https://doi.org/10.17221/91/2024-PPS

Monilinia fructigena air detection by two different rotorod samplers combined with quantitative real-time PCR

Miloň Dvořák[1], Dagmar Stehlíková[2], Tomáš Litschmann[3]*

[1]*Department of Forest Protection and Wildlife Management, Faculty of Forestry and Wood Technology, Mendel University in Brno, Brno, Czech Republic*
[2]*Department of Genetics and Agricultural Biotechnologies, Faculty of Agriculture and Technology, University of South Bohemia, České Budějovice, Czech Republic*
[3]*AMET – Litschmann & Suchý Corporation, Velké Bílovice, Czech Republic*

**Corresponding author: milon.dvorak@mendelu.cz*

Citation: Dvořák M., Stehlíková D., Litschmann T. (2025): *Monilinia fructigena* air detection by two different rotorod samplers combined with quantitative real-time PCR. Plant Protect. Sci., 61: 326–332.

Abstract: The brown rot of apple and stone fruits caused by *Monilinia fructigena* is a widespread disease causing serious losses in fruit production. The most common way the pathogen spreads is via airborne conidia. Therefore, air samplers can effectively monitor its occurrence. In this study, we have conducted a comparative sampling of two cheap air samplers – rotorod spore traps called ROTTRAP 52 and AMETRAP. An optimised quantitative real-time PCR assay with a hydrolysis probe evaluated samples. 14 concurrent samplings were positive in all cases, showing higher spore counts in almost all AMETRAP samples obtained under various weather conditions. The daily maximum air temperature was the only significant meteorological variable positively affecting the recorded spore counts. Both rotorod samplers are an efficient and economic option for horticulturists and researchers for *M. fructigena* air inoculum monitoring.

Keywords: spore trap; qPCR; moniliosis; silicone grease; air inoculum

Monilinia fructigena (Aderh. et Ruhl.) is the main causal agent of the brown rot of apple and stone fruits, which causes significant damage in pre-harvest as well as in post-harvest stage throughout Europe (Byrde & Willetts 1977).

Unlike the closely related species *M. fructicola* (G. Winter) Honey, *M. fructigena* does not reproduce sexually via apothecia, despite both mating types in Europe (Abate et al. 2018). The reproduction and present spread of the fungus is mostly intermediated via asexual conidia released from 1–2 mm wide sporodochia grown concentrically on the brown surface of rotten, later 'mummified' fruits (Byrde & Willetts 1977). Conidia are formed in branched chains, elongate-ellipsoid, limoniform or ovoid, hyaline, 15–25 × 12–16 µm (Batra 1979). Maturation of the conidia is determined by higher temperature and relative humidity. In contrast, the release of conidia is facilitated by wind, predominantly during afternoons, when the air humidity decreases and temperature increases (Bannon et al. 2009). Over short distances, within one tree, it can be spread by rain splash (Pauvert et al. 1969); however, the main ways of spread through

Supported by the Ministry of Agriculture of the Czech Republic, Project No. QK22010073 "Alternative methods of pest and disease control in potatoes minimising the negative impact on the environmen" and by the European Union's Horizon 2020 Programme for Research & Innovation under grant agreement No. 771271 (HOMED project, "Holistic Management of Emergent forest pests and Diseases").

https://doi.org/10.17221/91/2024-PPS

orchards and more distant areas are wind (Bannon et al. 2009), insects and humans (Lack 1989).

Protective measures to prevent the disease development are focused on chemical treatment of the trees and fruits (Byrde & Willetts 1977). Furthermore, a considerable decrease in the incidence can be achieved by removing the fallen, symptomatic fruits, which provide the source of inoculum throughout the season (Holb & Scherm 2007). The overwintered fruit mummies provide inoculum at the beginning of the season, a source of infection to the developing fruits. Early fallen fruits infect mature fruits, which react with the first symptoms after two weeks to two months (Holb & Scherm 2007).

Optimal control of the disease must be based on knowledge about seasonal and spatial dispersal patterns and the presence of current inoculum. Aerobiological methods can achieve these data. Air sampling by spore traps and quantitative molecular methods provides species-specific and highly sensitive detection. Various types of spore traps differ in price, efficiency, weight, precision, input power, and user-friendliness (Dhingra & Sinclair 2017). The cheapest option is to sample the air inoculum with passive spore traps, which consist of a piece of filter paper, microscopic glass, or a glass rod covered by a sticky layer. The filter paper is usually moistened by TE-buffer to prevent spore germination (Grosdidier et al. 2017). On these simple traps, air particles are trapped by passive settling with low efficiency (Dhingra & Sinclair 2017). On the other hand, volumetric spore traps (Hirst 1952) actively suck the air and sample at a known rate, mostly 10 L/min. The most common modification of the volumetric spore trap enables air sampling in real time, demanding the exchange of the trapping tape after seven days (Mehta et al. 1996). However, volumetric spore traps are expensive devices. A cheap option for sampling pathogenic fungi's inoculum are rotorods (Perkins & Leighton 1957). They consist of rotating arms with vertical rods covered by an adhesive medium. They offer very high sampling rates, even over 100 L/min (Dvořák 2022), but they can get overloaded (Lacey & West 2006; Chandelier et al. 2014; Dhingra & Sinclair 2017), and they are less sensitive to very small particles, which excludes them from experiments with pathogenic fungi spreading via too small conidia (Dhingra & Sinclair 2017).

As a part of elucidating the spore dispersal patterns, aerobiological methods are helpful in early detection of airborne pathogens, even in visually healthy areas, before the outbreak (Muller et al. 2023). Hence, the goal of our work was to optimise an economically affordable methodology and technology for *M. fructigena* air detection, useful for: *(i)* early detection of the pathogen, and *(ii)* describing the seasonal or spatial spore dispersal dynamics within and around infested orchards. For that purpose, we tested two different rotorod samplers with two different adhesives to reveal which is more efficient for detecting *M. fructigena* propagules. *M. fructigena* was chosen as a model pathogen because of its extreme incidence and economic impact in European orchards. The propagule amount was quantified by an optimised real-time PCR assay with a hydrolysis probe to achieve the highest sensitivity and specificity of the detection method.

MATERIAL AND METHODS

Rotorod air samplers. To sample the inoculum of *M. fructigena,* two similar rotorod samplers were used to assess their efficiency for detecting this pathogen in the air.

One of them was ROTTRAP 52 (RP) (Miloň Dvořák, Czech Republic; Figure 1), previously used for the detection of *Hymenoscyphus fraxineus,* the causal agent of ash dieback (Dvořák et al. 2023). Its construction, function and instructions for sampling are published by Dvořák (2022).

The other rotorod sampler, AMETRAP (AT) (AMET, Czech Republic, Figure 2), works on the same principle as RP, differing in the use of impaction rods. For AT, exchangeable medical injection needles were installed (length 40 mm, diameter 0.8 mm on one side and 1.2 mm on the opposite side, Becton Dickinson S.A., Spain), covered with

Figure 1. ROTTRAP 52 with square-profiled impaction rods coated with double-sided tape

https://doi.org/10.17221/91/2024-PPS

Table 1. Technical characteristics of the air sampling systems used in this study

Parameter	ROTTRAP 52	AMETRAP
Electric supply	9V DC	12 V DC
Revolutions per minute	2 067	3 000
Sampling rate	52 L/min	45 L/min
Rotating arm diameter	10 cm	6 cm
Impaction rod profile (length × width)	Squared (50 × 0.8 mm)	Rounded (40 × 0.8* and 1.2* mm)
Trapping medium	Double-sided, non-woven tape (Tesa, Germany)	Silicone grease (Onset, USA)
Minimal particle diameter	10.9 µm	**unknown

*diameter of the needle; **cannot be calculated with any known formula

a thin layer of silicone grease. The rotor of AT consists of a plastic fan of 7 cm in diameter. The exchangeable sampling needles were fixed to a bar mounted on the centre of the fan.

Further technical details of both rotorods are compared below (Table 1). Sampling rates and minimal particle diameter were calculated following the known equations for rotorods (Noll 1970; Dhingra & Sinclair 2017; Dvořák 2022). To calculate the sampling rate of AT, the sampling rod diameter was taken as the width of the sampling surface.

Field sampling. The experimental area was a private garden in Moravský Žižkov (South Moravia, Czech Republic, sampling spot's GPS: 48.8344 N, 16.9302 E; 192 m a.s.l.) surrounded by intensively managed orchards where apple, apricot and other fruit trees are planted for fruit production. The brown rot of apples and stone fruits is common and persistent. An Automatic meteorological station (AMET, Czech Republic) has been installed ten meters from the sampling spot to record air temperature, humidity, precipitation, global irradiance, leaf moisture and wind speed every hour.

Figure 2. AMETRAP with injection needles coated with silicone grease

The air sampling was performed 14 times from July 9 to September 3, 2022. The sampling period was 24 h, and one or two samplings were performed weekly. RP and AT were sampling simultaneously, installed 10 m from each other at a height of 1.6 m. After each sampling, the tape was removed from the rods of RP and inserted into 2 mL microtubes. The exposed needles from AT were returned to their original sterile tubes. Both tubes with samples were stored at –20 °C before further processing.

DNA extraction. The room temperature of the samples was recovered before DNA extraction. The grease on the whole surface of AT needles was manually wiped off with a 1 cm^2 piece of sterile paper tissue and inserted into 2 mL microtube. Further steps of DNA extraction of both types of samples followed the protocol published by Dvořák (2022), including the same materials. No internal control was used.

Real-time quantitative PCR. All the DNA samples were quantified by absolute quantification real-time PCR using the QuantStudio 6 Flex Real-Time PCR System (Life Technologies Holdings Pte. Ltd., Singapore) platform. A conidial suspension was prepared to obtain conidia counts in each sample. For this purpose, a few sporodochia from recently mummified apples were dipped in a petri dish with distilled water. Suspension was transferred into 2 mL microtube, vortexed and quantified microscopically in a haemocytometer. An appropriate amount of the suspension was pipetted into sterile 2 mL microtubes to obtain ten-fold serial dilutions of 10^5 – 10 conidia per microtube. The DNA of the suspensions was extracted from the field samples, as mentioned above.

Reaction mixture of each qPCR contained 15 µL Light Cycler 480 Probes Master (Roche Diag-

https://doi.org/10.17221/91/2024-PPS

nostics Nederland BV, the Netherlands), primers ITS1Mfgn1 and ITS4Mfgn1 and FAM-BHQ-1 labelled hydrolysis probe (P2_fgn/lx/ps) specific for *M. fructigena* (Ioos & Frey 2000; van Brouwershaven et al. 2010), 5 µL of template DNA and UltraPure™ DEPC-Treated Water (Invitrogen) to fill up the 25 µL final volume. Each sample was processed in three technical replicates in the wells of a MicroAmp Fast Optical 96-Well Reaction Plate with Barcode (Applied Biosystems, USA) and sealed (MicroAmp Optical Adhesive Film, Applied Biosystems, USA). The default qPCR program (10 min at 95 °C; 45 cycles at 95 °C for 15 s and 1 min at 60 °C) was set. The reaction mixture was optimised by gradient testing of the final volume of the primers and probe. Final volume 0.4 µM of each primer and 0.3 µM of the probe resulted in the lowest Ct values and the highest fluorescence intensities reliably detecting the lowest DNA concentrations, i.e. the 10 spores DNA standard. Consequently, all DNA samples were tested and data were analysed in StepOne™ Software (version 2.3).

Statistical analyses. Pairwise comparison of RP and AT numbers of spores was calculated in Excel with a level of significance $P < 0.01$. The non-parametric Wilcoxon signed-rank matched-pairs test was used based on low repetitions.

Relations between RP/AT spore count differences and meteorological variables were evaluated in NCSS 2024 (version 24.0.2) with a level of significance $P < 0.05$. Non-parametric Spearman rank correlation was used due to the lack of normality and the low number of repetitions.

RESULTS

Quantification of samples. The quantitative real-time PCR assay was optimised to reliably detect a standard sample containing 10 spores (Figure 3). All field samples from both spore traps tested positive for *M. fructigena*; however, the RP sampling conducted on August 17–18 yielded spore counts two to three orders of magnitude higher than the other 13 RP samplings. Consequently, this outlier from both traps was excluded from further analyses, leaving 13 valid paired samplings. Spore counts across these samples ranged from 58.60 to 4 162.64 (Figure 4) and were higher in the AT samples in 11 of 13 cases, demonstrating AT's significantly greater sampling efficiency than RP ($z = 2.90$; $\alpha = 0.002$). The maximum spore count (4 162.64 in a 24 h AT sample) corresponds to 64.8 m³ of sampled air, i.e. an average concentration of 64.23 spores/m³, while the minimum count (58.60 in an RP sample) over 74.88 m³ of air equals 0.78 spores/m³.

Meteorological determination of spore records. The only meteorological variable that correlated significantly with sampling differences was the daily maximum air temperature. The analysis showed

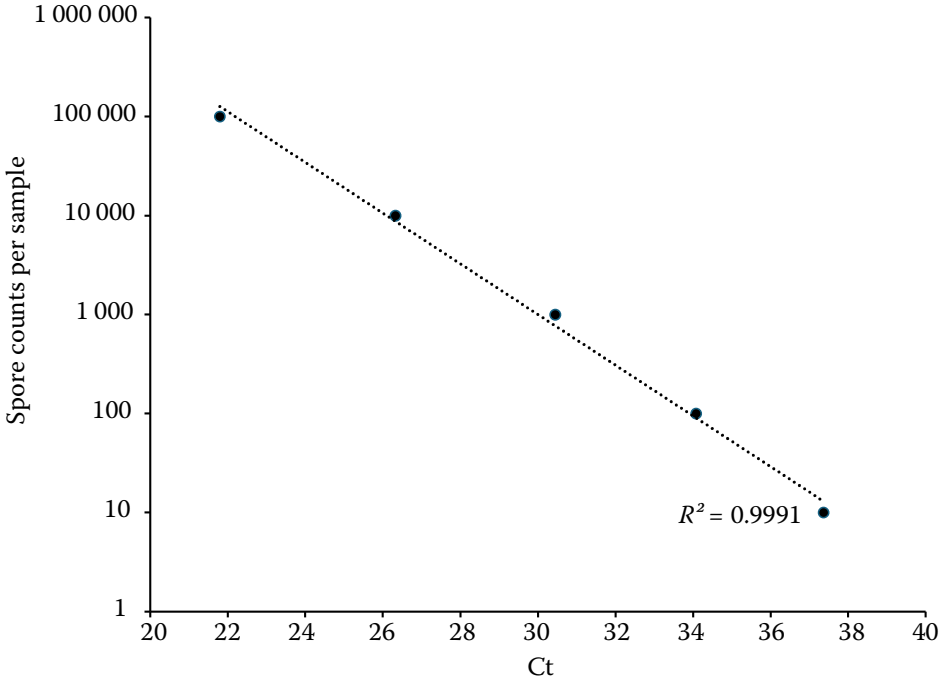

Figure 3. Standard curve set up from means of duplicate quantifications of conidial suspensions = samples with known spore counts

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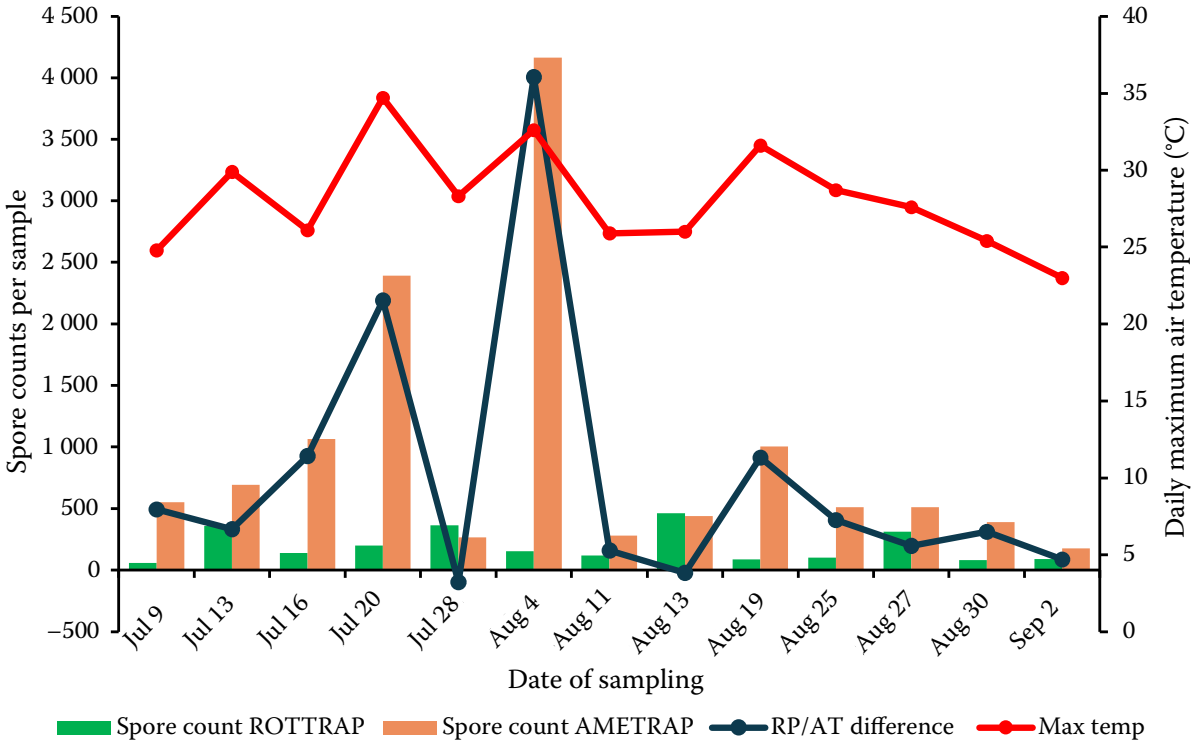

Figure 4. Spore counts were detected by both samplers during the experiment, with daily maximum air temperature as the only meteorological variable showing a significant correlation

that higher daily maximum temperatures were associated with greater differences in spore counts between traps, with the AT trap sampling more efficiently ($\rho = 0.555$; $df = 11$; $\alpha = 0.049$). A strong correlation was also observed between maximum temperature and AT spore counts ($\rho = 0.692$; $df = 11$; $\alpha = 0.009$), whereas the correlation between maximum temperature and RP spore counts was not significant ($\rho = 0.401$; $df = 11$; $\alpha = 0.174$). No significant correlation was detected between RP and AT spore counts ($\rho = 0.044$; $df = 11$; $\alpha = 0.887$).

DISCUSSION

Comparison of air samplers. The results demonstrate that both rotorod samplers tested in combination with qPCR analysis are effective tools for detecting airborne *M. fructigena* inoculum. This is the first report of rotorods being used to monitor Monilinia pathogens. Previously, 7-day volumetric spore traps were employed to characterise dispersal patterns of *M. fructigena* and *M. fructicola* (Luo et al. 2007; Bannon et al. 2009).

Bannon et al. (2009) used a Burkard sampler and microscopic counting to reveal diurnal patterns, finding a maximum of 73 spores per day – an average concentration of 5.07 spores/m³. In our study, the AT sampler recorded a peak concentration almost 13 times higher (64.8 spores/m³). This difference likely reflects methodological variation: Bannon et al. (2009) counted only limoniform conidia larger than 20 µm and could not reliably identify other *M. fructigena* conidia by morphology, leading to underestimation. Our species-specific qPCR assay ensures accurate detection and, together with both AT and RP samplers, achieves an approximately fivefold higher sampling rate. However, neither RP nor AT samplers provide real-time hourly resolution like volumetric traps.

Luo et al. (2007) focused on *M. fructicola*, applying qPCR without a hydrolysis probe to Burkard trap collections. They reported average daily concentrations up to 50 spores/m³, with occasional peaks of 300 spores/m³ – values that align with results of this study.

Our air-sampling systems cost only about 160 EUR each, making them roughly 30 times cheaper than commercial volumetric traps. This low cost allows deployment of multiple units for broader studies (Chandelier et al. 2014; Grosdidier et al. 2018) at minimal investment while maintaining high sampling efficiency.

https://doi.org/10.17221/91/2024-PPS

Despite similar nominal sampling rates for RP and AT samplers, AT outperformed RP in 11 of 13 paired samplings. Two factors likely account for this. First, the square profile of RP rods generates an "air cushion" that very small particles cannot easily penetrate, reducing impaction efficiency (Perkins & Leighton 1957; Noll 1970). In contrast, the rounded needles of the AT sampler are more aerodynamic, facilitating particle settlement. Second, DNA extraction from AT samples is more efficient: the sampling grease is readily removed and homogenised, whereas particles on RP's double-sided tape tend to adhere stubbornly, often rolling up during transfer and resisting elution (Dvořák 2022).

Influence of trapping adhesives and weather. Various adhesives have been evaluated for use on rotorods. Sometimes diluted with hexane, silicone grease consistently produces reliable spore captures (Solomon et al. 1980; Frenz & Guthrie 2001). Petroleum jelly (also called liquid grease, white petrolatum or medical vaseline), the standard coating for melinex tapes in Hirst-type volumetric traps (Hirst 1952; Calderon et al. 2002; Timmermann et al. 2011; Ganthaler & Mayr 2015; Thiessen 2023), has also been trialled on rotorods, but results have been ambiguous. Noll (1970) successfully used petroleum jelly in a complex rotorod sampler designed for different aerosol fractions. Yet, Solomon et al. (1980) reported that petroleum jelly on rotoslides was slightly, but significantly, less efficient than silicone grease under unspecified temperature conditions. In field tests, Čermáková et al. (2017) noted that petroleum jelly coatings often degraded during hot periods, causing the adhesive film to disappear (Čermáková, personal communication).

Our samplings spanned a wide temperature range from a maximum of 34.7 °C on July 20 to a minimum of 8.5 °C on September 3. Non-parametric correlation analysis showed that AT samplers outperformed RP samplers at higher temperatures. This confirms that silicone grease retains its adhesive efficiency even in hot weather. Indeed, AT spore counts correlated significantly with daily maximum air temperature, whereas RP counts did not. This differs from Bannon et al. (2009), who observed that higher air temperatures up to 100 h before sampling enhanced *M. fructigena* sporulation and maturation rather than immediate airborne concentrations. In our case, colder temperatures may slightly reduce grease adhesiveness, but not enough to compromise AT performance.

Rainfall had no significant effect on the relative performance of either sampler, confirming the robustness of both silicone grease (Frenz & Guthrie, 2001) and double-sided tape (Dvořák et al. 2023) as trapping adhesives under varying weather conditions.

CONCLUSION

In our study, we successfully employed and optimised two rotorod air samplers and performed qPCR evaluation of the air samples. The results support the AMETRAP rotorod sampler with injection needle impactors coated with silicone grease as the more efficient system under varying meteorological conditions. Thanks to the 160 EUR per sampler, researchers and practitioners can easily purchase and use it to monitor the brown rot of apple and stone fruits caused by *M. fructigena.*

Regular sampling during growing seasons and consequent modelling of the conidial spatial and temporal spread in particular localities can forecast brown rot outbreaks and/or shift disease management to a higher level using decision support systems. Moreover, these rotorod samplers present a promising tool for phytosanitary monitoring of airborne quarantine pests in compliance with Regulation (EU) 2016/2031 of the European Parliament and the Council of October 26, 2016, on protective measures against pests of plants.

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Received: June 5, 2024
Accepted: May 29, 2025
Published online: September 23, 2025