Evaluation of a decision making tools based on RT-PCR real time in *sclerotinia* stem rot control in oilseed rape

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Abstract

Preventive fungicide spray was systematically applied to control *sclerotinia* stem rot in oilseed rape until the late nineties. As *Sclerotinia* stem rot causes important yield losses once or twice every ten years only, many of the applied fungicide sprays are useless. In order to reduce the application of chemicals and its negative impact on the environment, a Petri dish based petal test was developed by CETIOM for rapid estimation of the percentage of infected flowers. A new set of experiments was carried out in 2006 in order to evaluate the interest of a real time PCR test developed by Cambrex Bio Science Clermont-Ferrand in comparison with the Petri dish based test. The sensitivity and specificity of both tests were evaluated by using disease incidence measurements obtained at harvest. Results of about one hundred samples of flowers demonstrated equivalency between the two test methods with respect to performance. According to these results, we intend to substitute the molecular biology based method to the Petri dish based test in our decision making scheme in order to save time in the analysis of oilseed rape flowers.

Key words: winter oilseed rape, *Sclerotinia sclerotiorum*, diagnostic test, real time PCR, decision making tools.

Introduction

Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* (Lib) de Bary is a major disease on winter oilseed rape in France. The disease incidence varies greatly among years and generally severe yield losses occur only once or twice a decade ( ). Until the late nineties the control of the disease was performed by application of a single preventive fungicide spray during flowering. But this practice has led to a large number of useless sprays and the emergence of resistance to currently used fungicides ( ).In order to reduce the application of chemicals and its negative impact on the environment, a reliable decision making for disease management is required.

Previous studies were carried out to define risk indicators for the control of *sclerotinia* stem rot based on petal test as considered in Canada (Turkington & Morrall, 1991) or risk point system (Maisonneuve & al, 1997). An improvement of petal test was obtained by replacing the medium by a more selective one and by plating flowers instead of petals under field conditions (Poisson & al, 2000; Taverne & al, 2003). A comparison of these various risk indicators suggested the use of the percentage of infected flowers as the more convenient (Makowski & al, 2005). Because of the measurement of the percentage of infected flowers remains difficult and time consuming for farmers, a new test of detection based on real-time PCR has been investigated. The aim of this study was to compare these two measurements of risk indicator under field conditions during the flowering season 2006 and to determine detection threshold for decision making.

Materiel and methods

A set of field plots were carried out by CETIOM with partners from various extension services located in three French regions - Centre, Burgundy and Lorraine - where rapeseed is used to be grown.

Rapeseed growers were requested to leave a 12m by 20-30 m plot unsprayed as a check. In this plot, the farmer or the technician of his extension service have performed two tests of diagnostic at the same time at early flowering. For both tests, 40 flowers were collected from 40 different plants picked in four sampling sites.

For the "Petal kit", people who have collected flowers plated them on 9cm diameter Petri dishes filled with a semi-selective agar medium (Steadman & al, 1994). The Petri dishes were incubated at 22-23°C for 4 days before each flower was scored for the presence of a colony of *S. sclerotiorum* (in this case, the blue medium have turned yellow) and the percentage of infected flowers was calculated.

For the real-time PCR, the flowers were collected into a tube that was immediately sent to the lab. The method consists in an extraction of DNA using a mechanical lysis. Specific primers of *S. sclerotiorum* were designed which are unable to amplify DNA from uninfected flowers and other plant pathogens fungi such as *Botrytis cinerea, Alternaria brassicae, Phoma lingam, Verticillium dahliae, Fusarium spp* and *Geotrichum* spp. Real-time PCR products were monitored using fluorescent DNA-intercaling dye SYBR Green. The methods of extraction and detection were developed by Cambrex Bioscience France. Amplification and detection of fluorescence were performed using LightCycler (Roche Diagnostics). The quantitative results were expressed as Ct value, the cycle number at which the fluorescence generated within the reaction crosses the threshold for

being significantly different from background signal. This Ct value is inversely proportional to the initial amount of DNA

Disease incidence was assessed at the end of the growing season, about one month before harvest. Two hundred plants were randomly selected in each untreated plot and were rated for symptoms of *sclerotinia* stem rot. Disease incidence was calculated as the percentage of infected plants.

A total of 97 field plots with both diagnostic tests were available for analysis. A ROC (receiver operating characteristic) curve analysis was carried out to compare these diagnostic tests which give two different risk indicators (Hugues & al, 1999). The 151 field plots were divided into two subgroups depending on a disease incidence threshold (D_{thresh}) of 10% infected plants which represent an injury level above which treatment is needed. Then each indicator value (I) was compared to another threshold I_{thresh} which represents a decision threshold above which treatment is recommended. The results were used to calculate the true positive rate (TP) and the true negative rate (TN). TP is the number of plots with $I > I_{thresh}$ in the subgroup of plots with $D > D_{thresh}$ divided by the total number of plots in this subgroup and represents correct decisions to spray divided by the total number of fields in need of spraying, TP is referred to as "sensitivity". TN is the number of plots with $I \leq I_{thresh}$ in the subgroup of plots with $D \leq D_{thresh}$ divided by the total number of plots in this subgroup and corresponds of correct decisions to not spray divided by total number of fields that should not be sprayed. TN is referred to as "specificity". ROC curves plot the sensitivity (TP) versus (1-specificity) at all possible decision thresholds. A ROC curve that passes close to the point (0,1) shows that the indicator has both desirable sensitivity and specificity characteristics. The diagonal line joining the points (0,0) and (1,1) is the no-discrimination line. The overall accuracy of the indicators is estimated using the area under the ROC curve (AUC).

Results

In 2006, 49.7% of field plots showed little or no disease with D_{thresh} <10% whereas 50.3% plot fields indicated more than 10% of infected plants, Consequently, the *sclerotinia* stem rot incidence in field plots was quite equilibrated.

Using the indicator "incidence of infected flowers" tested for 7 years, low *sclerotinia* stem rot is predicted if a field had less than 30% of infected flowers and spraying is not recommended. In the interval 30%-50% of infected flowers, the forecast was considered to be uncertain and if the decision threshold is above 50% or more spraying is recommended. At the lower decision threshold I < 30%, the error rates given by the percentage of fields that do not need fungicide spraying but reach more than 10% infected plants (false negative) were limited to 2,6% and was less than 5% for 7 years (Fig 1).

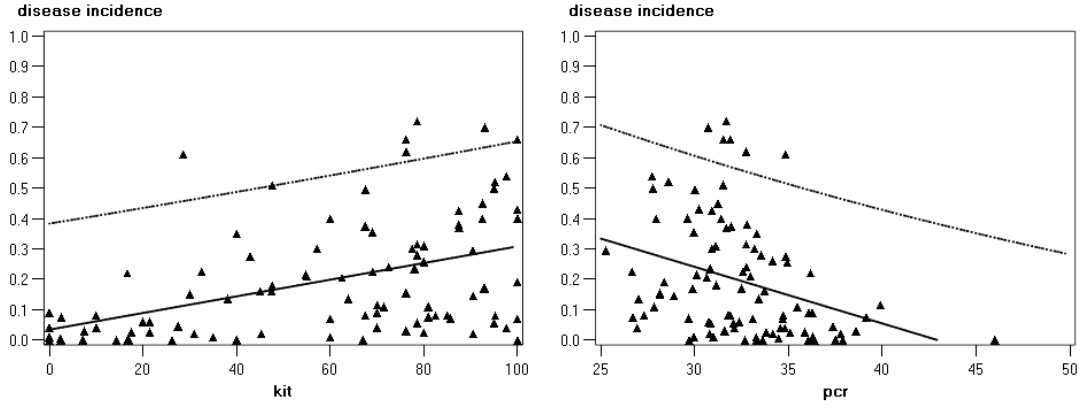

Figure 1: Relationships between risk indicators Kit (incidence of infected flowers) and PCR at early bloom and final disease incidence in 2006.

Real-time PCR values were less correlated with final disease incidence than the incidence of infected flowers given by the 'Petal kit' (Fig 1). Both PCR values and kit are linked with a negative correlation but no strong linear relationship was detected (Fig 2).

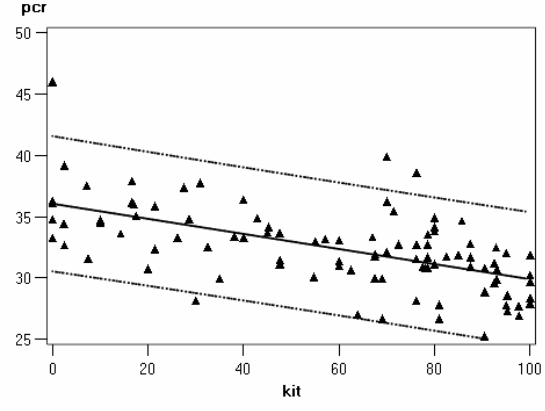

Figure 2: Relationships between incidence of infected flowers (Kit) and Ct values of real-time PCR.

Therefore the use of ROC analysis has allowed to assess the scores of these two indicators. The ROC curves for both indicators are positively above the "no discrimination" line, so both indicators are informative (Fig 3). The AUC are equal to around 0,7 for both infected flowers (kit) and Ct (PCR) and not significantly different at p=0.05. Consequently PCR could be similar in its ability to forecast a disease risk and the need of spraying.

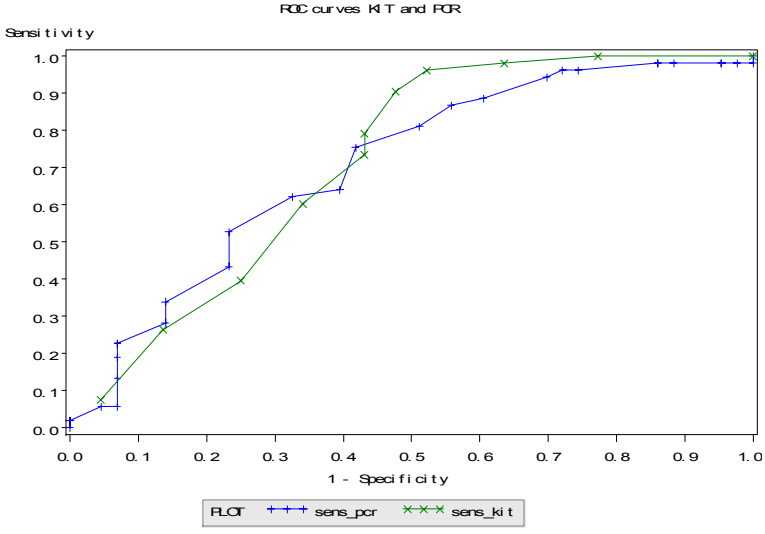

Figure 3: ROC curves for two risk indicators at disease incidence threshold of 10%.

Discussion

Semi selective medium and plating rapeseed flowers instead of petals have improved the method of detection of *sclerotinia* (Taverne, 2003). However the method still needed incubation of Petri dishes for 4 to 5 days at about 22°C before to detect colonies of *S. sclerotiorum* with sometimes were confusing with *Botrytis* or *Geotrichum* sp. So it was required a specific method that would be able to detect the presence of *S. sclerotiorum* more easily and quickly. A previous specific PCR assay demonstrated that it was possible to detect ascospores of *S. sclerotiorum* even in the presence of a large amount of other biological material (Freeman J, 2002). But it would be more interesting for risk assessment that the inoculum could be quantified on petals. Real-time PCR could afford a tool of quantitative diagnostic. The users need only to collect a sample of flowers, and to send it to a lab. Two days later their results could be available.

This study showed that the method based on real time PCR could be specific and sensitive enough under field conditions to determine a risk of potential *sclerotinia* stem rot. Further work is still needed to improve the real time PCR process. Some data have not used in our study because of high DNA concentrations that would have been inhibited the sites of polymerisation and amplification leading to a false diagnostic (Gilbert, 2006).

The comparison between real time PCR and petal kit did not provide the same information. While petal kit allows to estimate the incidence of infected flowers, real time PCR by quantifying DNA assesses the intensity of the inoculum. Moreover the quantitative PCR measures the whole DNA contents in a sample such viable or not ascospores but also mycelium that is developing into petals. This measure could lead to surestimate the risk indicator and consequently to recommend spraying while it would be not necessary.

Whatever the risk indicator used, it has just indicated a potential risk at early flowering stage but not the final disease incidence (Penaud & al , 2006). Previous studies on the risk indicator "incidence of infected flowers" have already indicated the development of the disease was dependant of weather conditions (Turkington & al, 1991). So it would be convenient to take account a long term meteorological forecasting for improving the decision making.

The use of the real-time PCR risk indicator requires more investigations towards the method itself and at the field level. Like the previous indicators, the scores of real-time PCR could vary among years. So new data sets will be performed before to determine a decision threshold.

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