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Brief Report

Inactivation of the Plant Pathogen *Pythium ultimum* by Plasma-Processed Air (PPA)

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Abstract: *Pythium* species are saprophytic or facultative plant pathogens that cause a variety of diseases. Usually, chemical anti-fungal seed dressing is applied in the conventional pre-harvest for seed protection. Nevertheless, recent legislative rules have created a ban on certain agrochemicals. Therefore, alternative eco-friendly methods have to be identified to ensure healthy field emergence and seedling development. In this study, a proof-of-concept was performed on the inactivation of *Pythium ultimum* Trow mycelia grown on potato dextrose broth agar (PBA) by plasma-processed air (PPA). Different plasma process parameters were applied using variation in gas flow of air through the microwave plasma generator and PPA exposure time. The PPA treatment was compared to the untreated and gas treated controls. The results showed a complete inactivation of *P. ultimum* mycelia after the PPA treatment. Inactivation efficiency was independent of the gas flow parameter and even shorter exposure times resulted in complete inactivation. To fully evaluate the potential of PPA as a possible seed hygiene measure, tests regarding the inactivation of *P. ultimum* after artificial inoculation onto seeds and/or studies using naturally infected seeds should be performed. This may be accompanied by monitoring the disease severity after the PPA treatment on a field scale.

Keywords: decontamination; plasma; plasma-processed air

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Citation: Wannicke, N.; Brust, H. Inactivation of the Plant Pathogen *Pythium ultimum* by Plasma-Processed Air (PPA). *Appl. Sci.* **2023**, *13*, 4511. https://doi.org/10.3390/app13074511

Academic Editor: Spiridon Mantzoukas

Received: 20 February 2023
Revised: 28 March 2023
Accepted: 30 March 2023
Published: 2 April 2023

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1. Introduction

Pythium ultimum Trow is a ubiquitous plant pathogen with a broad host range, often occurring as a complex of different *Pythium* species [1–3]. It is one of the most important plant pathogens and causes a variety of diseases including seed rot and damping-off, root- stem- and fruit rot, and foliar blights in lupine, corn, soybean, wheat, ornamental plants, and other crops [4–6]. Symptoms of infested plants include a reduction in seedling emergence, smaller and distorted first true leaves, wilting, plant stunting, reduced tillering, loss of fine feeder roots, crown rot, uneven plant growth, and, finally, lower yields. In combination with other pathogenic fungi and nematode pests, the annual losses to wheat and barley producers are estimated at over USD one million in e.g., Washington [7]. *Phytium* is predominantly soil-borne but can also be attached to seed surfaces as a mycelium [6]. Its life cycle is divided into two different sub-cycles, an asexual and a sexual cycle. The asexual cycle is characterized by the production of sporangia, which germinate either directly in liquid or on surfaces to produce a germ tube (direct germination) or may differentiate to form uni-nucleate, biflagellate zoospores (indirect germination). Plants are infected repeatedly at different stages of growth, because of the rapidity of the asexual life cycle of *Pythium*. The intracellular aseptate hyphae develop in branches through the plant tissue using nutrients from the host plant and creating a mesh of absorptive mycelium, from which sporulation occurs on the dying seedling and the disease cycle is repeated.

In the course of the sexual cycle, thick-walled oospores are produced which are adapted for over-wintering and survival under harsh environmental conditions. Fertilization occurs via the haploid structure of the antheridium, which produces and contains male gametes and the oogonium leading to the development of oospores. These oospores can

undergo extended dormancy and germinate under suitable conditions to produce single or multiple germ tubes. Finally, these germ tubes can form sporangia, thereby, recapitulating the asexual cycle of the pathogen [8].

Disease management of *Pythium* encompasses mechanical, biological or physical methods, as well as adapting cultural practices such as tillage and crop rotation [9]. Chemical treatment is also widely applied as seed coating against *Pythium* and other *Peronosporomycetes*. These chemicals include mefenoxam/metalaxyl (acylanilide class) which have been shown to provoke resistance in *Pythium* [10]. The mode of inactivation of this fungicide class comprises inhibition of RNA synthesis in the fungi by interfering with the RNA polymerase, as well as a second mode of action, which involves a loss of cell permeability [11].

Furthermore, fungicides of the carbamate class, which include propamocarb, are used as an active ingredient in a number of commercial products, which have received user approval in the USA in 2021, but not in the EU. The carbamate class adversely affects membrane permeability, as detected by the leakage of cellular constituents out of cells mostly when treated during growth [12].

The fungicide captan (phthalimide class) is used in single, as well as in mixture application. Captan is also applied to manage multiple pathogens at the same time i.e., *Pythium*, *Fusarium* and *Rhizoctonia* spp. in soybean seed treatment [13]. The main mechanism responsible for the fungicide effect of this class is the reaction with proteic and non-proteic thiol groups in the microorganisms, which provokes the reduction of overall enzymatic activity, respiration, and many other physiological and morphological changes in fungi [14]. Nevertheless, the general use of pesticides can have adverse effects on human life, as well as on ecosystems [15], including specific effects of fungicides on soil bacteria, actinomycetes and non-pathogenic fungi [16–18]. This also encompasses the development of resistance of target organisms, especially when overusing azoles as generalized fungicides. Resistance in this case occurs via mutation of the azole target, the sterol 14α-demethylase CYP51, a regulatory enzyme in the ergosterol biosynthetic pathway [19].

One potential alternative for seed treatment against fungal and bacterial pathogens is the application of cold atmospheric plasma using the direct or indirect exposure mode. It has been successfully tested on various crop species and pathogen types [20]. Plasma in general is considered as the fourth state of matter and contains charged particles, reactive oxygen and nitrogen species (RONS), excited molecules and UV photons [21,22]. Furthermore, electrical fields and temperature are important components especially in the direct treatment modes and influence the impact of plasma on microbial inactivation [23,24]. The mode of plasma exposure to the target can be direct or indirect via the generation of plasma-treated gas (e.g., plasma-processed air, (PPA)) or liquids (e.g., plasma-processed water) [22,25–28].

Indirect treatment using PPA based on microwave torches has proven to be efficient in microbial inactivation on the laboratory scale, as well as at the industrial scale for inorganic (e.g., glass), organic (e.g., plastic) and biological surfaces (e.g., fruit, vegetables, and meat) [29–34]. Moreover, the exposure of seeds from various plant species inoculated with the non-pathogenic spores of *Bacillus atrophaeus* has shown the bactericidal efficiency of the PPA treatment [35].

In this study, the objective was to perform a proof-of-concept on the efficiency of PPA for the inactivation of the vegetative mycelia stage of the plant pathogen *Pythium ultimum*, to elucidate the general potential of PPA as a seed treatment against the vegetative form of fungal pathogens. In addition to the untreated controls, further control samples were exposed to gas flow only to ensure that inactivation was not the result of drying out by using a gas flow within the PPA treatment system.

2. Materials and Methods

2.1. Cultivation of Pythium ultimum

A culture suspension of *P. ultimum* Trow containing a mixture of mycelia and spores originated from the German collection of microorganisms and cell cultures (DSM 62987, Braunschweig, Germany) was used. For each trial a fresh suspension of scraped-off mycelium in Potato Dextrose Broth (PDA, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was generated.

2.2. Artificial Inoculation of Agar Blocks and Determination of Recovery after PPA Treatment

Inoculation of PDA agar blocks with a size of 0.5 cm × 0.5 cm was carried out by application of 25 µL fresh hyphae and spore culture suspension. After 3 days of incubation at 22 °C, blocks with grown mycelia were picked for each of the four independent experimental trials. In each trial, six different treatments were applied to investigate the inactivation potential of PPA: (a) agar blocks with mycelia and no PPA treatment (positive control), (b) three PPA treatments with different gas flow and incubation times (see Section 2.3), and (c) two control treatments using two different gas flow/incubation time parameters without plasma ignition (gas controls, see below). To avoid contamination of laboratories and equipment with *Pythium*, agar blocks were sealed into gas-diffusive bags (CleanFlex® Tyvek®/HDPE, Bischof + Klein SE & Co., KG, Langerich, Germany). Previous experiments have shown that Tyvek® bags do not affect the PPA treatment process [34]. Bags with agar blocks were transferred to pressure resistant one-liter glass bottles (Duran, Germany) with seven replicates each. Agar blocks of PPA treatments (see below) and controls were placed onto the center of freshly prepared PDA agar plates and incubated at 22 °C. Radial growth of mycelia on each petri dish (diameter of 85 mm) was measured daily at three directions for 14 days and the average diameter was calculated. To evaluate the PPA inactivation efficacy, PPA treatments were compared to the untreated and gas controls.

2.3. Generation of Plasma Processed Air (PPA) and Agar Block Treatment

PPA was generated using a microwave driven discharge at frequency of 2.45 GHz, as well as the supplied power of 4 kW. Plasma was operated at a gas temperature of ~4000 K. For further details on the construction and composition of the device see Wannicke et. al. [35]. The flow rate of the compressed air was 63 and 73 slm (standard liter per minute). The exhaust PPA was cooled down to room temperature (about 23 °C) before entering the one-liter glass incubation bottles. After completing the flushing of incubation bottles with PPA, gas flow was stopped and PPA was exposed to the sealed agar blocks for different time intervals. The following combinations of process parameter were applied: 63 slm and 25 min, 73 slm and 15 min, 73 slm and 10 min. The two gas controls using compressed air only were applied with 63 slm gas flow and 25 min and with 73 slm gas flow and 15 min. To stop the exposure to PPA, the incubation bottles were refilled twice with untreated compressed air. The experimental set-up encompassed four independent runs on four different days with seven replicates for each run.

2.4. Statistical Analysis

Repeated measure one-way ANOVA (RM-ANOVA), with time as the subject and treatment as the factor, was used to check for differences in between treatments for mycelial radial growth over 14 days of observation. The Holm–Sidak method post-hoc analysis was chosen for pairwise multiple comparison to determine significant differences ($p < 0.05$) between treatments. Prior to statistical analysis, data were tested for normality and homogeneity of variances using Wilk–Shapiro and Brown–Forsythe tests. Statistical analysis was done using SigmaPlot 13 (Systat Software Inc., San Jose, CA, USA).

3. Results

Non-treated *P. ultimum* on PDA agar blocks (positive control, no PPA) displayed a logistic radial growth of mycelia within the course of 14 days (Figures 1 and 2). Exponential

growth occurred from day one to day five with an increase in mean mycelia diameter of 16 mm (Figures 1 and 2). Slower growth reaching stationary phase was detected from day six to day fourteen, which resulted in an increase to a final maximum mean diameter of 28.2 mm.

Gas controls were applied to exclude any dry-out effects of Pythium on agar blocks. However, similar growth patterns were recorded for the gas controls without PPA (Figures 1 and 2) compared to untreated controls; no statistically significant differences were detected for growth of untreated and gas controls (Table 1). Using the gas flow of 63 slm, exponential growth in the gas control was detected from day one to day five with an increase in mean mycelium diameter of 15.9 mm (Figures 1 and 2). Similar to the untreated controls, slower growth was observed from day six reaching a maximum mean diameter of 28.6 mm at day fourteen. For 73 slm gas flow control, the exponential growth phase extended till day six reaching a mean diameter of 16.3 mm. Logistic growth occurred from day seven till day fourteen resulting in a maximum mean diameter of 28.5 mm.

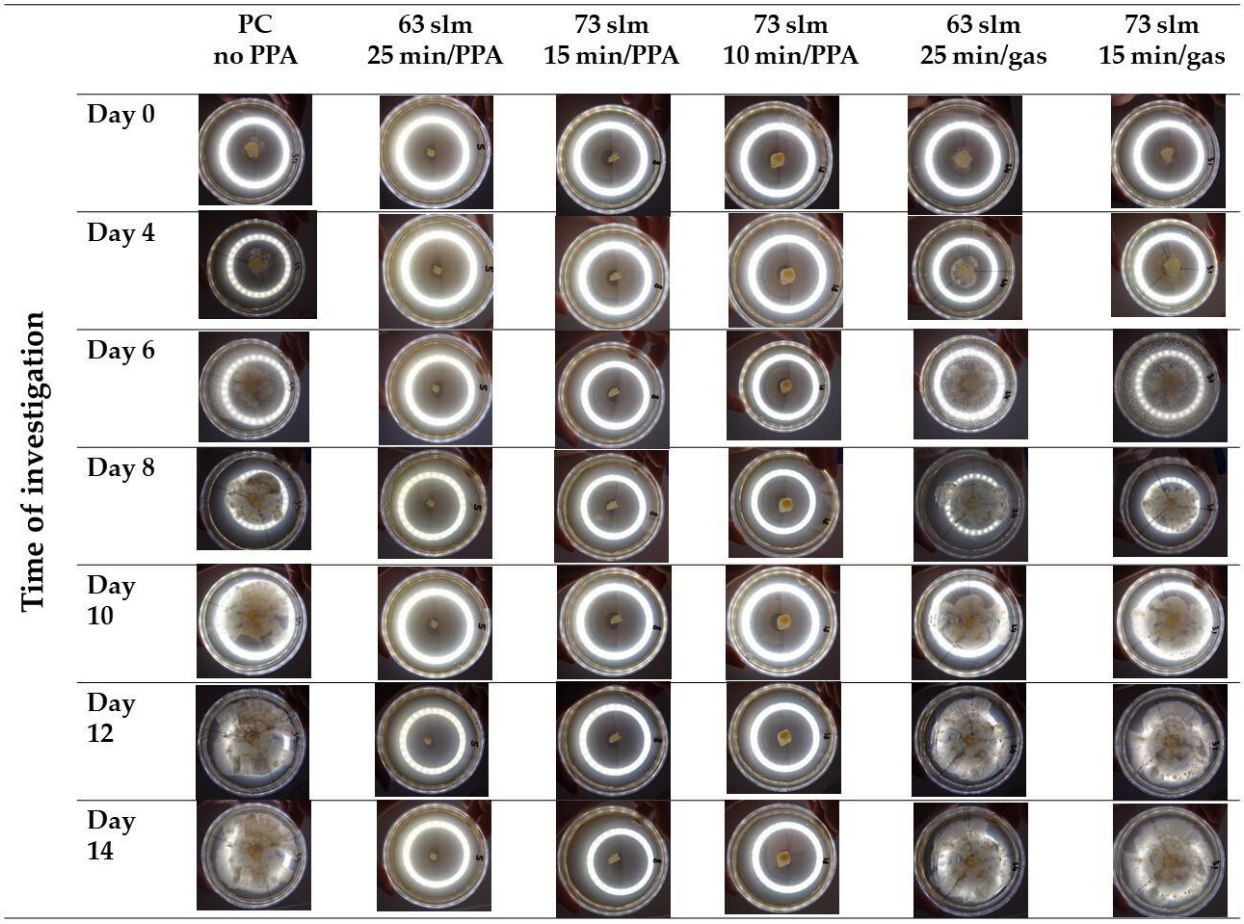


Figure 1. Radial growth of *Pythium* mycelia on PDA agar plates over 14 days of incubation. One representative replicate of seven replicates per run is displayed. All seven replicates per treatment showed similar results. PC: positive control, inoculated no PPA, two controls using gas flow of air only without microwave torch ignition (i.e., gas) and three different PPA treatment modes. For scaling classification note that petri dishes are 85 mm in diameter.

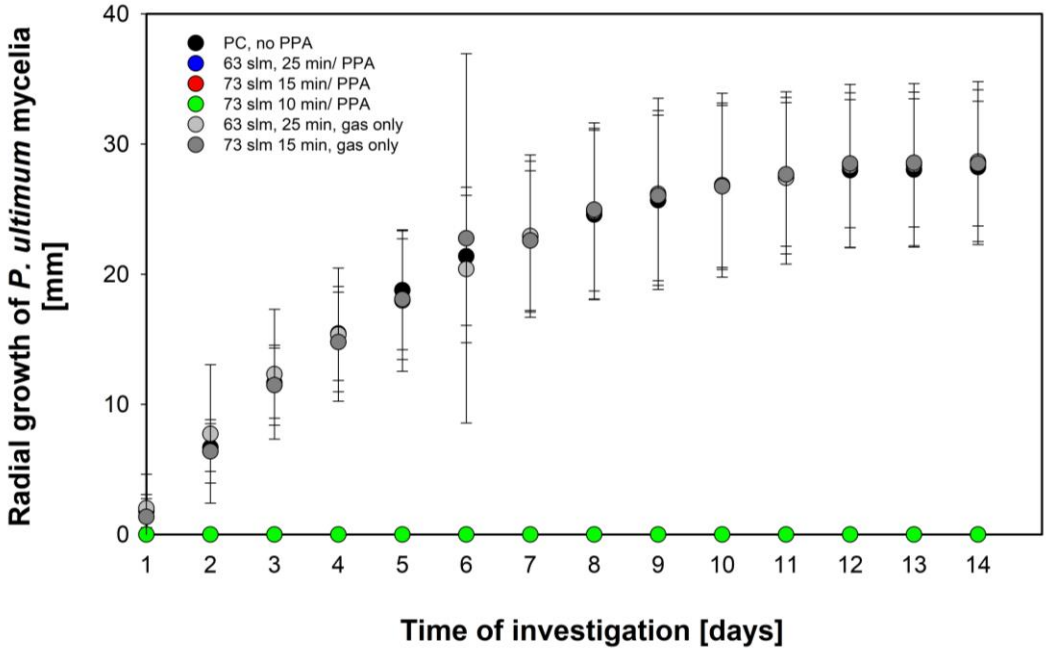


Figure 2. Growth of *Pythium* mycelia (mm + SE) over a 14-day period for the six different treatments. Four independent runs with seven replicates per treatment were applied (n = 28). PC: positive control, inoculated no PPA, two controls using gas flow of air only without microwave torch ignition (gas only) and three different PPA treatment modes. (PPA): Note that data points for PPA treatments (blue, red, green dots) are overlaying.

Table 1. Results of repeated-measure one-way ANOVA (RM-ANOVA), followed by Holm–Sidak comparison.

Source	**DF**	**Type III Sum of Squares**	**Mean Square**	**F**	***p***
Treatment	3	1461.292	1782.411	78.987	<0.001
Comparison	**Diff. of means**	**t**	***p***		
PC vs. 63 slm, 25 min/PPA	20.533	11.436	<0.001		
PC vs. 73 slm, 15 min/PPA	20.533	11.436	<0.001		
PC vs. 73 slm, 10 min/PPA	20.533	11.436	<0.001		
PC vs. 63 slm, gas only	0.136	0.0756	1		
PC vs. 73 slm gas only	0.0702	0.0391	1		
63 slm gas only vs. 63 slm, 25 min/PPA	20.669	11.511	<0.001		
63 slm gas only vs. PPA 73 slm, 15 min/PPA	20.669	11.511	<0.001		
63 slm gas only vs. PPA 73 slm, 10 min/PPA	20.669	11.511	<0.001		
73 slm gas only vs. 63 slm, 25 min/PPA	20.599	11.472	<0.001		
73 slm gas only vs. 73 slm, 15 min/PPA	20.599	11.472	<0.001		
73 slm gas only vs. 73 slm, 10 min/PPA	20.599	11.472	<0.001		
73 slm gas only vs. 63 slm gas only	0.0702	0.0391	1		
63 slm, 25 min/ PPA vs. 73 slm, 15 min/PPA	0	0	1		
63 slm, 25 min/PPA vs. 73 slm, 10 min/PPA	0	0	1		
73 slm, 15 min/ PPA vs. 73 slm, 10 min/PPA	0	0	1		

All PPA treatments, irrespective of applied gas flow rate or exposure time, resulted in 100% inactivation of *P. ultimum* on PDA agar. No detectable mycelial growth was observed over the entire 14 days of incubation (Figures 1 and 2). This resulted in significantly lower radial growth for PPA treatments relative to both the control groups (Table 1).

4. Discussion

All plasma process parameters applied for PPA treatment in our study resulted in the complete inactivation of the vegetative stage of *P. ultimum* on PDA agar blocks, potentially also preventing spore formation. No growth was recorded for mycelia in any of the PPA treatments over a period of 14 days. As no growth was detected in all the PPA treatments, the Holm–Sidak comparison during RM-ANOVA post-hoc analysis led to identical differences of mean, t and p statistics of the PPA treatments relative to the control and gas control. Furthermore, the growth in the control and the gas control was also comparable in magnitude, also leading to identical values for the Holm–Sidak comparison. Overall, increasing the gas flow or shortening the incubation time did not affect the inactivation efficiency. In future studies, exposure times below 15 min could be tested to identify the shortest exposure time still reaching complete inactivation. This would ensure quick processing of seeds during the pre-harvest preparation and would likely save energy costs.

One possible mode of action for the inactivation of hyphae is the destructive oxidation of the cell wall components and/or lipid peroxidation by the reactive species. This finally leads to loss of the cell integrity followed by morphological degeneration. It is also likely that after damage to the cell membrane, reactive species could have penetrated to the cell interior and affected intracellular enzymes and other cellular macromolecules. In future studies, microscopic analysis of *Pythium* hyphae after PPA exposure would give evidence on this and might also help to identify the lowest gas flow rate and lowest exposure time of PPA where morphological damage and inactivation are detectable.

Reactive nitrogen species play a crucial role in the inactivation of microorganisms by oxidation of the cytoplasmic membrane, protein, and DNA [36,37]. It has been shown that PPA generated by microwave discharge is composed of highly reactive nitrogen species including nitrogen dioxide, nitrous acid, and nitric acid. Schnabel et al. [34] published the qualitative gas composition of PPA generated by 16 slm flow of compressed air containing mainly six different molecules and radicals: nitrogen monoxide and nitrogen dioxide (NO•, NO_2•), carbon dioxide (CO_2), water (H_2O), nitric acid (HNO_3), and nitrous acid (HNO_2).

In general, indirect as well as direct treatment by cold plasma can lead to the inactivation of microorganisms including bacteria, viruses, and fungi [20] by morphological degeneration, including damage of the cell envelope structures. Previously published studies also gave evidence for damage of the cell envelope structures using a direct plasma exposure [38], which is among others related to lipid peroxidation of cell membranes and loss of cell integrity [39], and finally also reported necrotic death after direct plasma treatment [40]. In addition, morphological changes were reported for two agriculturally relevant fungal species, *Ascochyta pinodella* and *Fusarium culmorum* after direct DBD air plasma treatment [41]. In that study, increasing plasma exposure time led to higher inactivation and disruption of hyphae. Cracks and burst hyphae were visible in microscopic investigations leading to the hypothesis that oxidative reactions by reactive species resulted in the degradation of cell walls.

Two mechanisms are discussed in the literature for cell envelop disruption due to plasma exposition of microorganisms. Montie et al. [42] applied a glow discharge and suggested that the rupture of the bacterial cell wall is caused by alterations of its components due to oxidative effects generated by reactive species. Other authors also reported oxidative reactions of cell wall components [43,44].

Furthermore, Mendis et al. [45], who applied theoretical model calculation, proposed charge accumulation on the outer surface of the bacterial cell envelop and by that provided an important aspect of the inactivation mechanism. This was further underpinned by experimental data using a resistive barrier discharge (RBD) to treat bacterial cells [46].

Besides the efficient inactivation of pathogens, unimpaired seed germination has to be guaranteed after PPA exposure to ensure unaffected field emergence. Therefore, future investigations have to be performed with infected seeds (natural or artificial) of the relevant crop species to ensure both inactivation and seed germination after the PPA treatment. The effect of PPA exposure on the maximum germination of lupine, barley, wheat and rape

using similar process parameter as in this study was published in an earlier study [35], showing marginal effects on maximum germination in cereals and lupine.

5. Conclusions

To sum up, the proof-of-concept regarding the efficiency of inactivation of *Pythium* mycelia in our study was successful and displayed complete inactivation at all plasma process parameters. This has implications for the potential usage of PPA in the pre-harvest treatment of seeds to ensure seed hygiene in agricultural practice. To study the mechanisms of inactivation in more detail, microscopic investigations should be performed in future investigations. Subsequently, the efficacy of inactivation should be tested using either naturally infected seeds of relevant crop plants or artificial inoculation of hyphae or spores onto seeds, also making sure that seed viability is kept. Further studies also need to consider the field scale approaches that monitor seedling establishment along with the disease symptoms and disease severity of growing crops.

Author Contributions: Conceptualization, N.W. and H.B.; writing—review and editing, N.W. and H.B.; project administration, N.W. and H.B.; funding acquisition, N.W. and H.B. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by the by the German Federal Ministry of Education and Research (BMBF), FZK 03WIR2802B and FKZ 03WIR2805A.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding authors.

Acknowledgments: We kindly appreciate the experimental support by Jörg Stachowiak, especially handling of PPA treatment and acknowledge the experimental support regarding phytopathology given by Frank Brändle and Stefan Keil, IdentXX GmbH, Stuttgart Germany.

Conflicts of Interest: The authors declare no conflict of interest.

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