

An improved method to study *Phytophthora cinnamomi* Rands zoospores interactions with host

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Research Article

Keywords: *Phytophthora cinnammomi* Rands, zoospores, tomato, *Solanum Lycopersicon*, *Quercus* sp

Posted Date: March 29th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4125396/v1>

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Additional Declarations: No competing interests reported.

Abstract

The pathogen *Phytophthora cinnamomi* Rands (Pc) is one of the ten most widespread phytopathogens in the world causing crown rot, canker and root rot in multi-functional land plants. Pc has a disastrous impact on the surrounding environment and biodiversity of flora, fauna and soil. Pc infects more than 5,000 species, including *Quercus suber*, *Quercus ilex*, *Castanea sativa*, *Eucalyptus globulus*, *Persea americana* (avocado), *Zea mays* (maize) and *Solanum lycopersicon* (tomato). The efficient spread of Pc depends on the short survival of asexually motile zoospores (Zs), which move through water currents until they penetrate the host roots. Due to the complexity of the life cycle, the management of Zs in the laboratory has remained quite complex for decades. The production of Zs requires mimicking nature by following a complex protocol of circular steps of growth, drought and flooding. Similar to other pathogens, the artificial growth conditions induce a rapid loss of virulence, making it necessary to add additional steps of plant infection in between these other steps. In this work, the study of the survival of Zs under different conditions has allowed us to obtain a "ready-to-use" stable stock of Zs with a high capacity to infect the host by direct freezing in liquid nitrogen. Obtaining this stock prevents the loss of virulence of Pc between cultures, thus greatly simplifying the process of inoculating plants and studying Pc with their host.

1. Introduction

The oomycete *Phytophthora cinnamomi* Rands (Pc) is one of the most damaging phytopathogens described to date, infecting more than 5000 hosts among herbaceous, forest and non-forest species, causing destructive diseases and massive ecological and economic losses¹⁻⁴. Environmental factors associated with current global warming are contributing to the incidence of Pc^{1,5-10}. Recent studies of the global distribution of Pc have included world-scale data collected from natural ecosystems. While extreme drying and warming temperatures are not favorable for *Phytophthora* species and various other subspecies^{11,12}, Pc is becoming virulent in colder regions not previously affected by this oomycete^{1,9,11,13,14}. New methods are needed to facilitate researchers' studies of this oomycete to reduce its incidence in the coming decades.

Pc was first described as the causal agent of stripe canker of cinnamon trees *Cinnamomum burmanii* in Sumatra, giving the pathogen the name 'oomycete' to the pathogen¹⁵, and been associated with sporadic mortality of Proteaceae species^{16,17}. Pc has also been associated with *Phytophthora cambivora* infections in chestnuts and other hosts since 1966¹⁸, and in American chestnut (*Castanea dentata*) before 1910^{19,20}. More recently, however, the origin of the Pc species is not in Sumatra²¹ and may be Papua New Guinea²². More recently, its spread to other parts of eastern and southern Asia, New Guinea and Australia has been considered^{22,23}.

Pc has a high negative economic impact, including the death of native plants worldwide; environmental impacts include changes in vegetation communities; and we also see a reduction in botanical diversity

and food availability, which subsequently affects bird and small mammal populations²⁴. In the USA, Pc was detected in a commercial blueberry in Arkansas in 1978²⁵. Subsequently, Pc has been widely documented on American white oak (*Quercus alba*) in the Appalachian Mountains²⁶, and has also caused extensive damage to avocado trees in California²⁷. In southern regions of Europe, such as Portugal, Spain, France and Italy, Pc attacks native chestnut trees (*Castanea sativa*), mature cork oaks (*Quercus suber*) and holm oaks (*Quercus ilex*), among other species^{20,28–30}, sometimes causing significant market impact³¹. For example, the production of acorns from holm oaks in meadows, is the main feed for Spanish acorn-fed ham, highlighting the presumed impact of Pc into this market^{28,32–34}. With rising temperatures due to global warming, Pc is specifically moving into central, colder European regions in Germany and Poland, where Pc has recently been detected in the commercial sector. *Vaccinium corymbosum* (blueberry) nursery plants⁹. Therefore, the current situation makes the fight against PC a priority^{1,2,35,36}.

The most virulent form of Pc is the asexual biflagellate zoospores (Zs), which have a high capacity for dispersal and survival in water^{1,4,37–39}. In spring, and during the temperate and high humidity seasons, after short or long periods of drought, heavy rainfall can occur, sometimes causing soil waterlogging. Under these favourable conditions, Zs produced in sporangia move through flagella, in both forward and backward directions, swimming at high speeds, up to 250 μm/s before contact with the host plant^{37,40–42}, showing a high capacity to be attracted by root chemicals. Zs bind to the roots via annexin proteins and a complex, well-known mechanism involving a biofilm formation^{37,39,43,44}. Once the Zs bind to the root, the process of colonisation continues until produce the necrosis of roots, ultimately affecting the water supply to the aerial parts^{36,44}.

Despite the large number of research articles dealing with Pc^{4,45}, and the increasing incidence of this phytopathogen^{1,26,31,46}, the protocols developed in laboratories for handling this complex oomycete and Zs, have remained virtually unchanged since 1965^{6,24,47–52}. A high variability in laboratory conditions for obtaining Zs from Pc mycelium and in plant inoculations conditions was found. The two main protocols used involve inoculations of cut leaves, roots, shoots or explants with mycelial fragments or culture filtrates^{53–55}. Assays on cut plants are of particular commercial importance because of the high incidence of Pc in flowering ornamental plants^{53,56–58}. For the second type of protocols, assays were performed using freshly obtained Zs, inoculating plant seedlings, or plantlets previously obtained through months of micropropagation of forest and non-forest species^{29,59–63}. In all cases the protocols were very time consuming to obtain virulent Zs.

Within this study we have obtained a simple methodology for conserving stocks of Zs with high capacity to infect the plant host, allowing researchers to perform less time-consuming that preserve phytopathogen virulence. The results of this work have allowed us to add two additional steps to current protocols, facilitating the use of Zs stocks. The ability of Zs to infect the plants was tested in tomato, a poorly studied host of Pc^{64,65}, confirming us that tomato is a very useful host for making future studies.

2. Results

2.1. The protocol for the acquisition of Zs stocks

An extensive bibliographic study was first carried out to determine the state of the art. Based on previous studies, an agreed global protocol, shown in Fig. 1 (steps 1 to 8), was resumed. Two additional new steps to the protocol (Fig. 1) are included in this work, with a new step of rapid freezing of Zs, in liquid nitrogen, with subsequent storage at -80°C (Fig. 1, steps 7 and 8). The addition of these two new steps allowed us to obtain Zs as described in detail below. These two steps allowed us to obtain Zs supplies for ongoing host PC studies. Once the fresh Zs were obtained based on previously published work^{6,48,50–52,62,66} (summary of the protocol is shown in Fig. 1, see Section 5). The development of Pc cultures was followed by taking snapshots at different stages. A detail of fresh mature sporangia is shown at Fig. 1B, and chlamydospore and hyphal swelling after the step of sporangia induction with mineral salt solution is shown in Fig. 1C. A snapshot of the free living Zs obtained during the process is shown in Fig. 1E. The results and morphology are according to previous works describing Pc under similar growth conditions^{67,68}. They are also consistent to the identification of *P. cinnamomi* by the European and Mediterranean Plant Protection Organisation (OEPP/EPPO, Bulletin 34, 155–157, 2004, at PM 7/26).

2.2. Survival of fresh Zs measured by absorbance

The MTT method was used (see Section 5), which allowed us to measure Zs at concentrations as low as 10^6 Zs/ml, correlating with the results obtained by counting Zs dilutions into the Neubauer chamber (Fig. 2A). The MTT method proved very useful for measuring the survival of Zs at room temperature (considering 23°C), along the time. The survival of Zs remained quite stable up to 24 h hours, with a tendency of decreasing in survival, but with no statistical differences between the times measured (Fig. 2B). MTT was also useful to measure several samples and triplicates on the same assay using an absorbance plate reader (see section 5), and also to avoid the residual error obtained by counting Zs that do not turn to cyst during the vortexing in the Neubauer chamber. Both methods were used throughout this work.

2.3. Survival of fresh Zs measured by fluorescence

The ability of Zs to disperse under water and to adhere to host roots is directly dependent on their motility^{37,39,43,44,69}. Therefore, cell viability of sporangia and Zs in culture was monitored as shown in Fig. 3A (bright image) and by fluorescence signal (Fig. 3B, see section 5). As a control for live and dead cells, porcine spermatozoa were added to the medium (see section 5), where Zs are indicated by arrows (Fig. 3C and Fig. 3D). The viability of the Zs is shown at Fig. 3E and Fig. 3F; the F inset corresponds to a Zs scheme, which helps to recognize them in the snapshots. The data obtained by SQS[®] fluorescence (Fig. 3G, see section 5), showed a slight increase in the percentage of decayed cells after 24 h, related to the decay observed by MTT (Fig. 2B), attributed to residual mitochondrial activity still present on the red cells detected by SQS[®]. A good correlation was found between the green fluorescence signal and the viability and motility of Zs was found. All red fluorescing Zs showed motility along the assays, indicating

that this method allows to determination of Zs mobility and survival, similarl to estimates made with porcine spermatozoa. A comparison between the counting methods was made and it is shown in Figure S1A.

2.4. Survival and recovery of Zs to freezing point

To obtain stable stocks of Zs, we studied the best conditions for obtaining non-fresh Zs with the highest possible recovery capacity. The survival of NF Zs was inversely proportional to glycerol concentrations (from 0 to 30%), with the highest survival rate obtained in the absence of glycerol (Fig. 4A). A kinetic study up to 60 min at 4°C confirmed the decay of Zs in the presence of glycerol, which was due to glycerol and not to freezing or ice treatments, both on non-fresh Zs (Supplementary Figure S2A) and on fresh Zs (Supplementary Figure S2B).

The recovery of survival of Zs after freezing was determined by MTT (Fig. 4B). Liquid nitrogen and sequential freezing (RF) resulted in 80% survival, while the other methods reduced survival to less than 60%. Freezing of fresh Zs in a water solution, in the absence of glycerol, followed by freezing in liquid nitrogen, was selected as the best and quickest method to conserve Zs stocks.

In addition, between all temperatures tested (4, 22, 24 and 37°C), for defrosting Zs. The results in Fig. 4C, show that the Zs survived in higher percentage to defrosting at 37°C with a small but statistically significant survival at 4°C. However, defrosting Zs at 4°C (in ice) was considered the easier and faster way to defrost Zs in the laboratory, being a temperature that is less time and energy consuming, than 37°C which requires a water bath or thermoblock. Survival at -80°C of Zs stocks was measured by MTT from seven days to one year, showing a slight decrease in survival of 2.86% (± 0.5) per month (Fig. 4D).

2.5. Non-fresh Zs stocks retain the ability to infect plants after freezing.

To determine whether Zs stocks retain the original capacity to infect plants, we established a rapid method for inoculating tomato seedlings with Pc's Zs. Tomato has previously been described as a host for Pc ^{64,65,70,71} allowing us to rapidly determine the ability of Zs to infect a plant. Fourteen day old tomato seedlings were inoculated on soil with Zs stocks of previously frozen Zs. The Zs stocks produced high levels of root necrosis and secondary effects of chlorosis on primordial leaves after only 4 days of inoculation compared to water-treated controls (Fig. 5A and 5C correspond to controls and Fig. 5B and D to inoculated). Seedling necrosis was confirmed by trypan blue staining (Fig. 5E).

2.6. Measurement of Pc levels in plants by qRT-PCR

We compared the capacity to infect plants between fresh Zs and Zs stocks, by measuring Pc burden by qRT-PCR (see Section 5). A little increase on disease severity symptoms was observed on plants inoculated with Zs stocks (Fig. 5F), related to fresh Zs (supplemental Figure S3A and C for controls, and Figure S3B and D for inoculated plants). Trypan blue on seedlings confirmed necrosis on plants inoculated with fresh Zs, and Zs stocks (supplemental Figure S3E).

The oomycete biomass was quantified by qRT-PCR (see Section 5), on inoculated tissues with fresh Zs and Zs stocks, relating the oomycete biomass to the ng of plant tissue. A tomato *actin* housekeeping gene, and a *β -tubulin* gene from Pc were designed, and the efficiencies and melting curves for both primers were determined and confirmed before the analysis (see Section 5, supplementary Fig. 4A and B for *β -tubulin* gene and supplementary Fig. 4C and D for *actin* gene). Although slight differences were found on plant disease symptoms (Fig. 5F), no statistical significances were found into oomycete burden between the two kinds of inoculations (Fig. 5G).

A qRT-PCR was performed also to amplify mycelium recovered from inoculated tomato seedlings (see Section 5). Representative photos of mycelium growth of Pc on plates, obtained from the roots, after seven days, is shown at supplementary Figure S5A and Figure S5B. A photo of a current stock of Pc mycelium is shown at supplementary Figure S5C. Staining of mycelium, with TB, recovered from roots is shown in Figure S5D. Images were compared to current mycelium and with previous description of Pc hypha confirming the Pc identity (Ho and Zentmyer, 1977; Downer, Menge and Pond, 2001). The melting curve obtained by qRT-PCR, from amplification performed using Pc mycelium obtained from plate, or mycelium recovered from tomato roots, were identical, confirming a unique amplicon, showing that Pc mycelium recovered from tomato corresponds to Pc (supplementary Figure S5E and Figure S5F). Oomycete biomass quantified in ng is shown in supplementary Figure S5G.

3. Discussion

As part of this study, we carried out a biochemical and physiological study to determine the optimal conditions for obtaining live and virulent Zs in the laboratory. During this process, we found that each step required specific conditions that were in some way similar or related to the natural conditions under which Pc develops in nature. The four factors we found to affect Pc growth were the plant content of the growth medium, light and darkness, the solid or liquid phase of the medium, and temperature. In the first phase of mycelial growth, the high concentration of plant nutrients was essential to improve hyphal development. Filtering the growth medium completely inhibited mycelial growth, suggesting that high levels of specific plant nutrients favour Pc survival and growth on plates. In line with this, it was found that a specific soil composition strongly influenced Pc survival^{6,74}. V8 juice, the currently commercialised juice for human consumption, is the current medium used for Pc growth and contains eight different plant tissues. We observed that the growth of Pc in potato dextrose agar (PDA), with only potato derived nutrients, was much lower than in V8, which contains eight different plants. We conclude that Pc in the laboratory needs a high availability of plant nutrients in the growth medium, probably this observation is in line with the necrotic activity of Pc, performed by enzymes at different stages of development.^{11,14,37,41,43,44,69,75}. A well-known example is the necrosis caused by alpha-cinnamomin during the early stages of vascular colonisation.^{30,65,76–78}. Tests comparing Zs inoculations with mycelium in *Quercus ilex* showed that the increase in infection levels of Pc correlated with lower lignin and higher nutrient levels derived from necrotic tissue, suggesting that nutrient availability may contribute to the subsequent formation of chlamydospores.¹¹. In another paper, the increase in Pc growth was

correlated with the development of detritivores in very rich soil areas.^{79,80} Zs was able to prolong its survival time by colonising dead plant tissue, but the result varied depending on the host⁸¹. It may also be interesting to investigate how plant nutrients contribute to Pc virulence on the artificial medium used in the laboratory.

The second important factor to consider for growing Pc was the state of the medium, solid or liquid. The first steps for mycelium growth were carried out on a solid surface, keeping the mycelium on a filter paper disc, similar to the solid surfaces of roots where the mycelium is currently growing. The same steps for mycelium growth performed on liquid media inhibited mycelium development. In contrast, the steps to obtain Zs required liquid media, which inhibited Zs production on solid media (data not shown). These conditions corresponded to the natural growth conditions of Zs in water. The third factor considered important was light. Specifically, at the sporangia induction stage, some previous work highlighted the importance of light for sporangia induction, while others did not.^{47,82,83} In this study, the initial stages of Pc growth were carried out in darkness, in line with natural conditions in places where Pc grows naturally below ground. It was observed that low light levels in the forest contribute to the development of Pc.⁸⁴ Under our conditions, we observed that the presence of light was absolutely essential, especially for the induction of sporangia; these observations were confirmed by trypan blue staining during the process. The presence of light during the transfer of the paper disc with mycelium from the V8 solid plate to a liquid medium was necessary for the induction of sporangia. Continuous shaking was also essential. The absence of light or shaking significantly reduced sporangia production and later Z production. Some works^{85–87} determined that sporangium production using light periods alternated with dark steps increased growth of Pc. Some authors have observed that Pc can survive as a saprophyte on dead plant tissue.⁸¹, and we observed that the optimal conditions for Pc development overlap with the artificial greenhouse⁸⁶, and contribute to Pc occurrence. In addition, the abundance of Pc on Mediterranean forest soils has been associated with a predictable spatial structure related to the highly defoliated oak trees and light exposure in forests⁴⁶, but comparison with laboratory conditions is not meaningful. As contrasting conclusions have been reached about the influence of light on Pc sporangiogenesis, the influence of light remains to be properly investigated. The development of sporangia takes place in absence of light belowground under optimal conditions in nature⁸³.

The last important factor during the growth of Pc to obtain Zs was the temperature. The induction of the development of Zs in the sporangia was also carried out for the later step, adapting previously published methods^{42,47,52,88,89}. The highest efficiency for obtaining fresh Zs from sporangia was obtained by maintaining sporangia cultures in darkness in a cold water bath (see methods section 5). Again, the assays performed at room temperature or with light yielded much lower numbers of fresh Zs. These cold water conditions contribute in nature, especially in spring and autumn, to the development of Zs in the sporangia before Zs are released into above or below ground water.

Having determined the optimum conditions for growing Pc and obtaining fresh Zs, we studied the survival of Zs under different freezing and thawing conditions. Having determined the optimum

conditions for growing Pc and obtaining fresh Zs, we studied the survival of Zs under different freezing and thawing conditions. The MTT method, currently used for protozoa⁹⁰ has been used to quantify Pc oospores but never for Zs⁶. In the course of this work, we found this method very useful, especially when a large number of samples and replicates need to be counted, because MTT allows the measurement of Zs in a 96-well plate reader. We also measured the motility of Zs using SQS®, using two combined fluorescent dyes and in the presence of sperm cells. This method proved useful for determining the size of Zs and its survival under different conditions. Zs survived for 24 hours at room temperature (23°C), which is significantly longer than the 4 hours previously observed in other works.^{91,92}, this is probably due to the different conditions used to obtain Zs. Previous work measuring the persistence of Pc DNA and RNA in different types of soil showed that Pc RNA, a real-time marker of the metabolic activity of living Pc, remained measurable up to one day after soil inoculation, while DNA was still present at later stages of detection.⁸. Although the two studies cannot be compared because there are other spores of Pc than Zs in the soil, it must be considered that the survival of Zs in the laboratory and in nature may be longer than originally thought.

One of the more surprising results of this study was the observation of the high survival of non-fresh Zs under freezing and in the absence of cryoprotectants, particularly in the absence of glycerol. Pc is highly sensitive to extreme, but not warm, temperatures in nature^{5,6,9,31,32,89,93,94}. The survival of Zs in liquid nitrogen, in the absence of antifreeze, was explained by the discovery that the glycerol medium was toxic to Zs, especially to fresh Zs, being higher than to non-fresh ones (Figure S2). This result was surprising considering previous studies on Pc^{47,95–98}. Cryopreservation of *Phytophthora* sp. cultures in a liquid nitrogen atmosphere avoids the formation of ice crystals on tissues by prior immersion of samples in a cryoprotectant solution such as dimethyl sulfoxide (DMSO), 1,2-propanediol, glycerol or skim milk/glycerol.^{99,100}. In fact, the major collections saved for this specie use that method^{97,98}. Control of the freezing rate is critical to optimise the ice nucleation process and minimise cell damage. Because rapid freezing leads to intracellular ice nucleation^{101,102}, which is lethal to cells, we performed various tests to optimise the freezing method of fresh Zs. It was observed that slower freezing rates resulted in a higher incidence of cell death, probably due to the excessive 'pickling' effects resulting from long exposure to hypertonic solutions. Some similar results were observed with green macroalgae solutions of *Enteromorpha intestinalis*.¹⁰³. Our results suggest that intracellular ice formation in Zs may not occur or may not affect the recovery of Zs after freezing. Further experiments will be necessary to confirm this hypothesis, and the cell wall composition of Zs should be analysed to determine the effects of liquid nitrogen, which allows survival without cryoprotectants, as we have observed.

Pc has been described to induce changes in the mineral content of infected plant tissues, and these changes are quite different between susceptible and tolerant individuals^{104–108}. Specifically, in *Eucalyptus*, a decrease in metal content was observed in a susceptible host compared to a tolerant host, suggesting that Pc absorbs them from the plants.¹⁰⁵. It is plausible that the same capacity observed in Pc for uptake of plant nutrients, salts and minerals from the host may allow Zs to uptake salts and minerals from water, protecting Zs from water ice crystal formation during freezing and explaining the

high survival capacity of Zs in the absence of cryoprotectants. Again, however, this hypothesis should be confirmed experimentally.

The Zs stocks obtained in this work retained the ability to infect the plant host for long periods after freezing and thawing. Further analyses are underway to characterise the response of tomato to Pc at the physiological and molecular level.

4. Conclusions

To our knowledge, this is the first published study of obtaining stocks of Zs from Pc by freezing in liquid nitrogen and storing at -80°C in the absence of cryoprotectants. Frozen stocks of Zs maintained viability to infect plants similar to fresh stocks. Our research results will be useful for researchers to perform less time-consuming assays, saving money and avoiding the problems associated with loss of Pc virulence when growing on plates. MTT and SQS® staining methods will allow the survival of Zs to be monitored. We found that the survival capacity of Zs was higher than we had initially expected. Finally, we developed a protocol to study the interaction between Pc and tomato, a poorly understood plant-pathogen interaction.

5. Materials and Methods

5.1. Biological material

The tomato seeds (*Solanum Lycopersicum* L., var. *marmande*) were provided by Ramiro Arnedo S.A, La Rioja, Spain. The seeds were stored and maintained at 4°C until use. *Phytophthora cinnamomi* Rands (Pc) was kindly supplied by Beatriz Cuenca (TRAGSA), obtained from Solla's team from the Faculty of Forestry, Institute for Dehesa Research (INDEHESA), Universidad de Extremadura, Plasencia, Spain and obtained from the Center for Research and technology of Extremadura (CICYTEX), located in Mérida, Spain.

5.2. Detailed Protocol for obtaining Zs stock

The protocol was obtained from the specified bibliography^{5,51,59,66,109}, with little modifications. *Phytophthora cinnamomi* Rands (Pc) plate cultures, obtained from provider on a potato dextrose agar medium (PDA, Sigma-Aldrich, St. Louis MO, USA), were transferred to paper discs and grown on a V8 agar medium and were grown for 12–30 days under controlled conditions included 50% humidity (v/v), a temperature of 24°C during the day and 18°C during the night on plates protected from light with aluminum foil.

Pc mycelium obtained was transferred to potato dextrose agar medium plates (PDA, Sigma-Aldrich, St. Louis MO, USA), prepared with 39 g/L and autoclaved at 120° C for 20 min, and incubated at 24°C in darkness for three weeks within a Memmert incubator (INB 500, Madrid, Spain). For obtaining Pc subcultures a piece of 1 cm × 1 cm of mycelium was transferred onto a previously 1-mL-watered

Miracloth disk (Miracloth EMD Millipore Corp, Darmstadt, Germany) using a plate with a freshly prepared medium with 100 mL/L of fresh V8 juice (Campbell Soup Company, Camden, NJ, USA), 2 g/L of calcium carbonate (CaCO_3 , Sigma-Aldrich, USA) and 0.02 g of β -sitosterol dissolved in 5 mL of ethanol (Sigma-Aldrich, St. Louis, MO, USA), and 15 g/L of Difco agar. The pH was adjusted with sodium hydroxide (NaOH) or potassium hydroxide (KOH, Merck, Darmstadt, Germany) to 6.5, with 0.1 M HEPES (Sigma-Aldrich, St. Louis, MO, USA) ^{59,60}. Plates were closed then with Bemis™ Parafilm™ (St. Louis, Missouri, USA) and incubated in darkness at 24°C for 14 days ⁵⁹.

The diameter of mycelium as well as the high of it, was measured been between 0.5-0.9cm of high and 7–9 cm of diameter accordingly with previous works ¹¹⁰. To induce sporangia, the Miracloth disc was aseptically transferred to a 1-L Erlenmeyer flask, containing 100 mL of a 10% clarified V8 liquid medium. The medium was then filtered with one slide filter paper, and the filtrate was diluted 10 times with distilled water and autoclaved, following previous work¹¹¹. The Erlenmeyer containing the Miracloth disc, was incubated in an orbital shaker (100 rpm) at 24°C, under fluorescence light, for 48 h to induce sporangial production. These conditions were selected specifically, after the revision of different protocols ^{43,47,59,111,112}. The obtention of sporangia was confirmed via trypan blue (TB) staining via visualization under a light microscope (see TB details below). During this step we observed that the use of smaller Erlenmeyer flask (0.5L) reduced the oxygen availability for the Pc, thus decreasing survival. The centrifugation of the clarified medium also diminished the final yield of the process. The same step in the absence of light rendered no or significantly less available alive sporangia.

A second step of sporangia induction was subsequently performed based on previous work ⁴⁷, the clarified V8 liquid medium was decanted off, and the Miracloth disc was transferred with sterilized forceps onto a 9-cm sterilized plastic Petri plate (Fisher Scientific SL, Madrid, Spain). The disc was then washed by carefully adding 20 mL of a previously sterilized and fresh mineral salt solution (MSS). The MSS medium was prepared into an Erlenmeyer with 0.01 M of calcium nitrate (CaN_2O_6), 0.005 M of potassium nitrate (KNO_3) and 0.004 M of magnesium sulphate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), all chemicals were obtained from Sigma-Aldrich, St. Louis, MO, USA. The MSS was autoclaved and then supplemented with 1 mL/L of a chelated iron solution (CIS). To prepare CIS, 13.05 g/L of Ethylene-dinitrile-acetic acid (EDTA, Sigma-Aldrich, St. Louis, MO, USA) was mixed with 7.5 g/L of potassium hydroxide (KOH, Merck, Darmstadt, Germany), 24.9 g/L of iron sulphate (II), and heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 99%, both from Sigma-Aldrich, St. Louis, MO, USA). The CIS solution was filtered using a 0.22- μm diameter Millipore filter (Sigma-Aldrich, USA) before adding to MSS. 3MM (Micropore™, Neuss, Germany), and Bemis™ Parafilm™ (St. Louis, Missouri, USA) were used to avoid losing the liquid medium during orbital shaking at 100rpm for 24h at 24°C with fluorescence light. The obtention of MSS and CIS was performed following ⁴⁷. MSS and CIS mediums were found essential for sporangia induction. Again, variations of this step, including conducting the process in the absence of light, or transferring the disc to 1L Erlenmeyer using 100 mL of the medium, rendered, much lower numbers of final Zs (data not shown).

For the induction of Zs, the disc containing sporangia was transferred carefully with forceps to a 50-mL Falcon tube (Corning Inc, Corning, NY, USA) containing 20 mL of sterile water type I (available for molecular biology, water supplier Aristerra 2011 S.L., Pamplona, Spain), at 4°C. The tube was then placed in an orbital shaker and kept at 100 rpm at 4°C for 1.5 h and in the absence of light. The resulting solution (step six at Fig. 1A) was filtered through three slides of a sterilized gauze pad (Hartmann S.A., Heidenheim, Germany); this step was based specifically on ¹⁰⁹.

The occurrence of Zs releasing from sporangia was confirmed using the light microscope and via TB staining (see TB details below). The number of Zs per ml after release occurs, was measured for each assay, at Neubauer chamber (Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany), the quantification of Zs rendered always between 1.8 to 5 x 10⁸ zoospores/ml. A more restrictive filtering with Whatman filter paper for a 110-mm size pore, (GE Healthcare life science, Amersham, UK) rendered much lower quantity of Zs (data not shown). The number of sporangia per unit of area (mm²), was measured on each assay under the microscope, been between 5–10 sporangia/mm².

After previously described procedures for zoospore acquisition, the long-term maintenance and conservation of *Phytophthora cinnamomi* Rands mycelium, was made by taking 1x1 cm squares of mycelium from plates where Zs were obtained. These squares are carefully placed into 1.5 ml Safe-lock Eppendorf tubes (Eppendorf A.G, Hamburg, Germany), rapidly frozen in liquid nitrogen, and subsequently stored at -80°C. To initiate mycelium reactivation, the tubes are gently thawed on ice and the mycelium is transferred onto fresh Petri dishes containing V8 agar. The cultures are then maintained in darkness at 24°C for 7 days before start a new process for Zs obtention.

5.3. MTT procedure to measure the viability of Zs

Because fresh Zs move extremely fast, currently, for counting, the Zs needs to be fixed to avoid their mobility. This process currently involves killing the cells, or induction of cysts, by centrifugation or vortex ^{6,37,44,72}. At this point we observed that zoospores turned to cyst after shaking and remained alive, however, we still observed that not all of them turned to cyst and several remains alive moving at Neubauer chamber during counting.

Because that, and to ensuring a good correlation between the counting of alive cells and the final number of Zs available for inoculation assays, we used one additional method for counting Zs. A colorimetric assay, based on the absorbance signal produced by formazan, during the mitochondrial oxidative activity produced in the presence of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich, Rockford, IL, USA) production ^{90,113}. This reaction is produced only in viable cells ¹¹⁴.

This method is commonly used to also measure the cell viability in various species of protozoa, including the human pathogen *Leishmania sp.* parasite ¹¹⁵ and has been used to measure viability of *Phytophthora* oospores ⁶. Briefly, 100 µL of MTT at 0.5 mg/mL were added to 900 µL solution of Zs, incubating the mixture at 37°C for 2 h. Next, 100 µL of 10% Sodium dodecyl sulphate (SDS, Sigma-

Aldrich, Rockford, IL, USA) in distilled water was added and the mixture was kept at 37°C for 2 h. The absorbance of the solution was measured at 600 nm using a spectrophotometer (Hach DR 2000, Hach Co. Loveland, CO, USA). Controls were performed in the same manner but using water instead of Zs. The straight pattern showing the survival of spores at different dilutions measured via MTT is shown at supplemental Figure S1A.

5.4. SQS® Fluorescence procedure to detect the viability of Zs

Viability of Zs was conducted using the Swine Sperm Viability (SQS®) & Morphology Kit (Arquimea, S.L., Madrid, Spain). This methodology is currently used for determining the survival and detect the motility of different sperm cells¹¹⁶. The kit employs a dual staining approach that enables for the differentiation of live and dead sperm cells and flagella, as well as organ abnormalities¹¹⁷. To determine correlation between motility and viability of fresh Zs, the Zs were stained using this fluorescence dye, as controls for the alive and dead cells, pig spermatozooids were added at the medium with Zs. This method is based on the green or red fluorescence signal emission produced by live or dead cells, respectively, in real-time.

Briefly, a mixture of 1 µL of Duroc sperm cells, used for reference of size and survival, and 2 µL of a Zs solution (2×10^8 Zs/mL) was prepared and added simultaneously to the SQS® plate. Two distinct methods were utilized for analyzing and detecting the fluorescence signal. The first method involved an SQS2 semen analysis system machine (kindly provided by ARQUIMEA S.L., Madrid, Spain), coupled with a fluorescence microscopy-based sperm counter, integrated with a high-resolution CMOS camera with a high-power blue LED computer vision system. This system was used to evaluate the total number of cells taking snapshots at different times, using at least five photos per time. A SQS2 red led signal corresponded to Em617 nm/Ex536 nm, and a green led signal to Em526 nm/Ex500 nm. A second detection was performed by using a fluorescence stereomicroscope A292/21 Microscopy iScope IS.3153-PLFi/6 with Fluorescence–IS.3153-PLi/6,nEWF 10×/22; Plan Fluarex PLFi, 4×, 10×, 20×, 40×, 60×; 100× oil lenses that included blue, green, UV-DAPI, and red fluorescence filters; and a charge-coupled device (CCD) digital cooled camera A292/21 Euromex 20 MP USB 3.0 with a 1-inch CMOS sensor (Microsercon SLU, Madrid, Spain). The green led signal corresponded to Em570 nm/Ex510–550 nm, and the red led signal to Em690 nm/Ex620 nm. At least 10 photographs were captured per condition. Image processing and quantification of trypan blue were performed using the ImageJ® program and plug-in tools^{118,119} as previously¹¹⁸ (Figure S1 B to D).

5.5. Frosting and thawing of fresh Zs

The most frequent method for conserving microbial stocks for long periods of time within laboratories is glycerol imbibition prior to a fast freezing into liquid nitrogen afterwards^{99,120–123}. Cryopreservation methods were tested based on a previous studies made with liquid nitrogen^{100,124–130} and they also included the addition of glycerol as a cryoprotectant^{131,132}.

To study the direct effect of the glycerol on the Zs survival, two kinds of assays were performed with Zs stocks (non-fresh Zs or NF Zs) and fresh (F) Zs. Aliquots of 250 µL of Zs stocks were defrosted in ice (4°C), and mixed with 250µL of glycerol solutions in water, at final percentages of 0, 5, 25, and 50%. The survival of Zs was measured at 0, 30, and 60 minutes using MTT. In a second kind of assay, 250 µL aliquots of F Zs were treated as previously and were immediately placed into liquid nitrogen, before defrosting in ice. The recovery capacity was measured at times 0, 30 and 60 minutes, by MTT.

To determine the best method for frosting Zs, they were immersed in water, in the absence of glycerol, and frozen under different conditions. The conditions used were based on a previous work performed on *Phytophthora species hibernalis, infestans, lateralis and nicotianae*.¹³¹ A direct immersion into liquid nitrogen (– 196°C). A sequence ramp treatment at – 20°C and at – 80°C. A one degree decreasing ramp of freezing using the Nalgene® Mr Frosty™ Cryocontainer (C1562, Sigma-Aldrich, Rockford, IL, USA), used to achieve freezing with a speed of 1°C/min following the manufacturer's instructions, briefly, the container was filled with isopropyl alcohol before the samples were placed in a vial holder, with the assembly then being moved to a – 80°C freezer overnight. In addition, a ramp freezing was used for getting sequential water baths, performed over periods of two hours at 23°C, 18°C, 4°C, with a final freezing for 18h at – 20°C with a final step at – 80°C (RF). For determining the survival of Zs after different conditions of freezing, Zs were slowly thawed on ice, as well as thawed in water baths at 22, 24, and 37°C for 30 min. The recovery and survival rates for the Zs stocks after freezing were measured for twelve months. In all methods, 1.5 mL Safe-lock Eppendorf tubes (Eppendorf A.G, Hamburg, Germany) were used. The tubes were then stored in a freezer at – 80°C until evaluation of the viability of the Zs.

5.6. Plant growth and plant inoculation conditions

Tomato seedlings were grown following the methods of¹³³ with little modifications. Briefly, the seeds were germinated on soil twelve well microplates of twelve pots (Deltalab S.L, Barcelona, Spain), into a sterile mixture of Projar Professional Seed Pro 5050, NPK 14–16 – 18 plus microelements (Commercial Projar S.A., Valencia, Spain) substrate and vermiculite (3:1), with a granulometry measurement of between 0–3 mm (Commercial Projar S.A., Valencia, Spain), with 10 seeds per pot and twelve pots per plate, with a total of 120 seedlings per plate. In all assays, at least 120 tomato seedlings were used for each assay and condition. The plants were irrigated with a regular water supply and grown for 18 days in an Aralab® digital chamber (Lisbon, Portugal). Controlled conditions included 50% humidity (v/v), a temperature of 24°C during the day and 18°C during the night, with a 16 h light/8 h dark photoperiod and light intensity of 150 µE·m⁻² per second for all experiments; the photoperiods during the day were fixed and mimicked a spring day based on a meteorology statal agency (AEMET) profile, Madrid, Spain.

For plant inoculations, after fourteen days of growth seedlings were inoculated with a 500-µL of a sterilized distilled water suspension of fresh or non-fresh Zs (2×10^7 Zs/mL), detailed on each assay. Control samples were treated with 500 µL of sterilized distilled water. The inoculated seedlings were located into a digital growth chamber at same conditions for growth (Aralab S.L, Lisbon, Portugal) following disease symptoms.

5.8. Plant disease symptoms

Disease symptoms of tomato seedlings (*var. Marmande*) were followed from 0 to 12 days after inoculation or until plant decayed, currently apparent disease symptoms on roots and secondary symptoms produced on leaves, were observed after three or four days. The analysis of the seed germination rate and disease symptoms on tomato seeds and seedlings was made according to the Protocol for tests on distinctness, uniformity and stability established by Community Plant Variety Office (CPVO-TP/1-Rev-5, 01/06/2021). The plant disease symptoms produced by *Phytophthora cinnamomi* Rands were considered following the standards approved for diagnostic protocols for regulated pests by EPPO council (European and Mediterranean Plant Protection Organization), at PM 7/26, OEPP/EPPO Bulletin 34, 155–157, 2004). Those symptoms included principally root rot brown lesions followed by necrosis produced directly by the pathogen and secondary symptoms of decline producing leaf chlorosis and later necrosis and dead. The disease ratio was determined as the percent of disease severity symptoms present on the seedlings related to the total number of plants under the same treatment, this scale is detailed as follows: “0” no disease symptoms; “1” delayed growth observed for shoots and roots; “2” chlorosis on the aerial part; “3” significant chlorosis and necrosis both on leaves and roots; “4” high necrosis and decayed seedlings. For each scale, the percentages of disease severity symptoms were calculated related to the total number of plants (n), considering “n” the 100%. Delayed growth related to controls, when was observed by view on aerial parts or roots, was confirmed digitally by analysing snapshots from harvested seedlings, using Image J®^{118,119}. The cell death was detected by trypan blue (TB) staining, as previously described¹³⁴. Briefly, a TB solution was prepared with 10 mL of lactic acid (85%), 10 mL of phenol (TE balanced buffer, pH 7.5–8.0), 10 mL glycerol ($\geq 99\%$), 10 mL of distilled water, and 40 mg of TB (final concentration of 10 mg/mL). The plant tissue or Pc culture samples stayed for 20 min in the solution before been rinsed with 100% ethanol overnight and preserved in 60% glycerol at 4°C or mounted on slides until observation under bright-light microscopy using a stereomicroscope (Microsercon SLU, Madrid, Spain), to obtain digital photos and snapshots. Image processing of snapshots was performed, when necessary, using the ImageJ® program using the specific corresponding software and plug-in tools^{118,119} as previously¹¹⁸.

5.9. Measuring Pc biomass and oomycete burden in plants by qRT-PCR

In addition, at this work we were focus and interested in the quantification of pathogen biomass, in planta, useful to determine the oomycete burden into the tissue, because this is a crucial step when monitoring disease resistance^{135–138}.

Briefly, the tissues of tomato seedlings, growth on soil, infected or not, were harvested with eighteen days, corresponding to four days after inoculation with Zs (4 dpi), or treated with water on controls. The tissues were snap frozen and finely ground to powder with a Sylamat® (Ivoclar Vivadent, Liechtenstein, Austria). Total RNA extraction, including DNAase treatment, was isolated from 0.350 g of plant tissue, using at least two plants per triplicate, and at least three triplicates per treatment, following manufacture instructions (NZY Total RNA Isolation kit, Nyztech, Lisbon, Portugal). The quantity and quality of RNAs

were obtained using a nanodrop (UV-Vis ACTG Gene UVS – 99. 200 an 850 nm), and a Qubit 4.0 (Fisher Scientific, Madrid, Spain) respectively. RNA samples were visualized in 1% agarose gel stained with Gel Red (Nippon, Japan). First-strand cDNA synthesis was performed using an hexanucleotide random primer, following manufacture instructions of First-Strand Synthesis Kit (Amersham-Pharmacia, Rainham, UK). Briefly, a 1.5µL aliquot of the first-strand synthesis reaction was used as the template for PCR amplification.

The quantification of oomycete burden on inoculated and non-inoculated seedlings was determined by qRT-PCR using a previously obtained standard curves using samples of known ng of cDNA obtained from Pc mycelium as a positive control, a similar scale was performed to calculate plant tissue using know ng of cDNA. The relation between Ct numbers obtained measuring *actin* (tomato) and *β-tubulin* (Pc) amplification was calculated.

The program consisted of 10 min at 25°C, 30 min at 50°C, and 5 min at 85°C. The qRT-PCR was performed using a SYBR® Green qPCR master mix (Nzytech, Lisbon, Portugal), using a final volume of 10µL per well. Samples were run into a DNA Engine One-Step qRT-PCR machine (Thermofisher Scientific, USA). Gene-specific primers for detecting Pc was *β-tubulin* (selected from Pc genome database (PHYCI_418545, <https://fungidb.org/fungidb/app/>), which was designed considering the annealing temperatures of 56°C (forward) and 58°C (reverse), that amplified one product of 160 bp with an efficiency of the 97,4%. Pc-*β-tubulin* (forward): 5'-CGCTGTCCGTGCACCAGCTTG-3'/(reverse) 5'-CATGTCCGGCATCACCAAGTGC-3' (this work). The gene-specific primers for Tomato corresponds to *Solyc11g005330.2* (<https://solgenomics.net/>) from¹³⁹ with annealing temperatures of 60°C (forward) and 57°C (reverse), amplifying one product of 150 bp with an efficiency of the 98,01 %.Gene-specific sequences were Tomato-*actin* (forward): 5'-CAAGTTATTACCATTGGTGCTGAGA-3' / (reverse) 5'-TGCAGCTTCCATACCAATCATG-3'.

Data were acquired using the One-Step PCR Applied Biosystem Analysis software (Version 2.01), and changes in transcript levels were determined using the $2^{-\Delta\Delta CT}$ method¹⁴⁰. The primers were verified for specificity using a Basic Local Alignment Search Tool (BLAST at NCBI). The program of *qRT-PCR* consisted of 3 min at 95°C, 40 cycles of 30 seconds at 95°C, and 30 seconds at 60°C, with a final extension step consisting of 7 min at 72°C with a final dissociation melting curve. Data points were compared using t-tests. At least, three independent biological replicates obtained from different assays, were used with three technical replicates in each experiment.

5.10. Confirmation of Pc identity on inoculated seedlings estimated by qRT-PCR

To confirm Pc recovering from tomato, seedlings were growth and inoculated as previously, with 2×10^7 Zs/ml. After seven days, the roots were harvested, surface sterilized with water and transferred onto a Petri dish containing PDA medium. Subsequently, the dishes were placed into a digital chamber Aralab® (Lisbon, Portugal), under the current conditions of temperature described before. Plates were kept in darkness for seven days allowing mycelium growth. One 1cmx 1cm piece of agar containing the

mycelium from tomatoes was transferred onto a PDA plate following steps described previously, until mycelium plus sporangia and Zs were obtained. One piece of 1 cm × 1 cm of Pc culture was harvest from Miracloth paper, and dried into a sterilized filter paper, and snap frozen in liquid Nitrogen.

In parallel a fresh mycelium stock of Pc was obtained on plates, as previously. Again, one piece of 1 cm × 1 cm of mycelium was harvest from Miracloth paper, and dried into a sterilized filter paper, and snap frozen in liquid Nitrogen. In both cases, 0.135g of Pc obtained from culture and obtained from tomato roots proceeding culture, were broken to powder with a Sylamat® (Ivoclar Vivadent, Liechtenstein, Austria). The kits for RNA extraction and First-strand cDNA, and the conditions for the *qRT-PCR* were performed using the same protocols as previously. Specific primers for Pc *β-tubulin* were used to amplify Pc mycelium from both mycelium.

5.11. Statistical Analysis

The Stat Graphics Centurion XVI.II program (Stat Point Technologies, Inc., Warrenton, VA, USA) was used for all data analysis using a Variance check (p value > 0.05). Statistical significance was calculated for all the experiments by unpaired t-test ($P < 0.05$), indicated with one asterisk (*), bars with non-asterisk are not significantly different, according to one-way ANOVA, Bonferroni's multiple comparison test ($P > 0.05$). For qRT-PCR analysis, error bars indicate standard deviation (SD) ($n = 3$). Bars with the same letter are not significantly different according to one way analysis of variance (ANOVA), Bonferroni's multiple comparison test ($P < 0.05$). Linear trend estimation was calculated by t-test ($P < 0.05$), when the correlation coefficient is significantly different the linear trend is shown with gray dots, correlation degree ("r"), is shown at the corresponding figure legend.

Declarations

Author Contributions: Supervision, conceptualization, funding acquisition, writing M.B.-L.; data curation, formal analysis, methodology writing L.D.C.-G., S. S and N. D-G.; writing—review, project administration, funding acquisition J.A.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially funded by Ministerio de Agricultura, Pesca y Alimentación, Fondos Feader. European Agricultural Fund for Rural Development Grant, Spain, Contract number "TSA0069316" (TRAGSA). This research and L. D.C.G. contract was funded by Fundación Conde Del Valle Salazar, Spain. SS was funded by an international Ph.D. fellow from the Algerian Government from MHESR, Decree No. 14–196 (Collaboration UPM-Argelia). N.D.C-G; was funded by a PhD fellow DIN2018-009928 from Ministerio de Ciencia Innovacion y Universidades, Spain.

Acknowledgments: We thank Beatriz Cuenca (TRAGSA), and María del Mar Ruíz Galea (IMIDRA), for assistance and providing *Phytophthora cinnamomi* Rands culture and initial assistance with protocols; Rafael Núñez Ramírez (CIB, CSIC, Spain), for assistance with the ImageJ® specific plugging tools, Arquimea SLU., for providing the SQS® system; and Steven Lindow (Plant Biology Dpt. at Berkeley University) for made suggestions.

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

Authors declare any competing, financial or non-financial competing interests to become public after the publication of the manuscript.

Availability of data and materials: All materials generated in this study are available from the corresponding author upon request.

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Figures

Fig. 1

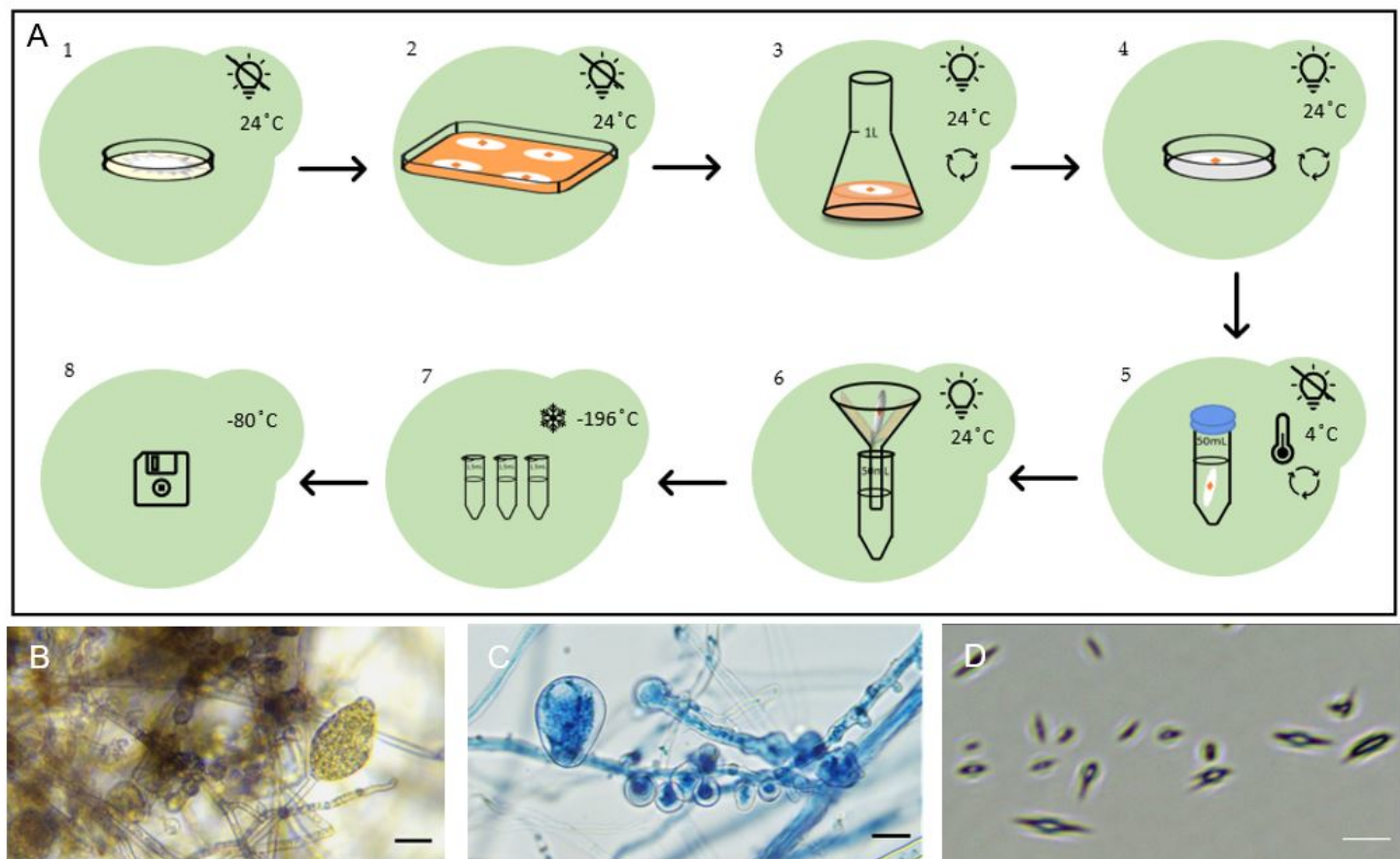


Figure 1

Obtaining *Phytophthora cinnamomi* Rands Zs . **A.1.** *Pc* growth in Potato Dextrose Agar medium (PDA) in darkness, at 24 °C for 3 weeks. **A.2.** Inducing mycelium growth by growing *Pc* on V8 agar medium in darkness, at 24 °C for 7 days on a miracloth disc . **A.3.**Inducing sporangia formation in V8 clarified liquid medium under fluorescence light and shaking, at 24 °C for 48 h. **A.4.** Second sporangia induction in mineral salt solution, enriched with chelated iron solution, under fluorescent light and shaking at 24 °C for 24 h. **A.5.** Inducing zoospore formation in sterile water under shaking, at 4 °C for 90 minutes. **A.6.** Filtration of Zs. **A.7.** Freezing of Zs in liquid nitrogen. **A.8.** Storage at -80 °C until use. **B.** Bright field of mature sporangium. **C.** *Pc* culture stained with trypan blue, corresponding to chlamydospore and hyphal swelling after the step of sporangia induction with mineral salt solution. Scale bar = 50 µm. **D.** Bright field snapshot image taken of obtained fresh Zs. Scale bar = 20 µm. Images were taken at 40x magnification.

Fig. 2

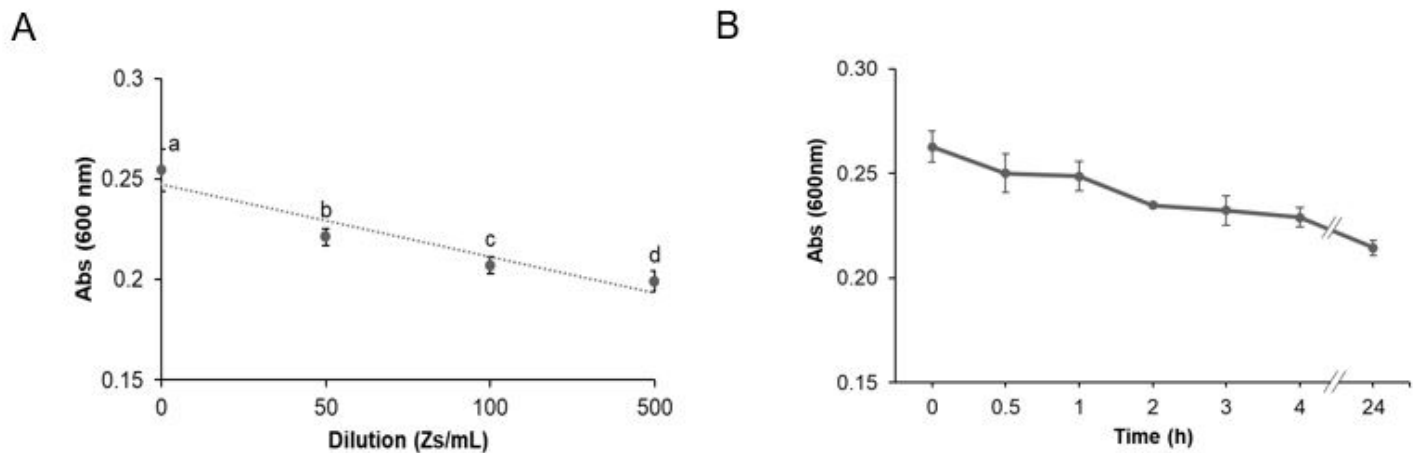


Figure 2

Viability of *Zs* measured via MTT and Neubauer chamber. **A.** The scatter plot shows the concentration of zoospores ($\times 10^8/\text{mL}$), at 50, 100 and 500 dilutions, counted using a Neubauer chamber plotted against the absorbance signal (measured at 600 nm using MTT; see section 5). Significant differences were based on one-way analysis of variance (ANOVA) with a variance check ($p < 0.05$). Lowercase letters (a, b, c and d) indicate significant differences ($R = 0.907$). Error bars indicate standard deviation (SD) ($n = 20$). **B.** Changes in zoospore survival at 23 °C measured via MTT. Significant differences were based on a non-parametric Krustal-Wallis's test with a variance check ($p < 0.05$). Error bars indicate standard deviation (SD) ($n = 14$). The data were analyzed using the Stat-graphics Centurion 19 program.

Fig. 3

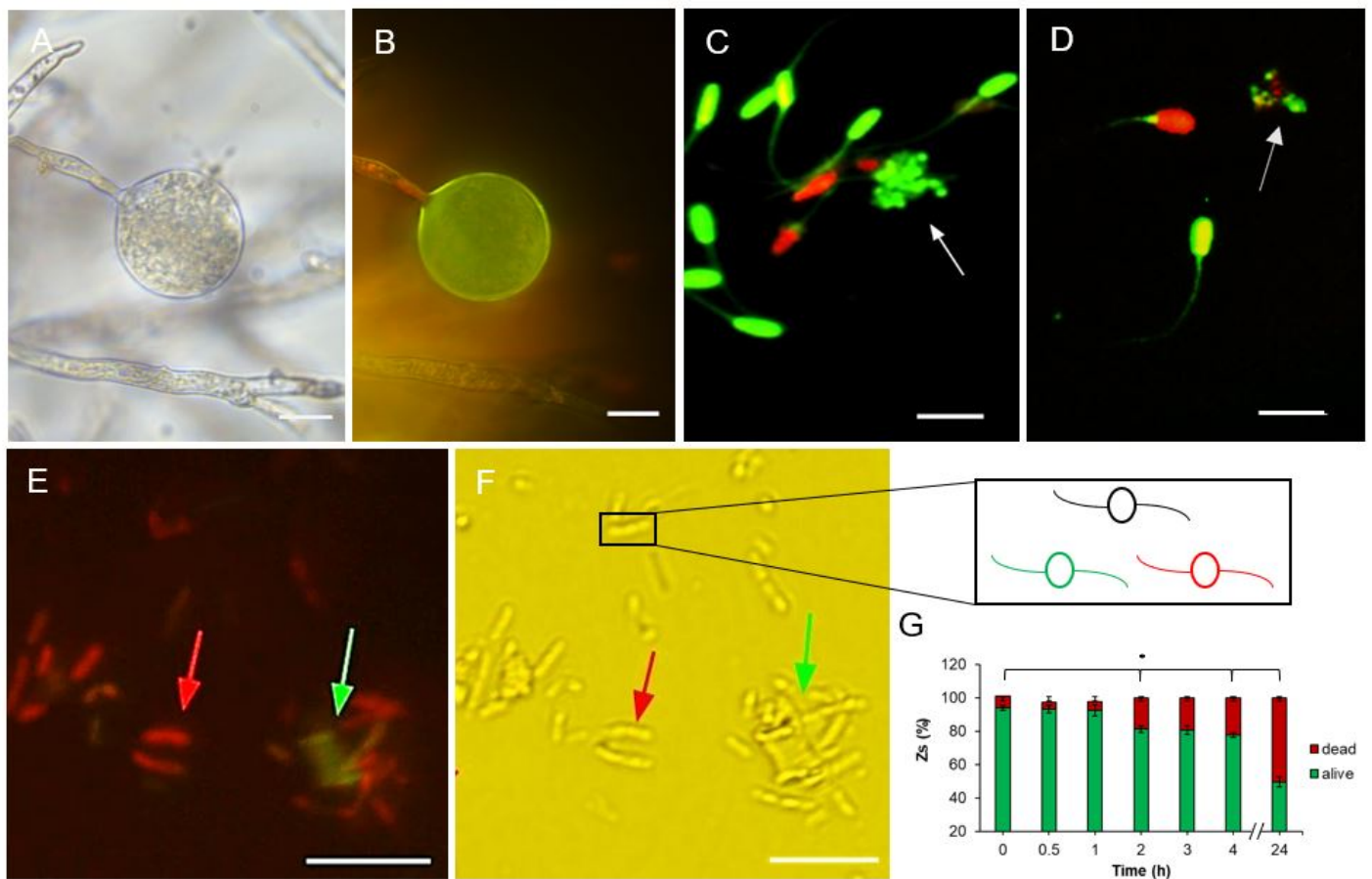


Figure 3

Viability of *Zs* determined by fluorescence. **A.** Bright image of sporangia full of *Zs*. **B.** fluorescent image of A. Scale bars = 50 μm . **C.** Snapshot of *Zs* (white arrowed), and Duroc sperm cells at time 0. **D.** Snapshot of *Zs* (white arrowed), and Duroc sperm cells after 1 hour. Scale bars = 30 μm . **E and F.** Bright (left), and fluorescence (right), images of *Zs*. **F inset.** Detail that alive cells are arrowed in green while dead cells are arrowed in red. Scale bars = 20 μm . Images with 40x magnification. **G.** Percentage of *Zs* survival after 0, 0.5, 1, 2, 3, 4, and 24 hours, determined by taking at least five snapshots per time, measured with SQS® machine and counted with ImageJ® Software (see Section 5). Error bars indicate standard deviation (SD) (n = 35). The data were analyzed with Stat-graphics Centurion 19 program, with a variance check ($p < 0.05$) indicated by one asterisk.

Fig. 4

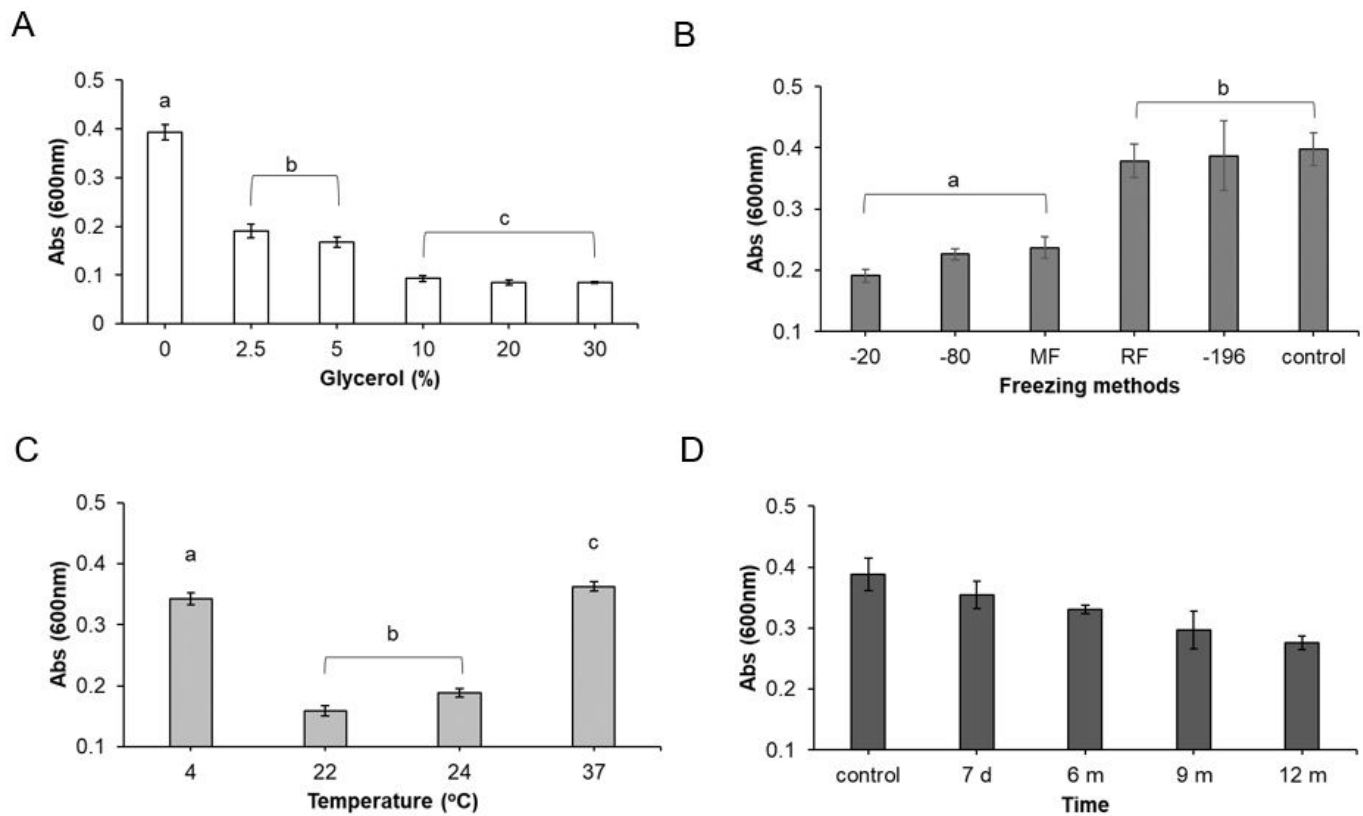


Figure 4

Survival of Zs to freezing and defrosting. A. Survival of Zs after freezing in glycerol (0, 2.5, 5, 10, 20, and 30%). Lowercase letters (a, b, and c) indicate significant differences. Error bars indicate standard deviation (SD) (n = 115). **B.** Survival of Zs using next freezing methods: -20 °C (-20), -80 °C (-80), Mister Frosty (MF), ramp freezing (for 22–18–4 to -20 °C) (RF), liquid nitrogen (-196 °C). For A and B significant differences were based on one-way analysis of variance (ANOVA) with a variance check (p < 0.05). Lowercase letters (a and b) indicate significant differences. Error bars indicate standard deviation (SD) (n = 16). **C.** Survival of Zs after defrosting at 4, 22, 24, and 37 °C. Lowercase letters (a, b, and c) indicate significant differences. Error bars indicate standard deviation (SD) (n = 30). **D.** Survival of Zs stocks at seven days (d) and six, nine and twelve months (m). The Zs stock analyzed was 2×10^8 /mL. At least four independent biological assays were performed with similar results. Error bars indicate standard deviation (SD) (n = 14). For C and D significant differences were based on Krustal-Wallis's test with a variance check (p < 0.05). All data were analyzed using the Stat-graphics Centurion 19 program.

Fig. 5

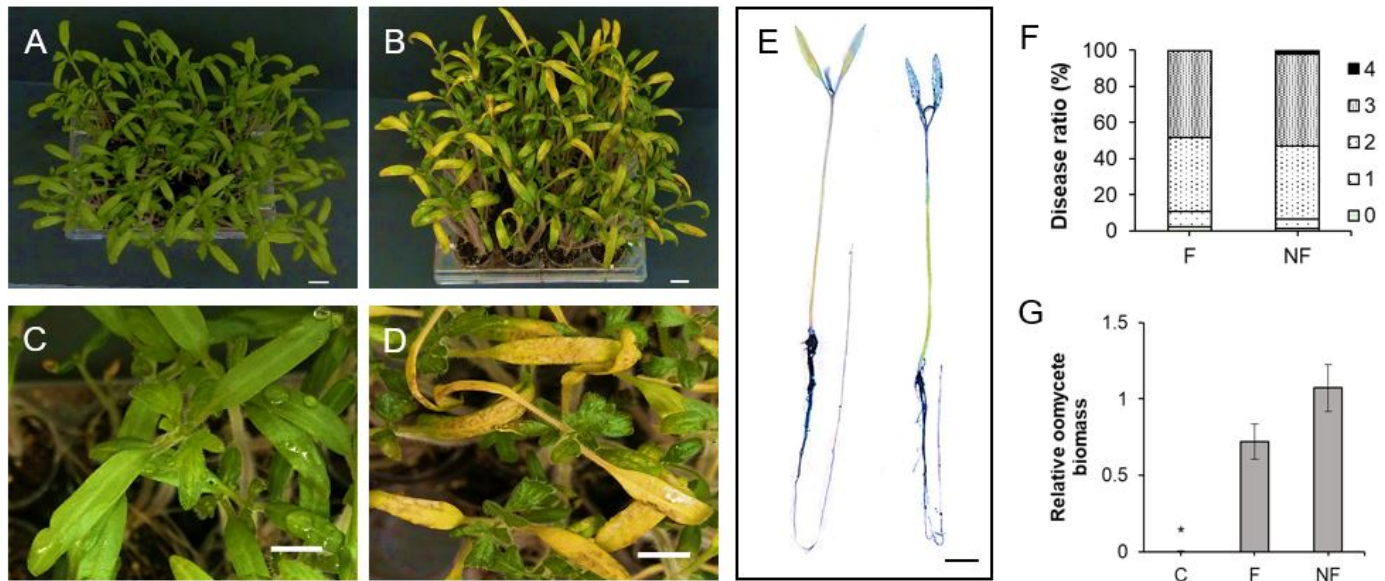


Figure 5

Symptoms of tomato seedlings inoculated with Zs stocks. Fourteen-day-old seedlings were grown on soil and inoculated with a final concentration of fresh Zs (F), or non-fresh Zs stocks (2×10^7 Zs/ml, see Section 5). **A and C.** Control plants (water). **B and D.** Symptoms of inoculated plants after four days of inoculation (4 dpi). Scale bar = 1 cm. **E.** Necrotic lesions detected by trypan blue staining, control (left image), and inoculated plant (right) at 4dpi. Scale bars = 1 cm. **F.** Disease ratio at 4 dpi, on seedlings performed with F, and NF. Symptom severity was assessed on a scale where “0” is absence of symptoms, “1” plant growth inhibition, “2” light chlorosis on leaves, “3” significant chlorosis on leaves and root necrosis, “4” high necrosis on leaves and roots and decayed seedlings. (n = 240). **G.** Oomycete biomass (ng), quantified into vegetal tissue at 4dpi, inoculated with F and NF, measured by qRT-PCR using specific primers corresponding to *Pc β -Tubulin* and tomato *actin* (see Section 5). The data were analyzed using the Stat-graphics Centurion 19 program, with a variance check ($p < 0.05$) indicated by asterisk. Error bars indicate standard deviation (SD) (n = 3).

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