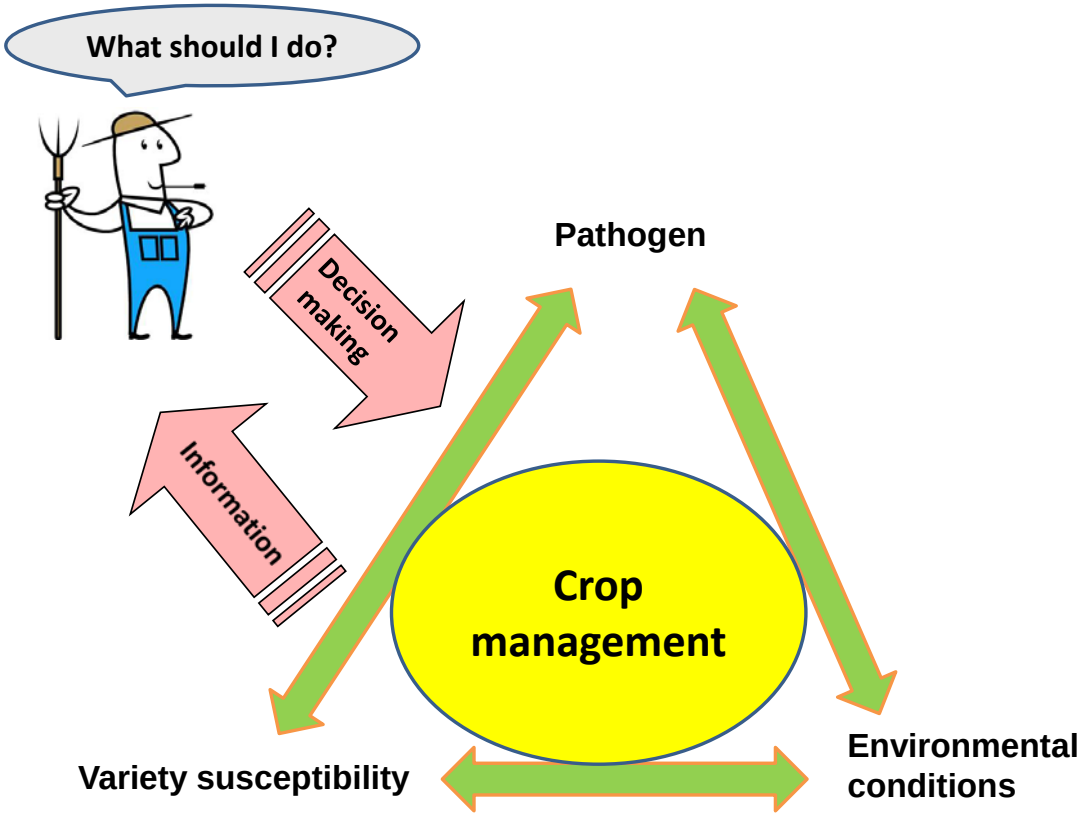
Developing an integrated management strategy for potato bacterial diseases, based on a real time PCR assay as a seed rot potential to determine the sanitary risk and preventive measures.



Seed rot potential

Real time PCR

Field expression



Real time PCR assay as a seed rot potential

Objetive

To Implement a seed potato tuber quantification technique based on a real time PCR assay as a seed rot potential to determine the sanitary risk

Methodology: Implementation of TaqMan assays

As *P. atrosepticum* and *P. carotovorum* subsp. *carotovorum* are main species of *Pectobacterium* present in Chile.

We decided to implement a TaqMan assays that detected *Pectobacterium* and *Dickeya* spp.

- After a search, we select primers and probes described by Humphris, S., *et al* 2015.
- Optimal amplification conditions were standardized in the laboratory: concentration primers, concentration probes and amount of DNA added to the reaction.

Methodology: Preparation of a standard curve ADN and its relation with total cells count of *Pectobacterium*

A culture of *P. atrosepticum* was grown in LB broth at 27°C with shaking for 18 h to give a bacterial density of 10^8 CFU/ml.

DNA extraction using 10^8 CFU/ml

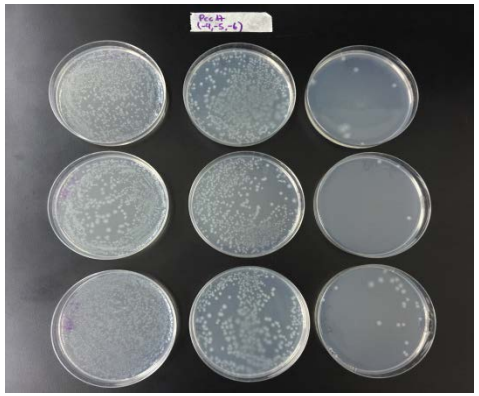
DNA quantification

Tenfold serial dilution

Real-time PCR assay using DNA dilution series as standards

Tenfold dilution series

Total cell counts in each dilution by plating on NA.

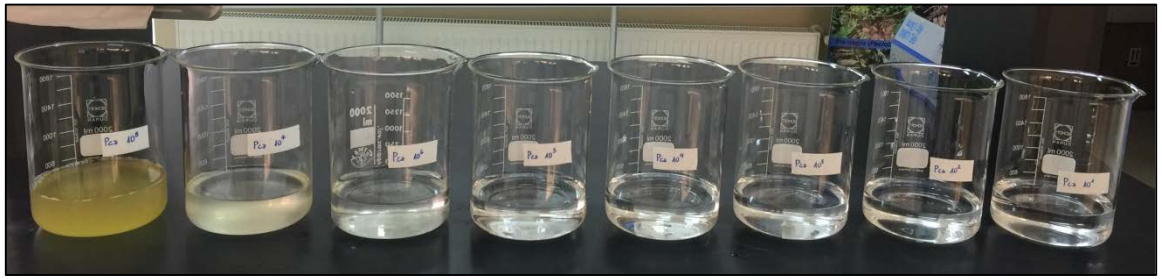


Methodology: Inoculation of minitubers with an increasing number of bacterial cells

Minitubers cv. Pukará washed and disinfected

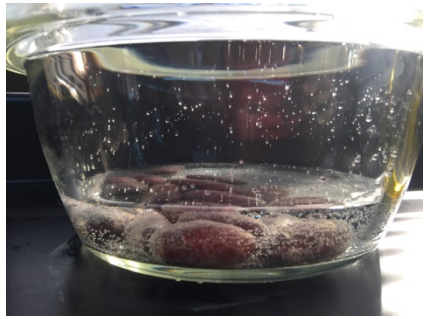
10^0 dilutions 10^{-7}

Standard broth: $5.8*10^8$ CFU/ml



Vacuum infiltration: 10 minitubers was completely immersed in each bacterial suspension. Vacuum of 0.7 – 0.9 bar was applied 4 times for 3 min.

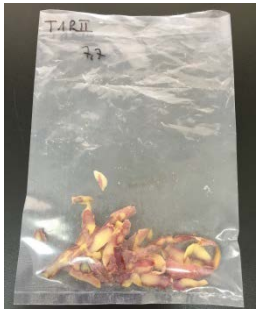
Treatments	Dilution
T1	without inoculation
T2	H_2O
T3	10^{-7}
T4	10^{-6}
T5	10^{-5}
T6	10^{-4}
T7	10^{-3}
T8	10^{-2}
T9	10^{-1}
T10	10^0



Methodology: Inoculation of minitubers with an increasing number of bacterial cells

Removed peel complete from 3 minitubers for each sample (three replicates)

Add Ringer buffer and ground sample (1g: 3ml buffer)



Pipette of the extract

Total cell count in plate (CFU/ml, CFU/gr peel)

Centrifuge

DNA extraction with commercial kit and Real-Time PCR (DNA/gr peel)

Humphris S., *et al* 2015

Results: Implementation of TaqMan assays

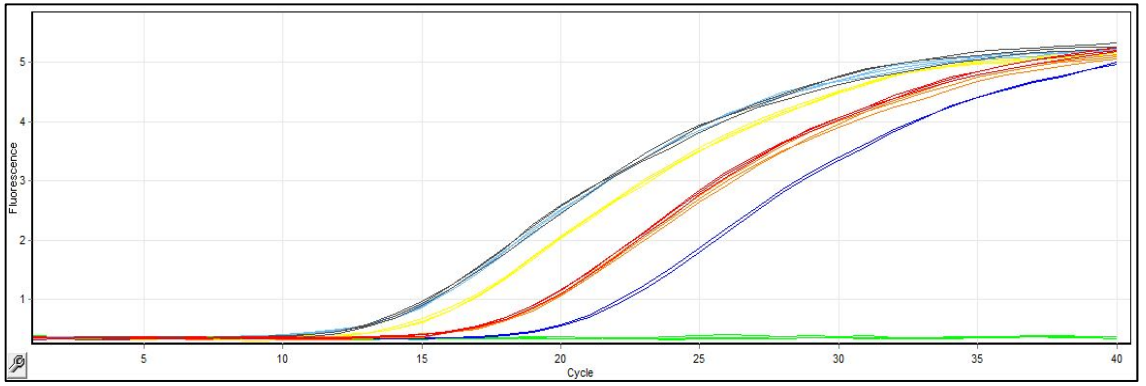
The qPCR reaction consisted of:

Reagents	Final concentration
Takyon kit 2X	1X
PEC-1F Primer	0,3 µM
PEC-1R Primer	0,3 µM
PEC-P Probe	0,1 µM
COX-F Primer	0,3 µM
COX-R Primer	0,3 µM
COX-P Probe	0,1 µM
DNA	20 ng/reaction

Reaction conditions		
Step	T °C	Time
1	95° C	3 min
2	95°C	5 seg
	60°C	40 seg

40 cycles

➢ qPCR evaluation with other pathogens

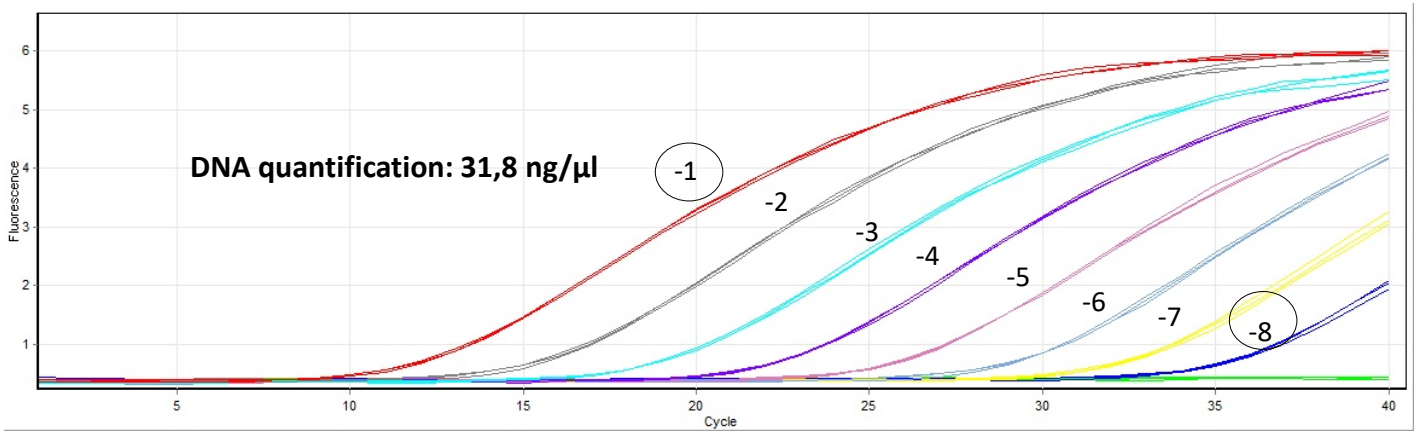


Only samples corresponding to *Pectobacterium* and *Dickeya* amplified.

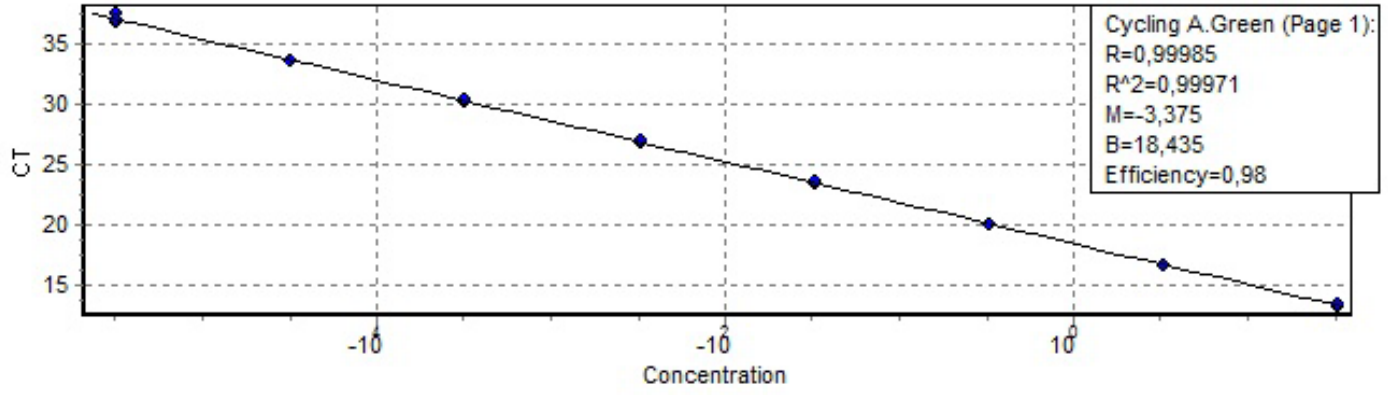
Standard samples: ***Rhizoctonia solani, Helminthosporium solani, Alternaria solani, Ralstonia solanacearum , D. solani, D. dianthicola, P. atrosepticum*, P. *carotovorum* subsp *carotovorum*.**

Results: Preparation of a standard curve

Standard curve for qPCR using serial dilution DNA of *P. atrosepticum*

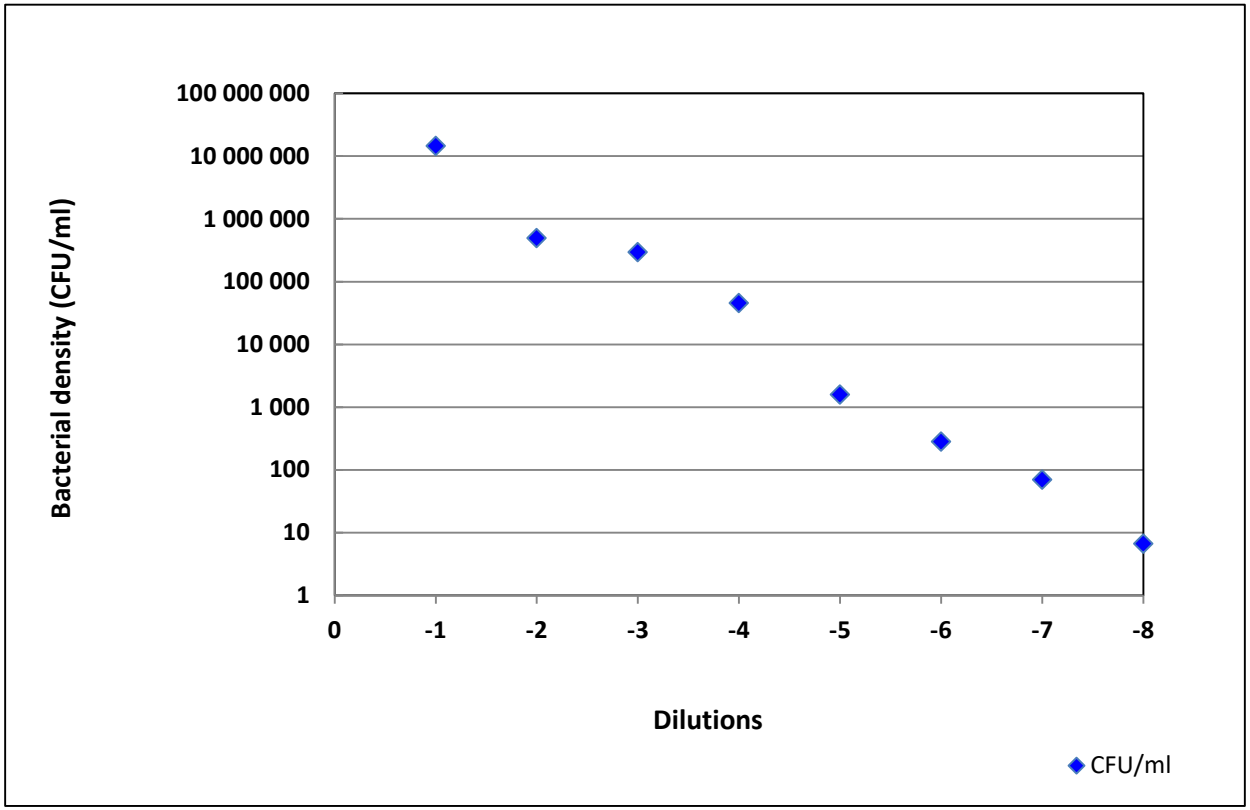


DNA quantification: 3,2 *10^{-6} ng/µl (3,2 fg/µl)



10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1} **Dilutions**

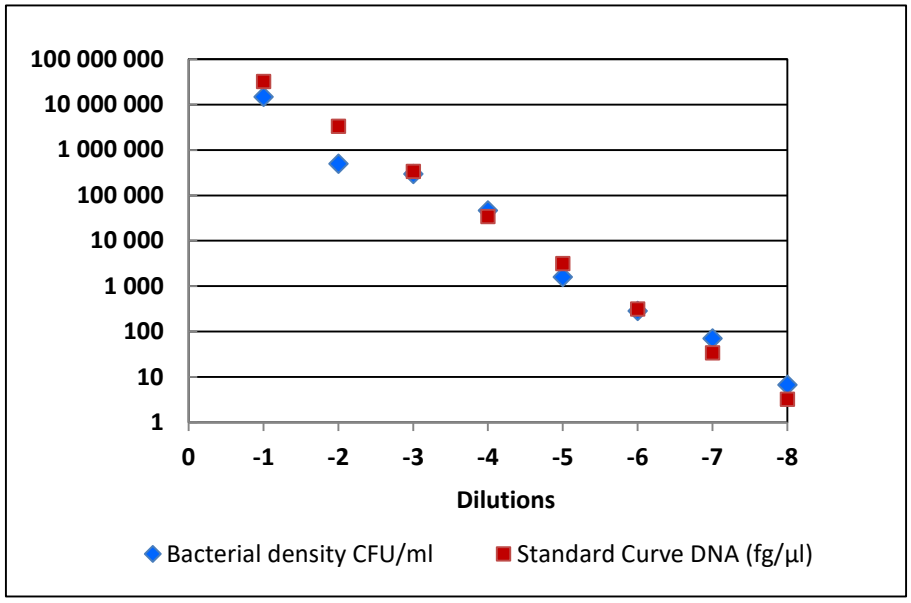
Results: Preparation of a standard curve and its relation with total cells count of *Pectobacterium*



*** Dilution -8: Of the 3 plates , there were colonies only in 2. However, in the same dilution analize by qPCR, all were positive.**

Results: Preparation of a standard curve and its relation with total cells count of *Pectobacterium*

Dilution broth	Dilution ADN	Ct qPCR	Quantification DNA Pectobacterium (ng/µl)	Quantification DNA Pectobacterium (fg/µl)	Bacterial density CFU/ml
-1	-1	13,37	3,2E+01	3,2E+07	1,5E+07
-2	-2	16,69	3,3E+00	3,3E+06	5,0E+05
-3	-3	20,04	3,4E-01	3,4E+05	3,0E+05
-4	-4	23,4	3,4E-02	3,4E+04	4,6E+04
-5	-5	26,88	3,1E-03	3,1E+03	1,6E+03
-6	-6	30,26	3,1E-04	3,1E+02	2,8E+02
-7	-7	33,51	3,4E-05	3,4E+01	7,0E+01
-8	-8	36,97	3,2E-06	3,2E+00	6,7E+00



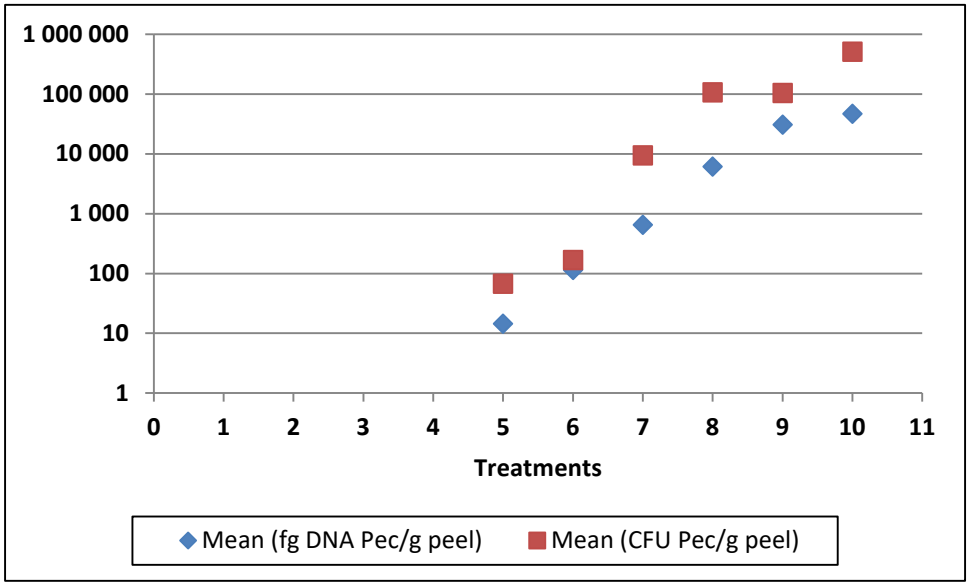
- **Minimum level of detection by real time PCR was 3,2 *10^{-6} ng/µl (3,2 fg/µl) with a mean Ct of 37.**

Results: Inoculation of minitubers with an increasing number of bacterial cells

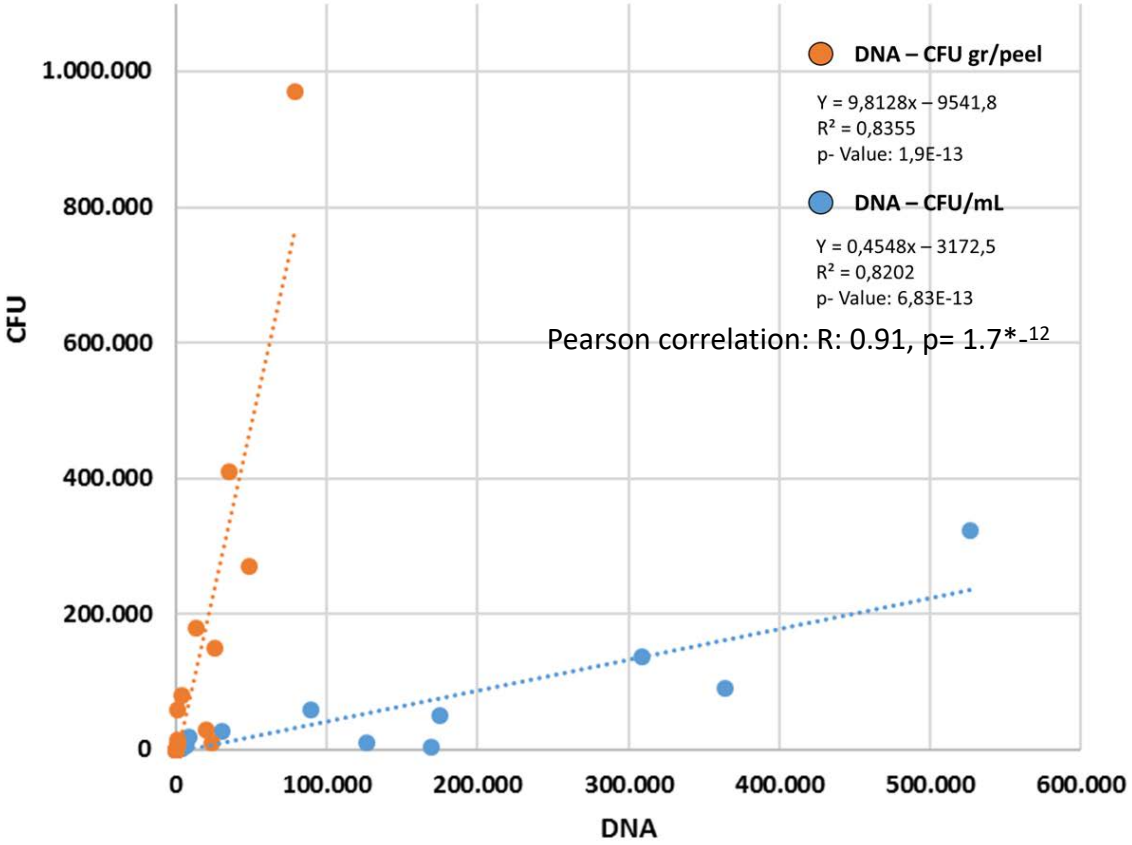
Standard broth: $5.8*10^8$ CFU/ml

Treatments	Dilution
T1	without inoculation
T2	H_2O
T3	10^{-7} $(5.8*10^1)$
T4	10^{-6} $(5.8*10^2)$
T5	10^{-5} $(5.8*10^3)$
T6	10^{-4} $(5.8*10^4)$
T7	10^{-3} $(5.8*10^5)$
T8	10^{-2} $(5.8*10^6)$
T9	10^{-1} $(5.8*10^7)$
T10	10^0 $(5.8*10^8)$

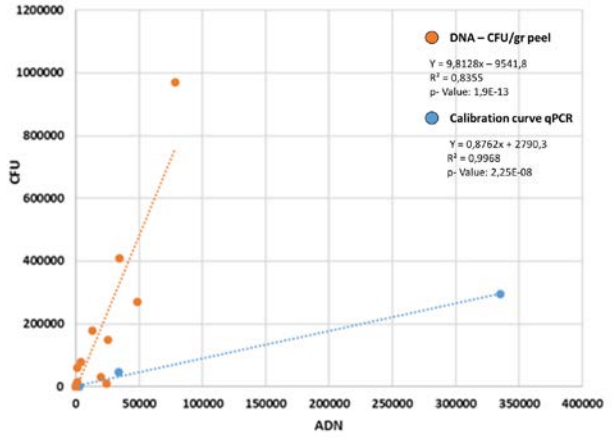
*three replicates in each treatment

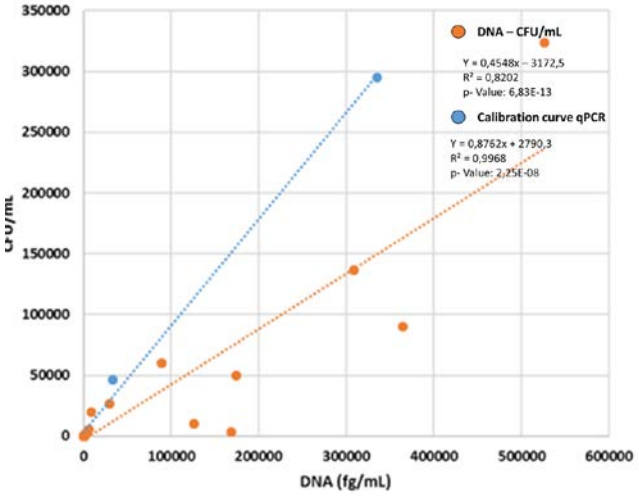


From treatment 5, we were able to detect *Pectobacterium* positive samples in the minitubers (67 CFU(gr peel) .
Quantification of *Pectobacterium* DNA increased with increasing bacterial inoculation density (14 fg DNA/gr peel).



Pearson correlation: R: 0.91, p= 1.7*-12





- A positive correlation was found between the amount of inoculum in the minituber and the bacterial quantification.
-This would be a promising methodology to determine seed quality under an integrated management approach.

Next…

3. Determine the risk of bacterial disease expression according to bacterial tuber infection, variety susceptibility, agronomic management and environmental conditions.

Main risk factors of blackleg and soft rot expression will be determined:

1. Management farmer survey
2. Seed rot potential determination
3. Evaluation of field expression of blackleg and soft rot considering results of real time PCR assay, agronomic management, variety susceptibility, environmental conditions.

Integrated management strategy → **Risk factors and their preventive management will be published on a platform as a DSS.**

Participants

- INIA Chile
- SAG Chile
- Chilean Potato Consortium
- Potato Seed Producers
- Chilean Potato Association

- Colaboration:
  - NDSU
  - Wageningen University
  - The James Hutton Institute

- Project Financie by Agricultural Innovation Agency FIA Chile.


¡Thank you!