PhD School on Agriculture, Environment and Bioenergy

(http://sites.unimi.it/dottorato_aab/)

(XXXVI cycle, 2020-23)

Project draft

1.Field of interest

*Indicare **il/i** settore/i scientifico disciplinari:*AGR/12 – Plant Pathology

2.Project title

Next-Generation Diagnosis: developing new methods for plant pathogen detection

3.Tutor

Prof. Piero Attilio Bianco

- Eventually: co-tutor/s

4.Relevance of the topic and state of the art:

In plant pathology, the certainty of detection of the presence of pathogens is a paramount necessity. As years passed, the techniques used to identify pathogens evolved in their speed and ability in determining the presence of specific pathogens, starting from the visual assessment of symptoms and the infection of test plants to the molecular biology techniques (ELISA, PCR) that are today's benchmark (Martin et al., 2000). Despite these advancements, the techniques currently employed to detect pathogens still have several limitations, such as how the specificity of the assay and the quality of the plant material can affect the result, leading to false negatives or false positive results (Lopez et al., 2009). Also, the speed of detection can be a limiting issue, especially when dealing with perishable goods on which several different pathogens must be searched.

Many of these limitations could be avoided by using Next Generation Sequencing (NGS) techniques for the identification of pathogens in plant material (Candresse et al., 2014). These techniques allow to obtain a large quantity of data on a sample and the microorganisms that are found within it. NGS can allow the characterization of all microorganisms present in a sample with a single assay, without having to set up a different assay for each pathogen (Hasman et al., 2014) and permitting the detection of pathogens in unusual hosts, in which they wouldn't be actively searched (Wu et al., 2015).

Currently, NGS data are not being considered for the detection of pathogens in plants, but the perspectives are that, as the cost and time needed for the obtainment of these data is progressively becoming lower, while the quality of the data obtained is rising, these techniques may become the new standard for diagnosis (Martin et al., 2017). In medicine, several studies have been carried out and testify the power of these methods in giving useful information for diagnosis and decisions on treatments in a timely fashion (Zhou et al., 2015; Deurenberg et al., 2017).

In this scenario, it is important that also in plant pathology detailed protocols for detection of plant pathogens using NGS are prepared so that, as it already happens for PCR- or ELISA-based protocols, all the phases of the process, from nucleic acid extraction to the data analysis are standardized, assuring the highest degree of sensitivity, repeatability, and reproducibility.

5.Layout of the project (draft)

5.1.Materials & Methods: da mezza pagina ad una pagina massimo

The project's main goal is to develop and validate diagnostic methods for the detection plant pathogens (bacteria, fungi, virus) based on NGS technologies. A diagnostic method, to be successfully employed, must have high levels of:

Specific sensitivity: the ability of the test to detect the pathogen in infected samples;

Specificity: the ability of the test to not detect the pathogen in non-infected samples;

Accuracy: a value that expresses how likely it is that the results of the test are correct, calculated from specific sensitivity and specificity;

Analytical sensitivity: the minimum amount of target that can be detected by the test;

Repeatability: how much the test results are in accordance between different replicates carried out on the same samples in the same conditions but at different times;

Reproducibility: how much the test results are in accordance between different replicates carried out on the same samples in different conditions.

In order to evaluate these parameters for an NGS DNA-barcoding based detection method, the project will carry out in parallel the NGS approach using well-known barcoding genes for bacteria (16S and *rpoD*) and fungi (ITS and COI) and a specific PCR-based detection method already employed for routine detection of each pathogen. These tests will be carried out on different samples prepared in the lab using the DNA of healthy plants spiked with known quantities of DNA from the pathogens (1:10, 1:100, 1:1000, 1:10000, 1:100000), as well as non-target samples spiked with DNS from non-pathogenic organisms. For viruses, the approach will be slightly different, and will be based on experimental inoculations of the viruses in greenhouse and using NGS to carry out a sequencing of small- and microRNA to identify the viruses present in the samples.

The NGS sequencing will be carried out both using the Illumina technology and the minion sequencer, in order to compare the results and design methods for both the benchmark technology for sequencing, and the most affordable and portable of sequencing technologies. The PCR-based techniques will use end-point PCR, real-time PCR, and digital PCR for the detection and quantification of each specific pathogen in the samples.

The pathogens to be studied in this project will be: *Phytophtora ramorum*, *Erwinia amylovora*, *Ralstonia solanacearum*, and *Xylella fastidiosa*, as well as the two viruses *Tomato spotted wilt virus* and *Impatiens necrotic spot virus*. Non-target organisms to include in samples will include other oomycetes of the *Phytophtora* genus, and bacteria belonging to the *Paraburkholderia*, *Pseudomonas*, and *Pantoea* genera.

It is planned that, after the validation step carried out in the lab, the project will move on to the analysis of real field samples to further validate the results obtained from the laboratory-prepared samples and test the NGS-based detection method on pathogens different from those considered in the lab conditions.

5.2.Schedule and major steps (3 years): mezza pagina max

1st Year:

Preparation of samples for diagnostic tests
Analysis of samples with both NGS-based (Illumina) and PCR-based methods
Data analysis
Comparison of data between methods (Illumina vs PCR)

2nd Year:

Analysis of samples with both NGS-based (Illumina, minIon) and PCR-based methods
Data analysis
Comparison of data between methods (Illumina vs minIon; minIon vs PCR)

3rd Year:

Sampling of plants from field/greenhouse
Preparation of samples
Analysis with NGS-based and PCR-based methods
Data analysis
Validation of methods.

6. Available funds (source and amount)
GARDING (GENI BARCODING: diagnosi di patogeni per un verde sicuro – € 120.000)

6. Literature: max 10 citazioni

Adams, I. P., Glover, R. H., Monger, W. A., Kemfor, R., Jackerviceince, E., Navalinskiene, M., Samuitiene, M., Boonham, N. 2009. Next-generation sequencing and metagenomic analysis: a universal diagnostic tool in plant virology. Mol. Plant Pathol. 10:537-545

Boonham, N., Kreuze, J., Winter, S., van der Vlugt, R., Bergervoet, J., Tomlinson, J., Mumford, R. 2014. Methods in virus diagnostics: from ELISA to next generation sequencing. Virus Res. 186:20-31

Candresse, T., Filloux, D., Muhire, B., Julian, C., Galzi, S., Fort, G., Bernardo, P., Daugrois, J. H., Fernandez, E., Martin, D. P., Varsani, A., Roumagnac, P. 2014. Appearances can be deceptive: revealing a hidden viral infection with deep sequencing in a plant quarantine context. PLoS ONE 9:e102945

Deurenberg, R. H., Bathoorn, E., Chlebowicz, M. A., Couto, N., Ferdous, M., Garcia-Cobos, S., Kooistra-Smid, A. M. D., Raangs, E. C., Rosema, S., Veloo, A. C. M., Zhou, K., Friedrich, A. W., Rossen, J. W. A. 2017. Application of next-generation sequencing in clinical microbiology and infection prevention. J. Biotech. 243:16-24

Frost, K. E., Groves, R. L., and Charkowski, A. O. 2013. Integrated control of potato pathogens through seed potato certification and provision of clean seed potatoes. Plant Dis. 97:1268-1280

Martin, R. R., James, D., Levesque, C. A. 2000. Impacts of molecular diagnostics on plant disease management. Annu. Rev. Phytopathol. 38:207-239

Martin, R. R., Constable, F., Tzanetakis, I. E. 2016. Quarantine regulations and the impact of modern detection methods. Annu. Rev. Phytopathol. 54:189-205

Pecman, A., Kutnjak, D., Gutiérrez-Aguirre, I., Adams, I., Fox, A., Boonham, N., Ravnikar, M. 2017. Next generation sequencing for detection and discovery of plant viruses and viroids: comparison of two approaches. Front. Microbiol. https://doi.org/10.3389/fmicb.2017.01998

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., Bolchacova, E., Voigt, K., Crous, P. W., et al (Fungal Barcoding Consortium). 2012. Nuclear ribosomal internal trnascribed spacer (ITS) region as a universal DNA barcode marker for Fungi. PNAS 109:6241-6246

Wu, Q., Ding, S. W:, Zhang, Y., Zhu, S. F. 2015. Identification of viruses and viroids by next- generation sequencing and homology-independent algorithms. Annu. Rev. Phytopathol. 53:425- 444