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SHORT COMMUNICATION

Sclerotization as a long-term preservation method for *Rosellinia necatrix* strains

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Abstract This work describes a simple protocol for long-term preservation of strains of *Rosellinia necatrix* based on sclerotia production combined with storage at 4°C in liquid substrate, without affecting the growth and pathogenic characteristics of the fungal isolates recovered. The sclerotization process was set up in both liquid and solid media, and the sclerotia-like structures (pseudosclerotia) obtained were preserved in liquid media or water at 4°C. *R. necatrix* pseudosclerotia viability after 6 years of preservation at 4°C was confirmed by growth and microscopic characteristics, with no differences when compared with the fungal strains routinely preserved by periodic transfers. Additionally, pathogenicity on avocado plants by the preserved *R. necatrix* strains showed no difference from those preserved by periodic transfers. The albino strain used in this study should continue to be preserved by periodic subculturing.

Keywords Avocado · *Dematophora necatrix* · Fungal storage

The fungus *Rosellinia necatrix* (anamorph *Dematophora necatrix*) is probably the most widely distributed of all *Rosellinia* species. This fungus can be found worldwide in temperate as well as tropical areas (Sivanesan and Holliday 1972) and is also considered to be the most destructive of all the *Rosellinia* species (Freeman and Sztejnberg 1992). *R. necatrix* has a very wide host range, including 170 plant species or varieties in 63 genera and 30 families of dicotyledonous angiosperms (Khan 1959; Ten Hoopen and Krauss 2006).

Under natural conditions, *R. necatrix* can develop sclerotia (Pérez-Jiménez et al. 2003), which are black, hard, spherical nodules several millimeters in diameter located mainly on invaded roots and connected from their base with the subcortical mycelium (Viala 1891; Khan 1959). Formation of these structures, similar to those of other fungi, could possibly be related to the survival of *R. necatrix* in the soil (Sztejnberg et al. 1980). When *R. necatrix* is cultured on synthetic culture media, the young mycelium of *R. necatrix* is initially white and cottony. With age, the mycelium could become brown black in color because of the presence of small microsclerotia (Sztejnberg et al. 1980). Microsclerotia are formed as irregular bodies of a compact mass of interwoven hyphae with high melanin content (around 98 × 130 μm in size) and tend to unite and form microsclerotial sheets. Red, blue, and fluorescent (daylight-type) illumination was found to induce microsclerotia formation, whereas near-UV light and darkness depressed the morphogenetic process (Sztejnberg et al. 1980).

In the Mediterranean area, *R. necatrix* causes white root rot (also called Dematophora root rot) in avocado and in many other crops (López-Herrera 1998; Pliego et al. 2011). Studies on avocado white root rot have led to construction of a small fungal collection composed of more than 55 undomesticated *R. necatrix* strains isolated from avocado white root rot in the Mediterranean area (Dr. C.J. López-Herrera, Institute of Sustainable Agriculture, IAS-CSIC, Córdoba, Spain). This collection has been traditionally maintained on potato dextrose agar (PDA) tubes, the fungal cultures being refreshed every year.

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Table 1 Selected fungal strains of *R. necatrix*, isolated from avocado white root rot, used in this study and provided by Dr. C.J. López-Herrera (IAS-CSIC, Córdoba, Spain)

Strain	Characteristics	Reference
CH53	(Former Rn400). Isolated in Almuñecar (Granada, Spain) in 1991. Virulent strain. Allowed genetic transformation	López-Herrera and Zea-Bonilla (2007), Pliego et al. (2009), Ruano-Rosa et al. (2010)
CH290	(Former Rn290). Isolated in Vélez-Málaga (Málaga, Spain) in 1990. Virulent strain. Allowed genetic transformation	Pliego et al. (2009), Ruano-Rosa et al. (2010)
CH12	(Former Rn12). Isolated in Salobreña (Granada, Spain) in 1988. Avirulent strain. Allowed genetic transformation. Albino strain	Pliego et al. (2009), Ruano-Rosa et al. (2010)

In this work, three representative *R. necatrix* strains (Table 1) from this collection have been selected and used to develop an easier long-preservation technique that avoids potential problems of subculturing, such as culture contamination or lack of desirable fungal characteristics, and will allow preservation of *R. necatrix* strains avoiding these risks. These three fungal strains were selected mainly based on their virulence (Ruano-Rosa et al. 2010), their ability to form sclerotia-like structures (pseudosclerotia), and their genetic ability to be transformed (Pliego et al. 2009).

A protocol of sclerotization has been set up for *R. necatrix* strains. Pseudosclerotia production by selected *R. necatrix* strains has been initially characterized on different synthetic media, such as corn-meal agar (CMA; Oxoid, Cambridge, UK), malt agar (AM; Difco, Lawrence, KS, USA), and potato dextrose agar (PDA; Oxoid). PDA disks 0.5 cm in diameter were taken from the border of a 5-day fungal colony grown on PDA at 25°C in darkness, placed in the center of fresh agar test plates, and incubated for 7 days at 25°C in darkness. Plates were then incubated at 25°C under fluorescent white light for 7–21 days, until the production of dark pseudosclerotia could be observed on the agar surface. Pseudosclerotia formation by *R. necatrix* CH53 and CH290 was observed on PDA after approximately 14 days of growth (7 days in darkness and 7 days under fluorescent white light) and in notably lesser amounts on AM and CM. For that reason, PDA was the media selected for further experimentation. The selected *R. necatrix* strain CH12 did not produce pseudosclerotia on solid media, even after 3 months kept under appropriate conditions to promote pseudosclerotia formation (under fluorescent white light at 25°C or at room temperatures under natural daylight on the laboratory bench). The colonial morphology of this "albino" *R. necatrix* CH12 strain is slightly different from the strains producing pseudosclerotia, showing whiter mycelia and more compact hyphae. In this sense, pseudosclerotia-producing *R. necatrix* strains showed less compact mycelia growth but faster radial growth of the colony (Fig. 1). The presence of "albino" strains has also been observed in other fungi, but the albino strain CH12 of *R. necatrix* used in this work was first reported by Ruano-Rosa (2006).

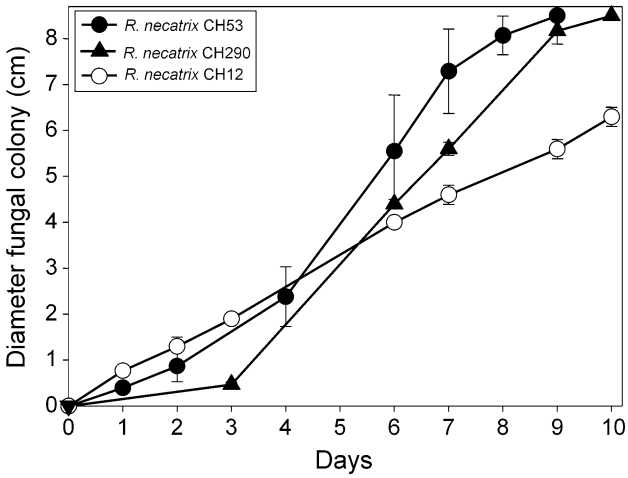

Fig. 1 Colony growth of *Rosellinia* (*R.*) *necatrix* colonies in potato dextrose agar (PDA). Measurements of each strain were performed using two perpendicular diameters of the colony

To obtain free pseudosclerotia structures, a procedure using liquid media has been developed (Fig. 2). Briefly, potato dextrose broth (PDB) was inoculated with *R. necatrix* mycelia from PDA plates. The Erlenmeyer flasks were incubated at 120 rpm at 25°C in darkness for 2 weeks to produce fungal mycelia, followed by 2 weeks under the same conditions under fluorescent white daylight-type light to induce pseudosclerotia formation. If necessary, this cycle was repeated two times (52 days in total) to obtain a sufficient quantity of pseudosclerotia. After growing for 14 days in darkness followed by another 14 days under natural daylight conditions at 25°C, black rod-shaped structures about 0.5 × 2 mm in size were observed at the bottom of the Erlenmeyer flasks for *R. necatrix* CH53 and CH290. These black pseudosclerotia structures were collected into a 10-ml plastic tube using a micropipette and stored in the dark at 4°C (Fig. 2). These black structures were allowed to grow on different nutrient media, and fungal growth was confirmed as *R. necatrix*. No pseudosclerotia structures were formed for the *R. necatrix* CH12 strain after 3 months of incubation, repeating the periodic growth conditions described previously.

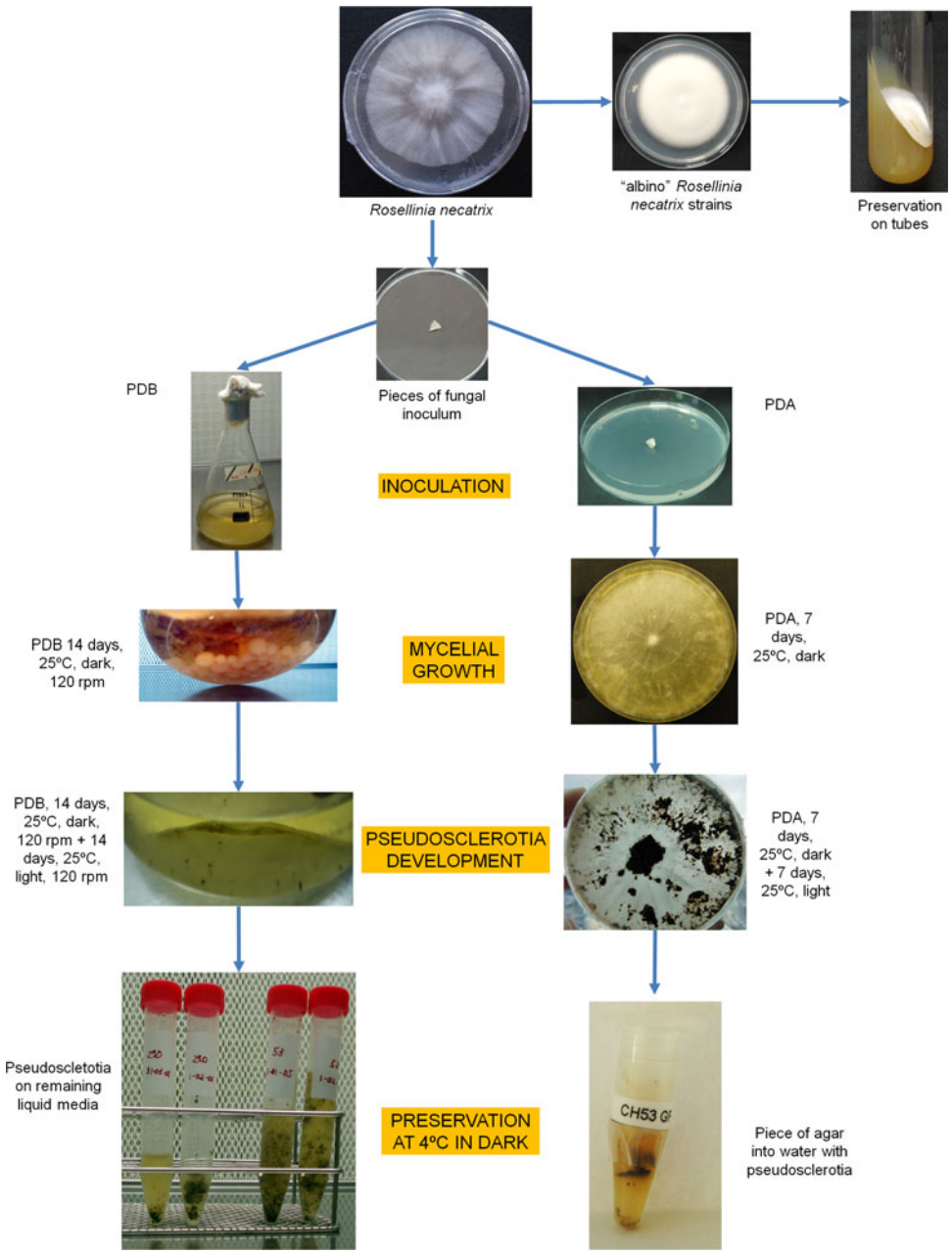

Fig. 2 Comparative schematic process of pseudosclerotia formation on solid and liquid media. Sclerotization can be induced after exposing the culture to white daylight. Preservation of pseudosclerotia was performed at 4°C in darkness. The strains that did not produce pseudosclerotia were preserved by periodic transfer on solid media

For preservation studies, it has been described that sclerotization procedures can be applied to some fungi that develop sclerotia or other long-term surviving propagules in culture (Daniel and Baldwin 1964; Singleton et al. 1992; Ohmasa et al. 1996), and preservation of such structures, usually at 3–5°C, is a good way to store fungal strains (Nakasone et al. 2004). On the other hand, according to the "species"-specific key to the determination of appropriate preservation protocols for fungi (Ryan et al. 2000), one of the best ways to preserve the *Rosellinia* isolates would be in water, which appears to suppress morphological changes in most fungi (Nakasone et al. 2004). Additionally, the storage of fungi on agar media under sterile distilled water has also been proven to be a reliable conservation method for many fungi (Ritcher and Bruhn 1989), avoiding the disadvantages of periodic subculturing, e.g., contamination, morphological or physiological changes, or reduction in ability to infect (Nakasone et al. 2004). Preservation in water has been used successfully to preserve oomycetes (Smith and Onions 1983), basidiomycetes (Ellis 1979; Ritcher and Bruhn 1989; Ritcher 2008), ectomycorrhizal fungi (Marx and Daniel 1976), ascomycetes (Nakasone et al. 2004), hyphomycetes (Ellis 1979), plant pathogenic fungi (Boesewinkel 1976), aerobic actinomycetes (Gelderen and de Komaid 1988), and human pathogens and yeast

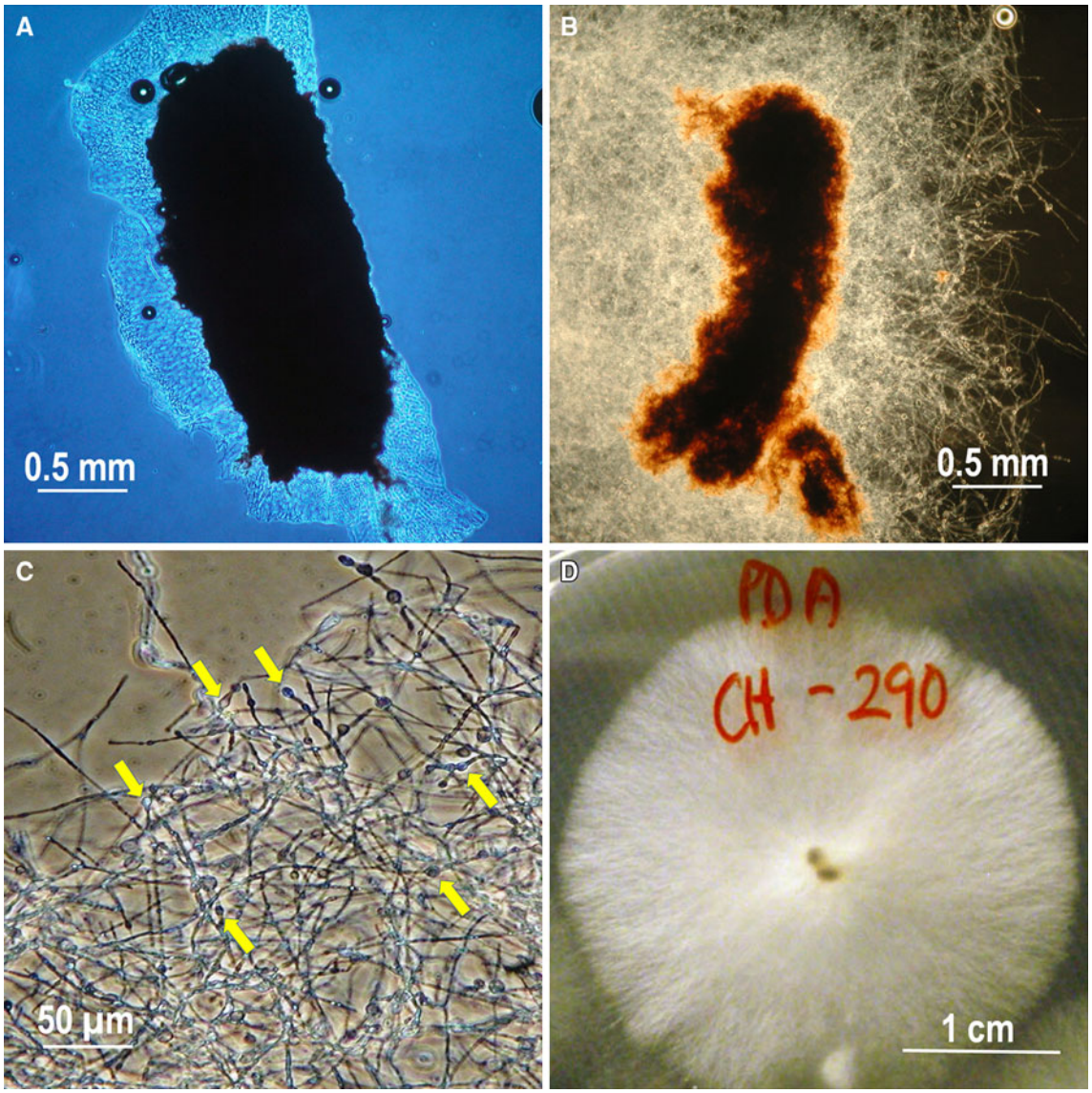

Fig. 3 Germination of pseudosclerotia of *R. necatrix*. **a** Pseudosclerotia from *R. necatrix* CH53 grown on potato dextrose broth (PDB), stained with aniline blue. **b** Pseudosclerotia from *R. necatrix* CH290 grown on PDB placed onto a PDA plate surface, after 24 h of incubation at 25°C. **c** Detail of the fungal mycelia growing from the pseudosclerotia present in **b**. *Arrows* indicate typical pyriform swellings for *R. necatrix*. **d** A 4-day-old colony on PDA resulting from pseudosclerotia germination, showing the typical features of *R. necatrix*

(McGinnis et al. 1974). By this method, authors have reported survival of several fungi stored in water for more than 12 years (Qiangqiang et al. 2009).

Based on previous knowledge, *R. necatrix* pseudosclerotia obtained in liquid media were placed into plastic tubes with some remaining PDB media and preserved in the dark at 4°C (Fig. 2). The black, crusted areas observed covering the PDA plate surface were also used. A small piece of agar containing media and the black bodies was placed in a plastic tube containing 1 ml sterile distilled water and preserved at 4°C. After 3–5 weeks, small black bodies (pseudosclerotia) at the bottom of the tube could be observed (Fig. 2).

After 6 years stored, *R. necatrix* pseudosclerotia were recovered from the plastic tubes and placed in the center of PDA plates for incubation at 25°C in darkness. Observation of fungal growth, colonial morphology after 5 days of growth, and presence of pear-like swellings on the fungal hyphae under the microscope was confirmed (Fig. 3) and used to characterize these fungi as *R. necatrix* (Pérez-Jiménez et al. 2002; Ten Hoopen and Krauss 2006). Radial growth of fungal colonies from pseudosclerotia preserved for 6 years was compared with the corresponding fungal strain preserved by periodic transfers, showing no apparent differences among them (Fig. 4). The fungal colonies covered the plate surface after 1 week of incubation.

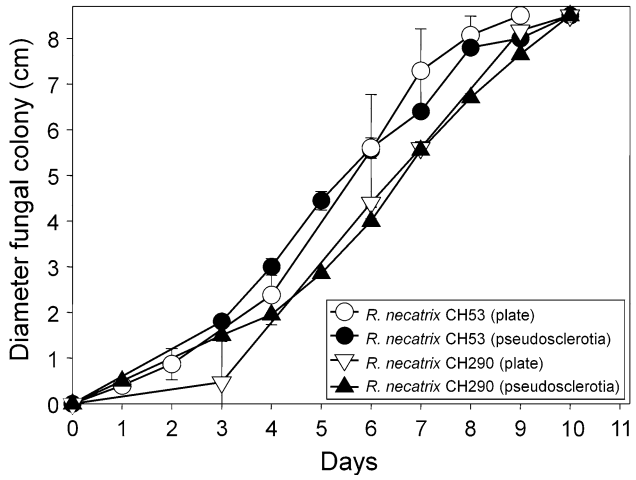

Fig. 4 Fungal colony growth of *R. necatrix* maintained by periodic plate transfer or from preserved pseudosclerotia. Measurements in each plot were performed using two perpendicular diameters of the colony

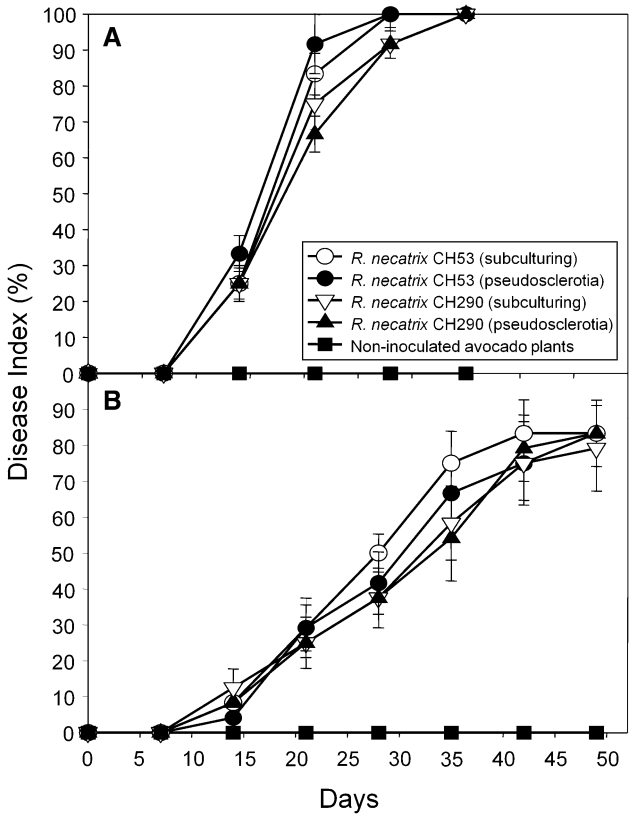

Fig. 5 Disease progress of *R. necatrix* strains obtained from periodic plate transfer or from 6-year-old preserved pseudosclerotia. Disease index was calculated in two independent experiments using 6-month-old avocado seedlings in potting soil (**a**), and using 2-year-old avocado seedlings in a commercial substrate infected with strains (**b**)

A few methods refer to long-time preservation of *R. necatrix*. The method described most recently (Ten Hoopen et al. 2004) resulted in successful storage of *Rosellinia* for periods of up to 2 years when using silica gel or by cryopreservation of mycelia in liquid nitrogen. Deep-freezing strategies have been described previously as successful ways to preserve fungal stock cultures (Kitamoto et al. 2002). However, preservation times slightly greater than 16 months were reported when *R. necatrix* was placed in sterile water (Ten Hoopen et al. 2004).

To determine whether pseudosclerotia formation and preservation have an influence on the avocado pathogenicity of these strains, artificial inoculation tests were performed. Pathogenicity assays using *R. necatrix* strains obtained from subculturing or from stored pseudosclerotia were performed as previously described (Cazorla et al. 2006) using 6- and 24-month-old commercial avocado plants (Brokaw España, Vélez-Málaga, Spain) growing in two different substrates. Six-month-old avocado plants were grown in a mixture of peat:coconut fiber:perlite (6:1:0.6 v/v substrate) and 24-month-old avocado plants were cultured in a mixture of sand:peat:silt (1:2:1 v/v substrate).

Inoculations with *R. necatrix* were performed using wheat grains infected with mycelia as previously described (Sztejnberg et al. 1980; Cazorla et al. 2006). A set of nine 6-month-old plants and four 24-month-old plants was tested per experimental strain. Avocado plants were placed in a growth chamber at 24°C with 70% relative humidity and 16 h daylight. Aerial symptoms were observed, and the disease index percentage of foliar symptoms was calculated using the symptom scale as previously described (Cazorla et al. 2006). After 2 weeks in a greenhouse, avocado white root rot symptoms were observed in all 6-month-old avocado plants inoculated with the fungal strains *R. necatrix* CH53 and CH290, and the disease index did not show apparent differences among the different fungal colonies used (Fig. 5a). When the experiment was performed using 2-year-old commercial avocado plants in a commercial substrate infested with *R. necatrix*, similar results were obtained (Fig. 5b). From those symptomatic plants, re-isolation from necrotic roots showed the presence of *R. necatrix*. These results confirmed that this method of preservation does not affect the fungal survival, vitality, and pathogenicity. Inoculation with *R. necatrix* CH12 did not show white root rot symptoms in these experiments, as was previously reported by Ruano-Rosa et al. (2010); this finding is in agreement with the observations that "albino" strains reported in other fungi also showed reduced virulence and loss of conidiation (Solomon et al. 2004).

These non-pseudosclerotia-producing fungal strains ("albino" strains) have sometimes been used as biocontrol agents when reduced virulence has been observed (Dixon et al. 1987; Held et al. 2003; Solomon et al. 2004). According to our observations, albino *R. necatrix* strains are avirulent and have different growth than that displayed by pseudosclerotia-producing strains (Fig. 1). However, further research has to be carried out to evaluate their

interest as biocontrol agents. Traditional preservation methods, by serial transfers every 6–12 months, can be used to preserve the albino *R. necatrix* strains.

These results showed that combination of two simple procedures applied to long-term preservation of fungi—sclerotization, and immersion in distilled water—can be used successfully to store *R. necatrix* strains without losing important characteristics with preservation times up to 6 years. Additional advantages of this procedure include that it is inexpensive and has a low-maintenance cost.

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