DEVELOPMENT OF DETECTION ASSAY FOR EARLY INFECTION OF LATE BLIGHT IN POTATO USING FLOW CYTOMETRY

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ABSTRACT: Late blight is the most devastating disease of potato caused by hemibiotrophic fungus *Phytophthora infestans*. The disease has two distinct phases of its life cycle: an early asymptomatic biotrophic phase and a late necrotrophic phase. An early biotrophic phase remains for about 72 hrs and is often difficult to detect. Here, we present the simple fluorescence activated cell sorter (FACS) based method to detect the *P. infestans* population at an early stage (before necrotrophic phase) of late blight infection using the same voltage gain settings which is generally used for DNA ploidy estimation of potato. An early detection of *P. infestans* populations in leaf samples will allow the sufficient time for the curative measures.

KEYWORDS: FACS, *P. infestans,* Modified HPI.

INTRODUCTION

Late blight is a major threat to potato production, causing an annual loss of € 12 billion (Haverkort *et al.,* 2009). The disease is caused by an oomycete *Phytophthora infestans* which exhibits biotrophic and necrotrophic phase depending on the stages of its life cycle (Lee and Rose, 2010). In the early stage of infection, pathogen remains in the biotrophic phase which generally lasts for 72 hrs in potato (Kelly *et al.,* 2010). In this phase, pathogen proliferates without showing any visual symptoms, either by suppressing the host defence system or programmed cell death (PCD) (Munch *et al.,* 2008). As pathogen proliferates asymptomatically in the early stage of its infection, it largely remains unnoticed in the potato fields. After completion of the biotrophic phase, the pathogen enters into the necrotrophic phase which is characterized by the tissue degradation and disease development. The control measures in the form of fungicides and other are generally applied once the disease symptoms appear on the potato plants (Kelly *et al.*, 2010). However, at this stage, when the plant's defence machinery has already completely hijacked by the pathogen, the application of control measures does not prove effective and control of the disease becomes difficult. Late blight disease can be managed efficiently if control measures are used at the biotrophic phase of the pathogen which remains undetectable as pathogen grows asymptomatically in the early stage of its infection. Hence detection of the active population of *P. infestans* in biotrophic phase is of prime importance. So far various PCR based methodology have been developed for the detection of pathogens but their application is limited because of generation of false positives through the detection of naked nucleic acids, non-viable microorganism, or through contamination in the laboratory. Efforts have been made by researchers to detect the potato pathogens with FACS such as *Phytophthora infestans* (Day *et al.*, 2002), *P. atrosepticum*, *Dickeya dianthicola*

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(Peters *et al.*, 2007), potato virus Y, potato virus X and potato leaf roll virus (Bergervoet *et al.*, 2008) using the DNA stains, such as 4′, 6-diamidino-2-phenyl-indole (DAPI), propidium iodide (PI) and ethidium bromide (EB). Day *et al.* (2002) used FACS to quickly detect and quantify *P. infestans* sporangia from air and water samples based on scatter and auto-fluorescence. Here we have developed the simple flow cytometry-based method to detect the *P. infestans* population in an early stage of disease development from potato leaf samples. The method is based on the basic principle of flow cytometry wherein *P. infestans* population in its biotrophic phase is detected in the infected potato leaf samples. FACS analysis is performed from the challenge inoculated diploid potato cultivar at 72 days post inoculation (dpi) and the active population of *P. infestans* is flow sorted using the reference DNA peak.

MATERIALS AND METHODS

Plant material and *P. infestans* strains

The diploid potato clone (SS1725) with known ploidy and mean position of DNA peaks of G_0/G_1 (16,932) and G_2/M (33,019) cell population on histogram was taken for the study. The DNA peaks of cell cycle phases (G0/G1, G2/M) were generated based on the propidium iodide (PI) fluorescence. The pure culture of the complex strains of *P. infestans* having general virulence was procured from the Division of Crop Protection, ICAR-CPRI, Shimla and used for generation of reference DNA peaks of G_0/G_1 and G_2/M cell population on the histogram and the same culture was used for the challenge inoculation of diploid potato clone.

Generation of reference DNA peak for G_0/G_1 and G_2/M cell population of *P. infestans*

Three days old *P. infestans* culture having the young mycelia was used for preparation of samples for FACS analysis. The modified HPI buffer (Krishnan *et al.*, 1975) was added to the mycelium and finely chopped with surgical razors in Petri plates kept on ice. The samples were filtered through nylon filters of 42-micron size and collected into the FACS tubes. The samples were incubated on ice for 15-20 minutes in dark to allow the binding of propidium iodide (PI) to the DNA. After incubation, the sample tube was loaded on the SIT flush of BD FACS Canto II for analysis. The samples were analysed on low flow rate with voltage (V) gain settings of FSC: 401 V, SSC: 398 V, FITC: 471 V, PE: 324 V, per CP-Cy5-5: 370 V and APC: 541 V. Maximum of 10,000 events were recorded and discretely observing population were gated for further analysis. The cell population in G_0/G_1 phase and G_2/M phase of the cell cycle were sorted and the histogram of fluorescence intensities was plotted with relative fluorescence intensities versus cell counts. The observed DNA peak position of the G_0/G_1 and G_2/M cell population on histogram was used as the reference for detecting the *P. infestans* population.

Challenge inoculation and sample preparation

Challenge inoculation was performed as per the methodology discussed by Siddappa *et al.,* 2014. The leaf samples were collected from the inoculated diploid clones at 72 dpi and immediately processed for FACS sample preparation as per methodology given in section 2.2.

Sorting the *P. infestans* population in inoculated potato

The samples were analysed using the same instrument gain settings on which reference DNA peaks for *P. infestans* were generated. Maximum of 10,000 events were recorded and discretely observing population were gated for further analysis. The diploid potato cell

population in G_0/G_1 phase and G_2/M phase of the cell cycle were sorted based on the known mean of the concerned population.

Similarly, the additional population on the flow channels was sorted using another gate. The additionally sorted population was then plotted on histograms and the generated DNA peaks were compared with the reference DNA peaks of the pure culture of *P. infestans* cell population. If the mean of the sample and the reference is comparable then it was assumed that the sorted population as same as observed in same peak population.

RESULTS AND DISCUSSION

Reference DNA peak for G_0/G_1 and G_2/M cell population of *P. infestans*

The sorted cell populations were plotted on the histogram with PE (R-phycoerythrin) area versus cell count. The *P. infestans* reference DNA peak of G_0/G_1 cells was plotted at 7,465 (Mean of PE area) with 2,252 cells (Fig. 1). Similarly, though less in cell count (184), DNA peak of G_2/M cells was plotted at 14, 998. The per cent CV for the PE area was around 3 (Table 1). The mean of PE area of DNA peak of G_0/G_1 cells becomes nearly doubles in the DNA peak of G_2/M cells. It is observed because cell undergoes DNA replication after the G_1 phase, so the observed DNA content in the G_2/M cells remains double.

It was also evident from the cell count that G_2/M cells were sorted less (22.5%) in number as compared to the G_0/G_1 (1.8%). The less cell count may be due to the phenomenon of cell cycle regulation wherein only a few cells that cross the cell cycle checkpoints are allowed to enter into the synthesis phase of

Table 1. Statistical analysis of the cell population of *P. infestans*.

Population	Events	% Parent	PE-A mean	PE-A %CV
All Events	10,000	###	12,283	221
G0/G1	2,252	22.5	7,465	3.1
G2/M	184	1.8	14,998	3.3

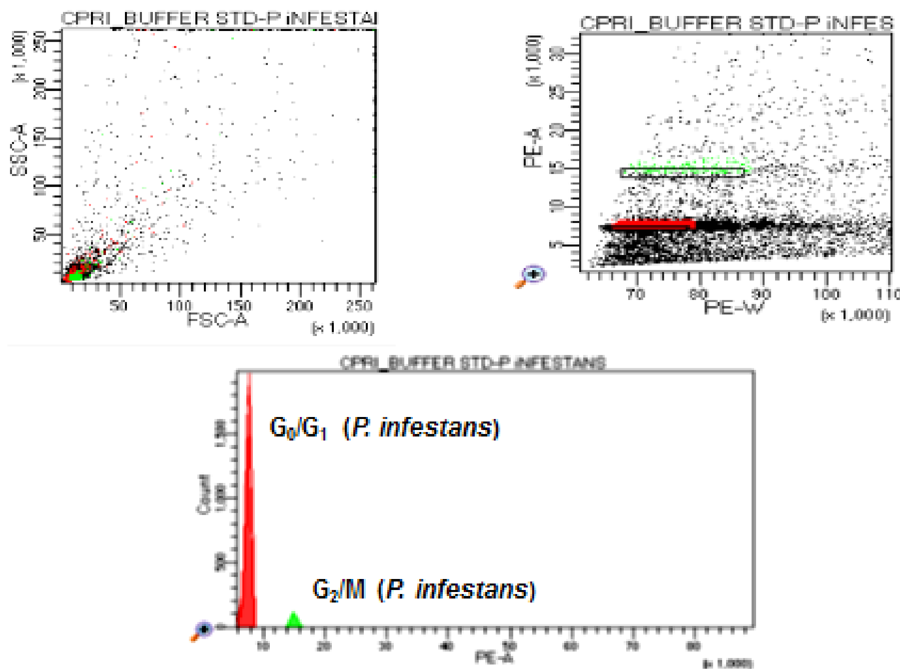

Fig. 1. FACS based reference peaks for the pure culture of complex strains of P. infestans

cell cycle (Dolezel *et al.*, 2007). The observed G_2/M cells are those cells which have crossed the checkpoints of cell cycle regulation before the S phase. Here in this study, we used the DNA peak of G_0/G_1 cells plotted at 7,465 (Mean of PE area) and DNA peak of G_2/M cells at 14,998 as *P. infestans* reference DNA peak on the histogram for detection of *P. infestans* population in the infected tubers.

Detection of the *P. infestans* population from inoculated diploid potato clones

The cell population from the infected potato samples was sorted with same instrument gain settings on which previously potato and *P. infestans* populations were individually analysed. The cell population was plotted with relative fluorescence (PE area) versus cell count and the observed DNA peak of the cell population was compared with the generated reference DNA peaks of *P. infestans* and potato. The three populations were sorted from the inoculated potato leaf samples. Based on the known reference DNA peaks, potato cells were sorted at mean PE area of 17,600 and 35,155 for $G_0/G_1$and G_2/M cells, respectively (Fig. 2). The reaming population was sorted using the additional gate and the population was plotted on the same histogram. The cells get plotted at 8,058 (mean of PE area) with the cell count of 41.5 per cent. The DNA peak position of this cell population was then compared with the reference DNA peak of *P. infestans* G_0/G_1cells. It was observed that the DNA peak position of the G_0/G_1 cells from the sample was 8,058 (mean of PE area) which was near to the reference DNA peak of *P. infestans* (Table 2). The deviation of DNA peak position of G_0/

Table 2. Statistical analysis of cell population of inoculated potato sample.

Population	Events	% Parent	PE-A mean	PE-A %CV
All Events	10,000	###	17,619	155.9
G0/G1 (SS1725)	1,642	16.4	17,600	2.8
G2/M (SS1725)	370	3.7	35,155	2.1
G0/G1 (*P. infestans*)	4,154	41.5	8,058	6.7

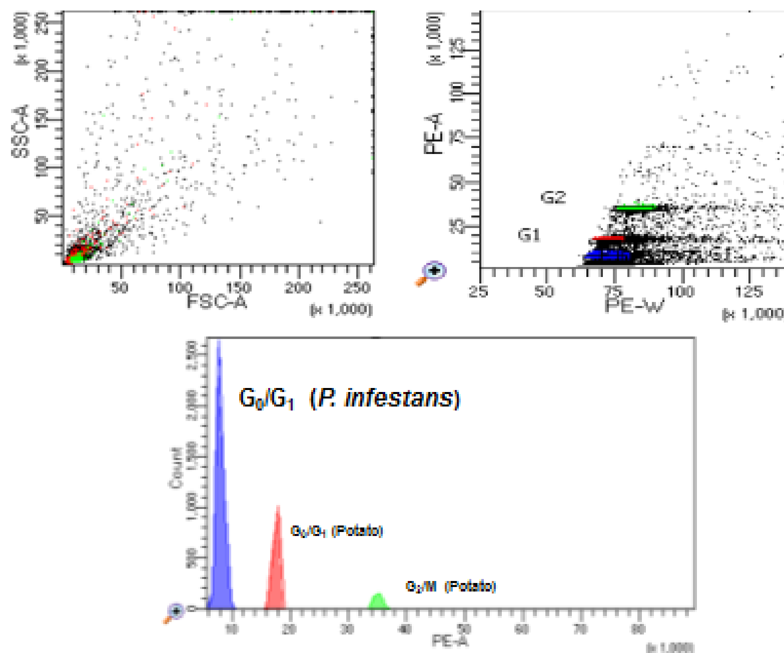

Fig. 2. Detection of P. infestans population from inoculated diploid potato clones

G_1 cells from sample and reference was only 7.3 per cent. With this, it was assumed that the observed population in this range was from the *P. infestans* growing biotrophically in potato leaf tissues.

CONCLUSIONS

Detection of *P. infestans* population in an early disease development stage is necessary to apply the curative measures. Here we have developed a simple flow cytometry-based method to detect the P. infestans population in an early stage of disease development. This methodology has also a potential to detect P. infestans population from seed tubers. Apart from this, the methodology can be further exploited to detect multiple pathogens based on the differences in the DNA ploidy level and use of different fluorochrome.

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