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ORIGINAL ARTICLE

Diagnosis of *Synchytrium endobioticum*: the importance of sample pretreatment to determine the infectivity of resting spores in bioassays

Hana Tlapák[1,2] | **Friederike Chilla**[1] | **Anne-Kristin Schmitt**[1] | **Peter Büttner**[3] | **Kerstin Flath**[1] | **Anna Pucher**[1]

[1]Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Field Crops and Grassland, Julius Kühn-Institut, Kleinmachnow, Germany

[2]Unit of Sexually Transmitted Bacterial Pathogens and HIV, Robert Koch – Institut, Berlin, Germany

[3]Bayerische Landesanstalt für Landwirtschaft (LfL), Institut für Pflanzenschutz, Freising, Germany

Correspondence
Anna Pucher, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Field Crops and Grassland, Julius Kühn-Institut, Stahnsdorfer Damm 81, 14532 Kleinmachnow, Germany.
Email: anna.pucher@julius-kuehn.de

Abstract

Potato wart disease, caused by the soil-borne, obligate biotrophic fungus *Synchytrium endobioticum*, is one of the most important diseases of cultivated potatoes. The fungus does not produce hyphae, but produces very thick-walled sporangia (resting spores), so an infection with *S. endobioticum* results in a long-term contamination of fields. Chemical control of the disease is not possible; consequently, potato production on infested fields is prohibited for many years. Reliable diagnosis of *S. endobioticum* is essential to preserve cultivation areas. A critical step is to determine the infectivity of resting spores. The new implementing regulation (EU) 2022/1195 recommends a bioassay based on soil samples in addition to microscopic analysis. The aim of this study was to evaluate soil-based bioassays for their ability to determine the infectivity of resting spores in infested soils. It was shown that the resting spores could be in a dormant state, in which they do not infect potatoes. In some cases, this dormancy could be broken by a certain sequence of moistening and aerating the soil. Reactivation of soil is therefore recommended prior to soil-based bioassays. Microscopic analysis could not distinguish between dormant and infectious resting spores.

KEYWORDS
dormancy, potato wart disease, reactivation, soil-based bioassay, *Synchytrium endobioticum*

Diagnostic de *Synchytrium endobioticum* : L'importance du prétraitement des échantillons pour déterminer l'infectiosité des spores dormantes durant les tests biologiques

La gale verruqueuse de la pomme de terre, causée par le champignon *Synchytrium endobioticum*, est l'une des maladies les plus importantes de la pomme de terre cultivée. Le champignon ne produit pas d'hyphes, mais produit des sporanges à paroi très épaisse (spores dormantes), de sorte qu'une infection par *S. endobioticum* entraîne une contamination à long terme des champs. Le contrôle chimique de la maladie n'est pas possible. Par conséquent, la production de pommes de terre dans les champs infestés est interdite pendant de nombreuses années. Un diagnostic fiable de *S. endobioticum* est essentiel pour préserver les zones de culture. Une étape critique consiste à déterminer l'infectiosité des spores dormantes. Le nouveau Règlement d'exécution (UE) 2022/1195 recommande un test biologique basé sur des échantillons de sol en plus de l'analyse microscopique. Le but de cette étude était d'évaluer la capacité des tests biologiques basés sur le sol à déterminer

l'infectiosité des spores dormantes dans les sols infestés. Il a été démontré que les spores dormantes pouvaient être dans un état de veille dans lequel elles n'infectent pas les pommes de terre. Dans certains cas, cet état de veille pourrait être rompu par une certaine séquence d'humidification et d'aération du sol. La réactivation des sols est donc recommandée avant les tests biologiques sur le sol. L'analyse microscopique n'a pas permis de distinguer les spores en état de veille des spores dormantes infectieuses.

Диагностирование *Synchytrium endobioticum*: важность предварительной обработки образцов для определения инфекционности покоящихся спор при анализе проб

Рак картофеля, вызванный почвенным облигатным биотрофным грибом *Synchytrium endobioticum,* является одним из наиболее серьезных заболеваний культивируемого картофеля. Этот гриб не образует гиф, но формирует толстостенный споридий (покоящиеся споры), поэтому инфекция *S. endobioticum* приводит к долгосрочному заражению полей. Химическая борьба с этим патогеном невозможна, поэтому, выращивание картофеля на зараженных полях запрещено в течение многих лет. Достоверное диагностирование *S. endobioticum* является ключевым фактором для сохранения посевных площадей. Определение инфекционности покоящихся спор является важнейшим элементом анализа. Новый Имплементационный регламент (EU) 2022/1195 рекомендует в дополнение к микроскопическому анализу проведение анализа проб на основе образцов почвы. Целью данного исследования была оценка анализа почвенных проб на предмет его способности определять инфекционность покоящихся спор в зараженных почвах. Результаты показали, что такие споры могут находиться в состоянии покоя, когда они не инфицируют картофель. В некоторых случаях состояние покоя может быть нарушено определенной последовательностью увлажнения и аэрации почвы. Именно поэтому перед осуществлением анализа почвенных проб рекомендуется проведение реактивации почвы. Оказалось, что при проведении микроскопического анализа невозможно отличить инфекционные покоящиеся споры от спор, находящихся в состоянии глубокого покоя.

1 | INTRODUCTION

Potato wart disease is one of the most important diseases of cultivated potatoes. The causal agent of this disease is the quarantine pest *Synchytrium endobioticum*, an obligate biotrophic, soil-borne fungus (Obidiegwu et al., 2014), which belongs to the order Chytridiales in the phylum Chytridiomycota (Obidiegwu et al., 2015). *Synchytrium endobioticum* is categorized on the EPPO A2 list and is recommended for regulation (EPPO, 2017). The fungus infects certain meristematic tissues of the potato plant via zoospores. It can infect the underground parts of the potato plant (except the roots), as well as the shoot and leaves (Artschwager, 1923; Langerfeld, 1984). In most cases, symptoms are visible on the tubers. Infection with *S. endobioticum* leads to proliferation of the cells surrounding the infection sites. This induction of cell division results in tumour-like tissue, which is a typical symptom of the disease. Warts vary in size from a few millimetres up to several centimetres and contain the growing fungus and its sporangia (Ballvora et al., 2011). One peculiarity in the biology of *S. endobioticum* is that it does not produce any hyphae or mycelia but it does produce resting spores (Abdullahi et al., 2005; Pratt, 1976). Upon decomposition of the wart tissue the resting spores are released into the soil and can remain viable for more than 30 years (Obidiegwu et al., 2015).

Resting spores have a typical golden brown colour and a diameter of 25–75 μm (van Leeuwen et al., 2005) and have an irregularly thickened cell wall with ridges. To assess viability, the content of the resting spores has to be examined. It is agreed that a viable resting spore has a homogeneous content that largely fills the sorus. In case of doubt, resting spores should be classified as viable (EPPO, 2017). Owing to the difficulty of resting spore evaluation, assessment of viability should only be done by experts.

Because of the persistence of the resting spores of *S. endobioticum* and the fact that chemical control of potato wart is not possible (Obidiegwu et al., 2015), potato production on infested fields is prohibited for many years. The major economic losses caused by *S. endobioticum* infestation are due to the prohibition of potato production and the yield losses directly related to the disease symptoms (Ballvora et al., 2011; Obidiegwu et al., 2015; Stachewicz & Langerfeld, 1998). Spread of sporangia occurs mainly through infected tubers and contaminated

agricultural machinery and tools (Ballvora et al., 2011). Certain environmental factors favour infection with potato wart disease. These include slightly acidic mineral soils with a not too high humus content, a water capacity of 60%–70% and an annual rainfall of >600 mm. Rainfall in the first 3 weeks and 7–10 weeks after planting also favours infection (Stachewicz & Langerfeld, 1998). Langerfeld (1984) reports that an average soil temperature between 15.5 and 18°C favours infection with *S. endobioticum* but emphasizes that rainfall and temperature need to be considered together.

It is well known, that crop rotation is a critical point in disease management for many soilborne pathogens, and *S. endobioticum* is no exception. However, the most effective ways to combat potato wart are the cultivation of resistant potato cultivars and strict phytosanitary measures (Ballvora et al., 2011). In the European Union *S. endobioticum* is listed as a Union quarantine pest in Annex II B of Regulation (EU) 2019/2072 (European Commission, 28.11.2019). In July 2022, the Commission Implementing Regulation (EU) 2022/1195 establishing measures to eradicate and prevent the spread of *S. endobioticum* (Schilbersky) Percival was published and its measures are now mandatory in the EU (European Commission, 19.7.2022). An important factor in the diagnosis of *S. endobioticum* is the viability of resting spores found in infested fields. This is even more important as the partial release of infested fields is possible under certain conditions. The implementing regulation recommends a bioassay in the form of a pot test to assess the viability of spores, in addition to microscopic analyses. For the bioassay, a potential issue arises owing to resting spores, which may be in a dormant state and therefore not infectious at the time of the bioassays.

In addition to the pot test, the Spieckermann test (Spieckermann & Kotthoff, 1924) is a well-established bioassay in which infested soil is used as an inoculum. Although this is not specified in the implementing regulation for assessing the infectivity of resting spores, it is a commonly used test in diagnostic laboratories and was used in this study to test the infectiousness of contaminated soils (Spieckermann & Kotthoff, 1924).

The aims of the current study were to test the reliability of bioassays using contaminated soil as inoculum as a tool for assessing the viability of resting spores, and to assess if soil treatment prior to the bioassay can have an effect on the infectivity.

2 | MATERIALS AND METHODS

2.1 | Plant material

Tubers of the fully susceptible potato cultivars Tomensa (Europlant Pflanzenzucht GmbH) and Tessa (Norika Nordring- Kartoffelzucht- und Vermehrungs-GmbH) were used for all bioassays. To induce germination of the tubers, they were stored at room temperature and in dim light (partially covered boxes were stored in daylight) for approximately 1 week until sprouts of 1–2 mm length were visible.

2.2 | Wart inoculum

Bioassays were performed with two types of inoculum of *S. endobioticum*. Wart compost from the JKI collection was used as the inoculum for pathotype 6(O1). The JKI wart compost was produced in 2007 by regularly stirring and moistening finely chopped warts mixed with sand (30% quartz sand) for 6 months. The compost was air dried before long-term storage. Wart compost for pathotypes 2(G1) and 18(T1) was provided by the Bavarian State Research Center for Agriculture (LfL), and was produced under more natural conditions. In closed containers, non-infested field soil was mixed with fresh wart material on an annual basis for a period of 15 years and left to decompose in similar conditions to infested fields until 2022. Fresh warts from laboratory tests were added regularly for continuous rotting of wart material to elevate the infectious level of the soils. In addition, susceptible potatoes were grown annually in the containers for wart production and rotting.

The average concentration of spores per gram of soil was determined using the wet sieving method (see below). The composts were stored in closed boxes at 4–6°C. All composts were pre-tested in pot tests or using the Spieckermann test to determine the infectivity. In the pre-test, the composts of pathotypes 2(G1) and 6(O1) were categorized as non-infectious, and the compost of pathotype 18(T1) was classified as slightly infectious inducing less symptoms (score 4) on fully susceptible cultivars. Those pre-tests were conducted in the late summer.

2.3 | Reactivation of soil samples

To reactivate the soil samples, the soils were moistened and aerated at specified intervals (Table 1). An untreated control was maintained for each pathotype and treatment. The untreated control was kept at the storage temperature of 4–6°C until the bioassay started.

Soils (2–3 kg) were transferred to open boxes (30 × 40 cm), moistened with a sprayer, and then stirred. The composts were stored at 16°C during reactivation.

2.4 | Extraction of resting spores

Extraction of resting spores from untreated soils/composts was performed by wet sieving, with some modifications to the sieving method B described in the EPPO PM 7/28 (2) (EPPO, 2017). In addition to 25 and 75 µm

TABLE 1 Different reactivation treatments of soils. Samples were moistened and aerated differently for treatments A, B and, C for 2, 3 or 4 months.

Treatment A		Treatment B		Treatment C	
Term	**Frequency of moistening and aerating the samples**	**Term**	**Frequency of moistening and aerating the samples**	**Term**	**Frequency of moistening and aerating the samples**
Month 1	Once per week	Month 1	Once per week	Week 1+2	Once per week
Month 2	Once per week	Month 2	Three times per week	Week 3+4	Twice per week
Month 3	Three times per week	Month 3	Three times per week	Month 2	Three times per week
Month 4	Three times per week				

sieves, a 125 µm sieve was used for the sieving process. The samples were centrifuged at 2300 ***g***.

2.5 | Bioassays

To determine the infectivity of soils, the Spieckermann test (EPPO, 2017) was used with slight modifications. Instead of 1.0–1.5 g of inoculum per eye plug, the eye plugs were covered with a 1–2 cm thick layer of compost. The boxes were cultivated at 16°C (darkness), and the symptoms were evaluated after 6–7 weeks. The reactivated soils and untreated controls were used as inoculum. Ten tubers per cultivar were used per treatment.

The SASA method (EPPO, 2017) was used with some modifications to test the infectivity of the extracted resting spores. In the SASA method, extracted resting spores are sprinkled on a water surface and incubated for 21 days to stimulate germination before inoculation. The resting spores are thus in contact with water and oxygen, comparable with the reactivation process. Contrary to the standard protocol, germination of resting spores was induced at a reduced temperature of 16°C (in the dark). To determine the germination rate after 21 days, an aliquot of 25 µL was analysed microscopically. All full resting spores and all germinating resting spores (with a germination vesicle) were counted. Infection of fully susceptible potato cultivars according to the EPPO protocol took place after 21 days. After inoculation, the resting spore suspension was left on the sprouts until it evaporated or for a maximum of 10 days to avoid rotting of the eye plugs.

2.6 | Microscopic evaluation

After 6–7 weeks, the eye plugs were examined under a stereomicroscope (Stereomikroskop Zeiss Stemi 2000-C, Zeiss, Germany) for disease symptoms and resting spores. The evaluation was carried out according to the rating scheme of the EPPO Standard PM 3/88 (EPPO, 2020). To determine the infectivity of the inoculum, we differentiated between non-infected eye plugs, slightly infected eye plugs (score 4) and highly infected eye plugs (score 5). Score 4 is defined by the presence of resting spores but no wart formation. Score 5 is defined by wart formation.

Extracted resting spores were characterized as viable or non-viable using a microscope (Axioscope 5/7 KMAT, Zeiss, Germany) at 400× magnification. Resting spores were counted as viable/ infectious by detecting the known criteria (homogeneously filled, intact sorus wall). Ambiguous resting spores were not taken into account. To determine the number of viable resting spores in soils, two aliquots of 25 µL each were prepared after sieving of 100 g of soil. The number of viable spores was counted and the number of viable resting spores per gram of soil was calculated.

2.7 | Statistical analysis

A chi-square test was conducted to determine if there is a significant difference between the infectiousness of reactivated and non-reactivated inoculum samples. For this purpose, the results of the cultivars Tomensa and Tessa were grouped. Further, a chi-square test was used to determine if there is a difference between the two cultivars and between the different reactivation treatments. For all chi-square tests, the infectiousness was categorized in three scoring groups (non-infected; 4, slightly infected; 5, wart formation). The significance level was set at $\alpha = 0.05$.

3 | RESULTS

Resting spores extracted from three different composts, previously considered non-infectious (P2(G1) and P6(O1)) or with low infectious potential (P18(T1)), were examined under the microscope. The question was whether the differences in infectivity could be explained by a difference in the amount or absence of viable resting spores in the samples. The total number of resting spores varied slightly between the samples, but completely filled spores with a homogeneous content were detected in all samples (Figure 1a–c). While the number of viable resting spores was almost identical for the composts of pathotype 2(G1) (54 viable resting spores per gram of soil) and pathotype 18(T1) (56

viable resting spores per gram of soil), only the compost of pathotype 18(T1) showed infectious potential in the pre-tests. For the compost of pathotype 6(O1), the number of viable resting spores was more than 10 times higher than for the other composts (639 viable resting spores per gram of soil). However, this compost did not show any infectious potential in the pre-tests.

To verify that the spores tested in previous bioassays were indeed viable and possibly in a dormant state, the soil samples were reactivated through repeated steps of aeration and moistening. As the ideal time for reactivation was unknown, three different procedures were tested with reactivation periods of 2, 3 and 4 months (Table 1). After reactivation, the infectivity of the reactivated soils was tested against the untreated controls in Spieckermann bioassays. Evaluation of the Spieckermann bioassays after 6–7 weeks showed that there was little or no infection in the eye plugs inoculated with the untreated control. Eye plugs inoculated with the reactivated soils, showed clear symptoms (reaction types 4 and 5) on two of the three composts tested. An example of this is shown in Figure 2 for cultivars Tomensa and Tessa inoculated with compost of pathotype 2(G1), which had been reactivated for 3 months (treatment B).

The reactivated samples showed strong wart formation on most of the eye plugs tested for pathotypes 2(G1)

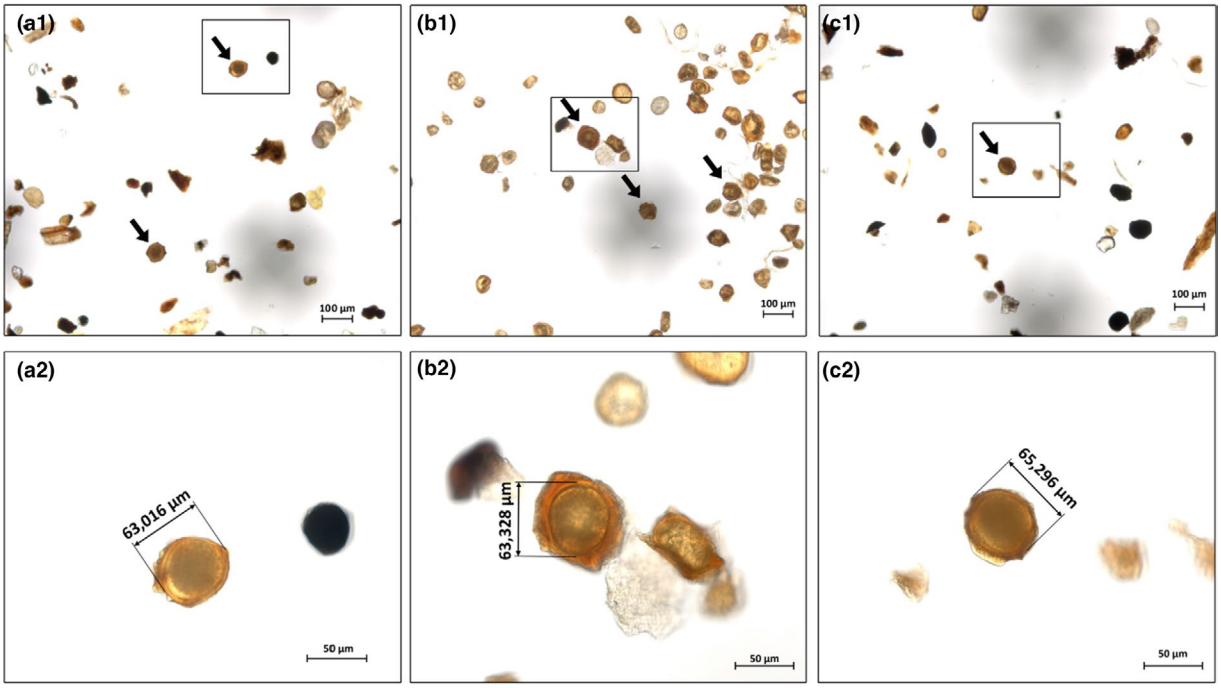

FIGURE 1 Microscopic examination of resting spores extracted from soils of pathotypes 2(G1), 6(O1) and 18(T1). a = pathotype 2(G1), b = pathotype 6(O1) and c = pathotype 18(T1). a.1, b.1 and c.1, 100× magnification of spore samples; a.2, b.2 and c.2, 400× magnification of viable spore samples. Black arrows indicate viable spores; squares in the top row indicate the area magnified in the images in the bottom row.

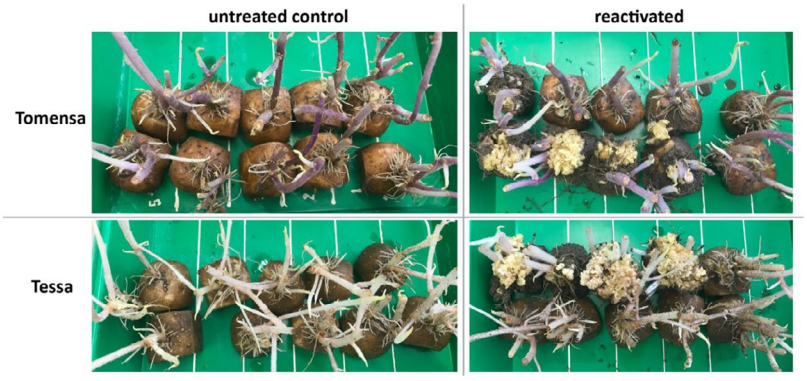

FIGURE 2 Comparison of susceptible cultivars Tomensa and Tessa after incubation with reactivated and untreated control compost using the Spieckermann bioassay. Compost of pathotype 2(G1) was reactivated for 3 months (treatment B) and used to inoculate eye plugs of the susceptible cultivars Tomensa and Tessa in comparison with the untreated control. Eye plugs inoculated with the reactivated compost showed warts, whereas those inoculated with the untreated control did not.

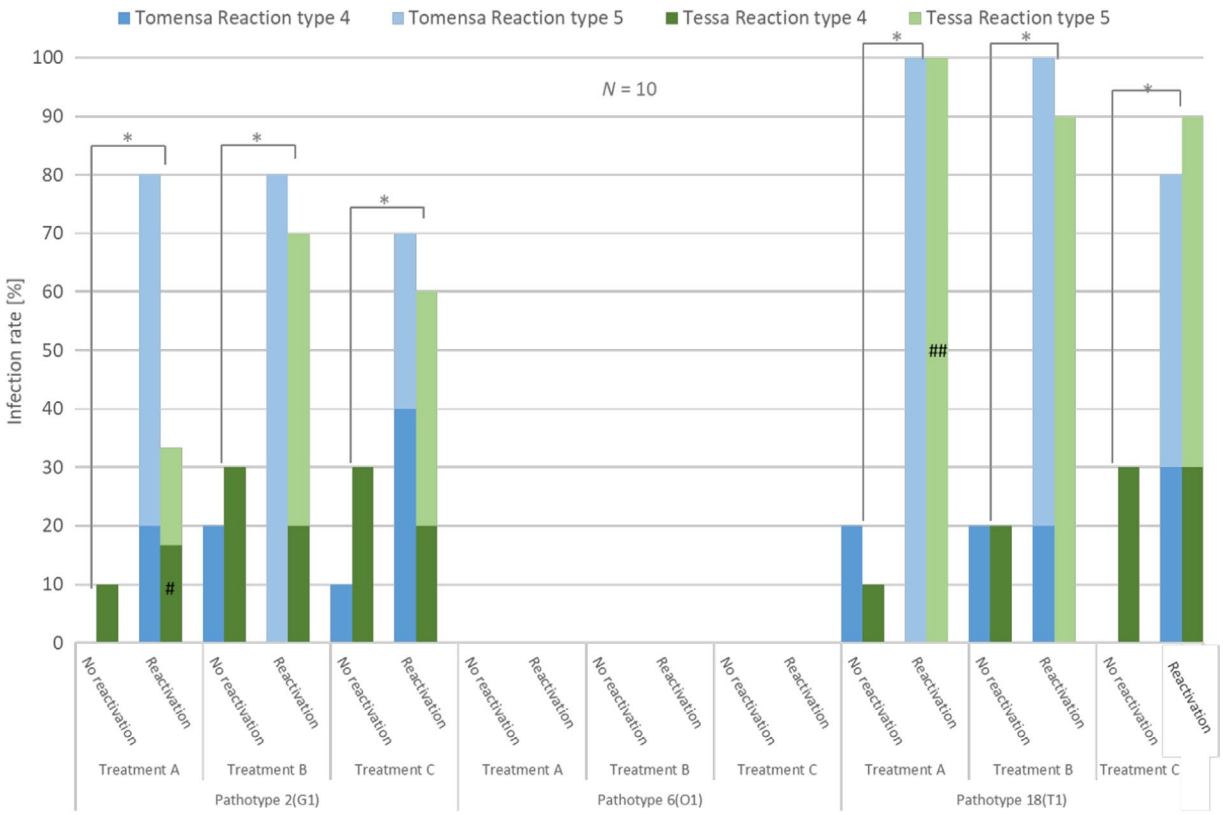

FIGURE 3 Percentage of infected eye plugs after a Spieckermann bioassay with differently treated composts (reactivation) compared with untreated control (no reactivation). Treatment A, 4 months of reactivation; treatment B, 3 months of reactivation; treatment C, 2 months of reactivation. Reaction type 4, slightly infected; reaction type 5, highly infected. Ten tubers per cultivar and treatment were tested and used for infection rate calculation, except for # with $N=6$ and ## with $N=3$ (seven tubers did not germinate). * Significant difference between groups, tested with a χ^2 test at a significance level of 0.05.

and 18(T1) for all reactivation procedures. Pathotype 6(O1) showed no increase in infection levels after reactivation (Figure 3).

Only a small proportion of the eye plugs inoculated with the untreated compost of pathotype 2(G1) scored 4, meaning that some resting spores were observed in the plant tissue but no wart formation. All three treatments used in the reactivation procedures (A–C) showed a significantly positive effect on the infectivity of the pathotype 2(G1) compost compared with the untreated control (Figure 3).

Although we counted the highest number of viable resting spores microscopically, no signs of infection could be detected on any of the eye plugs inoculated with the pathotype 6(O1) compost for either the untreated control or any of the reactivated samples.

Compost of pathotype 18(T1) infected 10%–20% of the eye plugs in the untreated control showing score 4 symptoms without wart formation. After reactivation, the number of infected eye plugs increased in all treatments tested. Treatment B had the highest number of wart-producing eye plugs with 80% for Tomensa and 90% for Tessa. Summarizing the results of the different reactivation protocols, treatment B with a duration of 3 months seems to be the most effective. The chi-square test indicated that there was no significant difference between the treatment groups A and B, or between A and C. However, treatment B was found to be significantly more effective than treatment C. However, it should be noted that it did not work for all tested composts (P6(O1)).

There were minor differences in the infection success between the cultivars Tomensa and Tessa, with Tomensa producing more infected eye plugs than Tessa; however, this difference was not significant. The infection rates for the three non-reactivated control samples of pathotype P2 and pathotype P18 varied, which can be explained be the different time points of the bioassay and therefore development stages of the potato tubers.

We also tested the infectivity of spores extracted from the untreated soils in a bioassay using the SASA method to see if the results were comparable with those from the Spieckermann bioassays or if the SASA method could be an alternative to bioassays with soil samples, avoiding the need for reactivation (Table 2).

Only 10% of the eye plugs inoculated with pathotype 2(G1) showed a type 4 reaction. This means that resting spores were visible in the tissue, but no wart formation was seen. All other eye plugs showed defence necrosis or no reaction at all. No susceptible reactions (score 4 or 5)

TABLE 2 Evaluation of the SASA bioassay with resting spores from untreated composts of pathotypes 2(G1), 6(O1) and 18(T1). Percentage of infected eye plugs scored 4 (detection of resting sori) and 5 (wart formation) at 3 weeks after inoculation. Culture conditions: 4 days at 10°C (in the dark) followed by 17 days at 16°C (in the dark).

			Infection rate (%)	
Pathotype	Cultivar	Total number of eye plugs	Reaction type 4	Reaction type 5
2(G1)	Tomensa	20	10	0
6(O1)	Tomensa	20	0	0
18(T1)	Tomensa	20	0	0

were recorded for pathotypes 6(O1) and 18(T1). All eye plugs showed no infection or defence reactions (Table 2). These observations indicate that the results of the SASA bioassay are comparable with those of the Spieckermann test for the untreated soils.

4 | DISCUSSION

Assessing the infection potential of soils contaminated with *S. endobioticum* is an essential part of controlling the spread of potato wart disease. With the implementation of the Commission Implementing Regulation (EU) 2022/1195 in July 2022, soil-based bioassays have become even more important, as they are part of the process for revocation of the measures of demarcated fields. Although it is known that resting spores of *S. endobioticum* can persist in the soil for many decades (Obidiegwu et al., 2015), it is not known what factors break the state of dormancy that some spores appear to be in. These unknown factors could significantly affect the results of bioassays or might lead to false negative results. In the present study, it was shown that soil samples previously considered non-infectious or that showed low levels of infection in pre-tests could be reactivated with specific intervals of moistening and aeration. After reactivation, these soils were able to induce severe symptoms in many cases. Three different reactivation periods of 2, 3 and 4 months were tested (Table 1).

It must be mentioned that the compost of P2(G1), which was not infectious in the pre-test, showed few symptoms in the control variant for reactivation (Figure 3). The different results can be explained by the physiological stage of the potato tubers (seasonal effect), as the pre-test was carried out in late summer and the bioassays of the reactivation samples in late spring.

All treatments tested showed a positive effect on the number of infected eye plugs (composts P2(G1) and P18(T1)) when reactivation was generally possible. No infections were observed for compost of P6(O1), thus in this case the tested treatments showed no effect. The unsuccessful reactivation of compost P6(O1) is most likely not attributable to the pathotype, but rather to its age (14 years old).

A comparison of the treatments revealed that the reactivation treatment B with a 3-month period was more effective than treatment C (2 months). Treatment A was not significantly different from the other two treatments. It is known from the literature that the presence of oxygen is essential for the germination of resting spores in soils (Esmarch, 1928; Langerfeld, 1984). Stirring the soils increases oxygen availability in the composts and promotes germination of resting spores with release of zoospores for infection. It is also known from an early study that moistening soils promotes germination of resting spores. In this study, the best infection rates were obtained by moistening the soils for 15–18 days before starting the bioassay. The increased infectivity of the soils was observed for the following 6 days (Thiede & Wierling, 1960). Longer moistening intervals were tested in the present study and it is known from previous observations (data not shown) that enhanced infectivity can be maintained for a longer period if the soils are further moistened and stirred three times a week. The reactivation protocol presented in this study combines the beneficial effects of oxygen and water availability to promote germination and break the dormancy of resting spores. In the case of the SASA method, the availability of oxygen and water is also provided. However, the infection rates observed in this study were very low, suggesting that the conditions to induce germination in the SASA method do not have the same effect as the reactivation treatment. Therefore, the SASA method, as used in this study, cannot be used as an alternative to reactivation of soil samples.

To characterize the resting spores of the untreated inoculum, they were extracted by wet sieving and analysed microscopically. All three composts contained resting spores, which by definition must be characterized as viable. As shown in this study, a high number of resting spores meeting all definitions for a classification as viable may be detected in a soil sample (P6(O1)), but in bioassays the soil may not lead to infection even after reactivation. The number of viable resting spores counted per gram of soil did not explain the variability in infectivity. It can thus be observed that the assessment of vitality through the utilization of microscopy and the bioassay (even following reactivation) may yield disparate outcomes.

This could mean that microscopic differentiation between viable and non-viable resting spores is very difficult and carries the risk of a false positive result, as previously discussed by other authors (Hinrichs-Berger & Zegermacher, 2022). Conversely, the findings indicate that a bioassay may yield false negative results if the factors influencing the bioassay are not optimized. In addition to the resting spore germination, which is stimulated by reactivation, the seasonal effect, potato variety and tuber quality are also critical factors.

Furthermore, field samples typically exhibit a markedly lower spore concentration as used in this study, rendering the bioassay particularly challenging.

The experiments presented here only cover a small sample size and limited factors to break the dormancy of resting spores, i.e. aeration and humidification. It is plausible that other factors or longer periods of reactivation may prove effective in achieving a comparable outcome with respect to sample infectivity. Further experiments with soils from different sources and stored for different periods of time are needed to verify the results shown here and to determine these factors.

The present study, along with numerous discussions on the viability of resting spores within in the potato wart community, demonstrates the need for an improved methodology. Recently, Vossenberg et al. (2024) introduced a promising alternative in the form of an mRNA-based molecular test. Nevertheless, this method also requires further validation and comparison with a bioassay. The use of reactivated samples for the validation of the mRNA-based test can enhance the reliability of the comparison results derived from the bioassay.

5 | CONCLUSION

The results presented offer a significant improvement in the quality of diagnosis for the detection of vital potato wart resting spores in infested soils. The Commission Implementing Regulation (EU) 2022/1195 was published in 2022 and recommends soil-based bioassays (pot tests) to determine the viability of resting spores in infested soil samples (European Commission, Directorate-General for Health and Food Safety, 2022). These pot tests are part of the procedure for revocation or partial revocation of infested fields. To obtain more valid results, we recommend that the reactivation protocol presented in this study is used or another adapted protocol to account for the potential dormancy of resting spores. This procedure can help prevent false negative results in bioassays, which could lead to further dissemination of *S. endobioticum*. The reactivation protocol can be used for all types of soil-based bioassays such as pot tests or Spieckermann tests. The use of the current reactivation protocol in combination with a soil-based bioassay leads to an extended duration until results are available, but these results are more reliable. It has been shown that microscopy of isolated resting spores can indicate infectivity that cannot be confirmed by bioassays. To diagnose *S. endobioticum* and to assign its viability, a combination of different techniques is still required and the development of new tools is an important aspect of current and future potato wart disease research.

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