

Survival and dispersal of *Puccinia psidii* spores in eucalypt wood products

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Abstract The rust, caused by *Puccinia psidii*, is one of the most important diseases of *Eucalyptus* and other Myrtaceae. The disease can be spread by windblown fungal spores, infected root cuttings, seedlings, pollen and other host tissues that increase the risk of its introduction to rust free countries as well as of spreading new pathogen lineages. To assess the risk of disease spread by transport of spores on wood products, we evaluated the effect of height of traps installed in different eucalypt timber and wood pulp storage areas on rust spore capture. We also evaluated *P. psidii* spore dispersal in the field and survival of *P. psidii* urediniospores and teliospores under different temperatures (15, 25, 30, and 35°C) and relative humidity (35, 55, 70 and 87%) for up to 120 days. Spore viability was evaluated *in vivo* by germination on leaves of a susceptible hybrid *E. urophylla* x *E. grandis* clone and by staining with fluorescein diacetate. The number of *P. psidii* spores captured in timber and wood pulp storage areas was significantly lower than in eucalypt

plantations that contained infected plants. A combination of high temperature and high relative humidity decreased spore survival. Urediniospores and teliospores survived 90 days at 15°C and 35% relative humidity, but only 10 days at 25 to 30°C and 70% relative humidity. The results of this study indicated that although rust spores may reach wood products (timber and pulp) storage areas in very low numbers, the adverse environmental conditions encountered in these areas and during overseas transport do not favor spore survival. Thus, the risk of spread of this pathogen into new areas in the absence of infected host plants is considered extremely low to inexistent.

Keywords *Eucalyptus* · Quarantine risk · Invasive disease · Rusts disease

Introduction

Puccinia psidii is an important pathogen of many Myrtaceae species in which it may infect juvenile leaves, buds, flowers, shoots or fruits. This pathogen causes necrosis, deformation and hypertrophy of infected organs, prolific branching, galling death of the apical portions, and reduced plant growth in eucalypts. Selection and breeding for resistance is the main method of disease control in eucalypt plantations in Brazil (Alfenas et al. 2009; Glen et al. 2007). *P. psidii* appears to be native to South America (Coutinho et al. 1998), but is found in Central America and the Caribbean (Maclachlan 1938; Laudon and Waterston 1965; Di Stefano et al. 1998), Florida (Marlatt and Kimbrough 1979; Rayachhetry et al. 1997), Hawaii (Uchida et al. 2006), and more recently in Japan (Kawanishi et al. 2009) and Australia (Carnegie et al. 2010). In Australia, given the absence of a telial stage, the disease was originally attributed to *Uredo rangelii* and named as

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“Myrtle Rust” (Carnegie et al. 2010). However, the telial stage of the so called “Myrtle Rust” has recently been found in Australia, which is morphologically similar to the fungus originally described as *P. psidii* (Carnegie and Cooper 2011). There is also an unconfirmed report of eucalyptus rust (*P. psidii*) in Taiwan (Wang 1992; Glen et al. 2007).

To date the pathogen has not been found in South Africa, where *Eucalyptus* spp. are among the most important forest tree species and accidental introduction of *P. psidii* to this country could cause serious forest industry losses (Glen et al. 2007). *P. psidii* appears to move easily to new environments and its recent introductions to Florida, Hawaii, Japan, and Australia indicate that it continues to spread around the world and will therefore likely spread to regions that are currently rust free, such as South Africa and South East Asia.

Puccinia psidii is a biotrophic pathogen that cannot grow in the absence of a live host (Alfenas et al. 2009). This pathogen can easily be spread by wind borne spores or contact with infected plant material as well as contaminated clothing and footwear (Langrell et al. 2008). However, little is known about *P. psidii* spread in wood products. This fungus may be present in the wood products industry, but spore survival is probably reduced by unfavorable conditions of temperature, relative humidity and light. These environmental factors are known to affect patterns of spore release and disease progress in the field (Ruiz 1988; Blum and Dianese 2001). As eucalypt trees grow, their ability to escape infection increases, and generally plants more than 3 to 4 m tall are no longer infected by *P. psidii*. Zauza (2007) found that above this height, microclimatic conditions (mainly leaf wetness) and low inoculum densities reduced the chance of infection.

In this study we evaluated the relationship between trap height and capture of rust spores in eucalypt plantations and in lumber yard and wood pulp storage areas. We also tested *P. psidii* urediniospore and teliospore survival under different conditions of temperature and relative humidity and evaluated *P. psidii* spore dispersal in the field to base risk analysis of fungal spread to currently rust free countries.

Material and methods

Wind dispersal of *Puccinia psidii* urediniospores

Fungal spore traps were placed at four different locations in North Espírito Santo and South of Bahia states (Fig. 1) during the period from October 2006 to November 2007 to study *P. psidii* spore dispersal. The traps were installed at the following locations: A1—a saw mill (Fig. 2); A2—a eucalypt plantation containing five month old infected

plants (Fig. 3); A3—a pulp mill (Fig. 3); and A4—a harbour used for export of eucalypt lumber and pulp (Fig. 3).

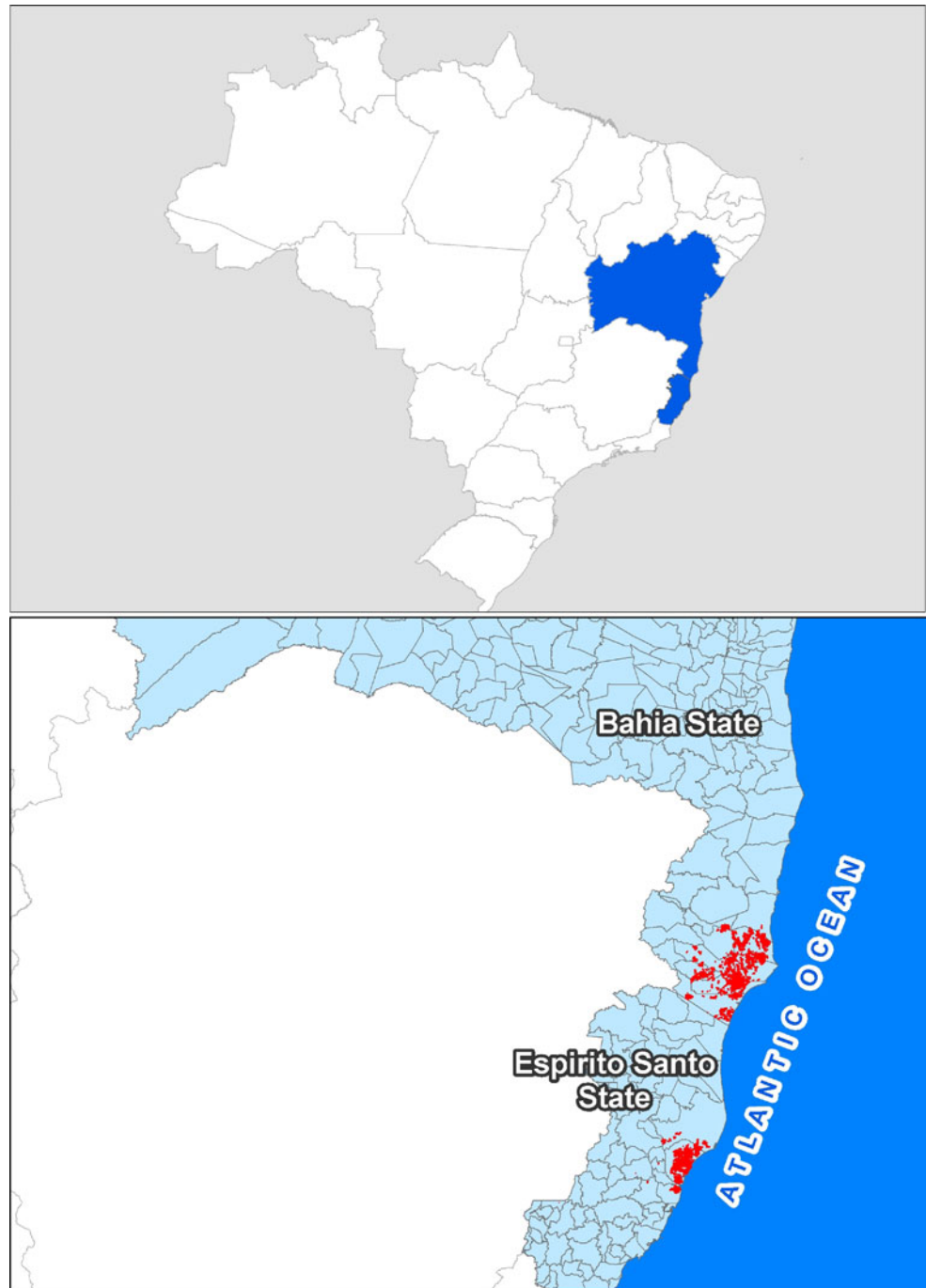
The traps were adapted from the model proposed by Reis and Santos (1985) and were made from 10 cm diameter white plastic tubes with one end cut at a 45° angle, so that they were 38 cm long on top and 25 cm long on the bottom. A glass microscope slide was placed in a hole 19 cm from the front (angled) end so that it was protected from rain and sunlight (Zauza et al. 2010). An acrylic wind vane was attached to the back end to keep the trap pointed in the direction of the wind. The microscope slide was greased with silicon and exchanged weekly during 14 months to count the trapped urediniospores (microscopic slide 26 × 76 mm) under a light microscope (200×). The traps were installed at heights of 0.8, 1.2, 1.6, and 2.0 m above the ground. The urediniospores were quantified weekly. A randomized factorial design, composed of four locations, four heights, and 14 months, containing four replicates per treatment was used. Non-parametric tests were used to compare treatments. Time effects were compared using the standard error of the mean and location and height effects were compared using the Kruskal-Wallis test (Fig. 4). Pearson correlation analysis ($p < 0.05$) was used to determine the influence of environmental variables (Table 1) on spore capture.

Spore germination and viability

To produce urediniospores a single pustule (isolate UFV2, race 1) was multiplied on young expanding leaves of *Syzygium jambos* that were incubated for 24 h in the dark, at 25°C under intermittent mist. The infected plants were transferred to a growth chamber and kept at 22°C under a 12 h photoperiod of 40 μ moles.s⁻¹.m⁻² light intensity. Spores were collected after 12 days of incubation. Teliospores were produced using the same procedure, except that rooted cuttings of a susceptible *E. urophylla* × *E. grandis* hybrid clone were used instead of *S. jambos*.

Spore samples (7 mg) were stored in 1.5 mL Eppendorf® vials at different temperatures (15, 25, 30, and 35°C) and relative humidity (35, 55, 70 and 87%) during 10, 20, 30, 40, 50, 60, 90, and 120 days. The Eppendorf® vials were kept uncovered during the storage period in 50 mL Falcon tubes (Falcon®) containing 5 mL saturated salt solution (Dhingra and Sinclair, 1995) to maintain the relative humidity. A randomized factorial design (4 × 4 × 8) with four replicates per treatment was used. Half the stored spore samples (3.5 mg) were suspended in 0.01% mineral oil and used in the leaf tissue (LT) spore germination test. In this test, 4 μ L of the spore suspension were deposited on the abaxial surface of the third expanding detached leaf from branches of an *E. urophylla* × *E. grandis* hybrid clone, which is highly susceptible to the strain of rust used here. The branches were

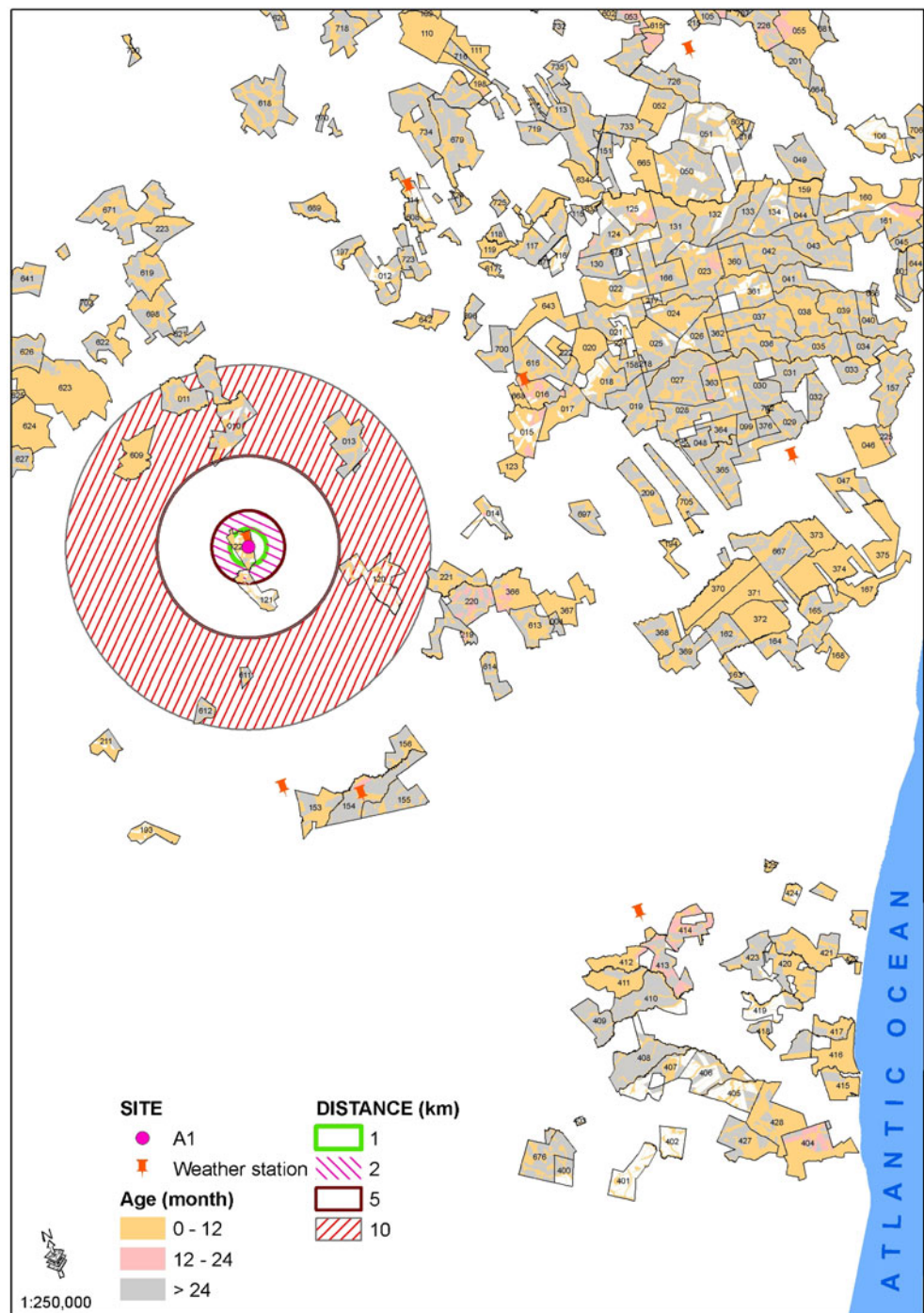
Fig. 1 Locations of Brazilian eucalypt plantations selected for installation of *Puccinia psidii* spore capture traps



kept in a Gerbox® chamber containing two sheets of paper towel water-soaked in distilled water to control humidity. The base of each branch was wrapped in water soaked cotton to maintain leaf turgor. The chambers were covered with aluminum foil and incubated at 22°C for 48 h according to preliminary tests. After incubation, leaf sections (0.7×1.0 mm) containing spores were treated with chloral hydrate solution (5:2 v/v) for 5 days, and then with lactophenol for 5 more days. The samples were transferred to microscope slides containing three drops of lactophenol and

aniline blue (20 g phenol, 16.7 mL lactic acid, 32 mL glycerin, 0.01 g of aniline and 31.33 mL water). The slides were covered with a cover glass and examined under a light microscope (200×) for spore germination. Spores that presented germ tubes of length equal to or longer than their width were considered germinated. For each microscope field observed, 30 randomly distributed spores (120 spores/slide) were evaluated. In the fluorescein diacetate (FDA) solution spore viability test, 0.5 mL of the stored spore samples were added to 0.5 mL distilled water

Fig. 2 Location of trap installed at a saw mill showing eucalypt plantations in the South Bahia State, Brazil



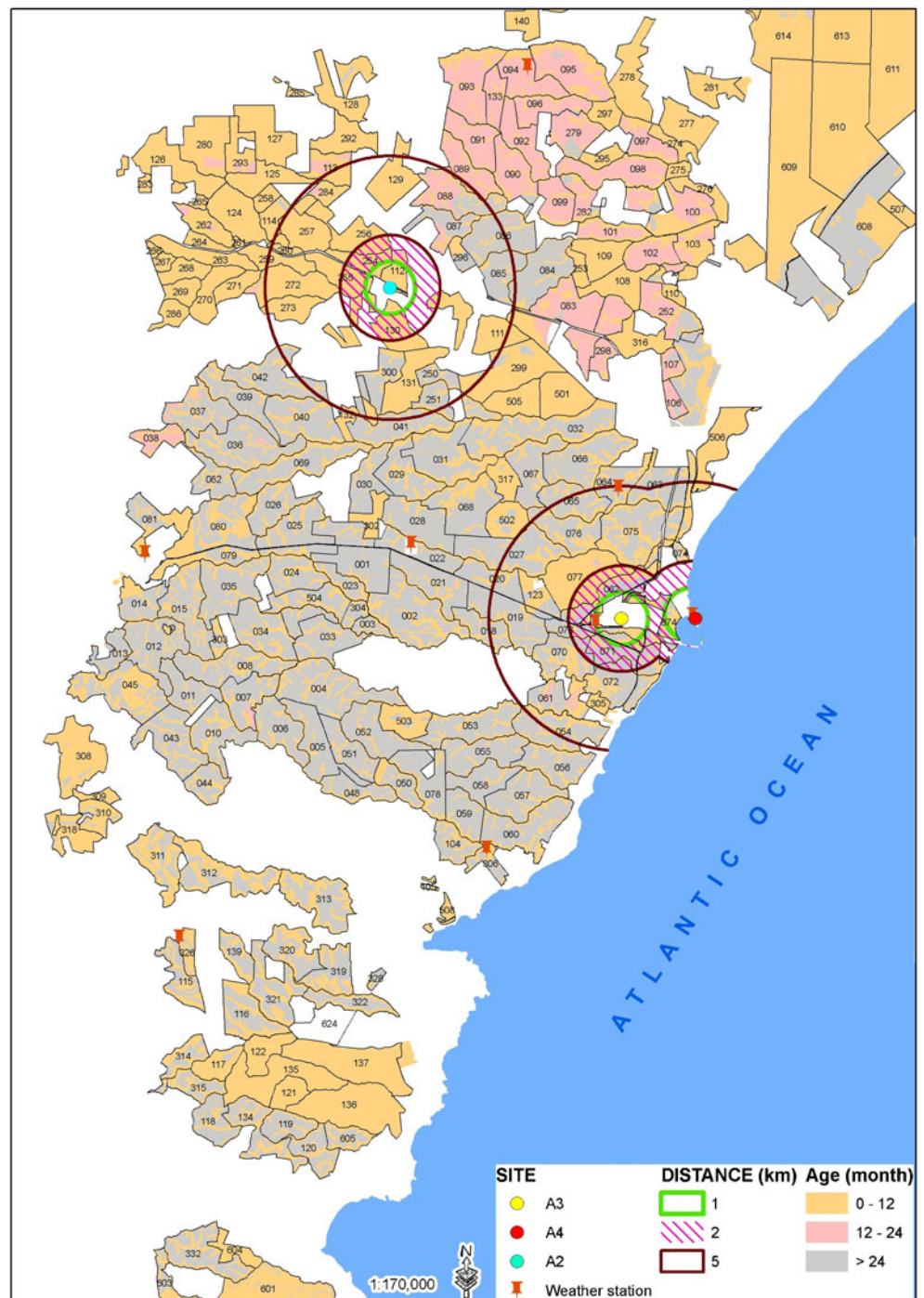
containing 10 μL of fluorescein diacetate (FDA) solution (2 mg fluorescein diacetate/mL acetone) in the dark. After gentle manual mixing for 5 min, 30 μL of this mixture were placed on microscope slides and covered with a cover glass. The number of viable (fluorescent) and unviable (non-fluorescent) spores in a total of 100 spores per repetition were determined using a fluorescence microscope (200 \times).

Results

Wind dispersal of *Puccinia psidii* urediniospores

More spores were captured at location A2 where rust infected plants were present than at the other three locations (Fig. 4). However, no significant differences ($p < 0.001$) were found

Fig. 3 Locations of traps installed near a eucalypt pulp mill and a port used for export of wood and wood pulp in the Espírito Santo State, Brazil



among numbers of spores captured at different heights. Urediniospores were found in traps installed at all heights, with the mean spore count ranging from 6.63 to 7.04 urediniospores (Fig. 4). After five months no more urediniospores were captured at any of the locations (Fig. 5). Teliospores were not detected at any of the four locations.

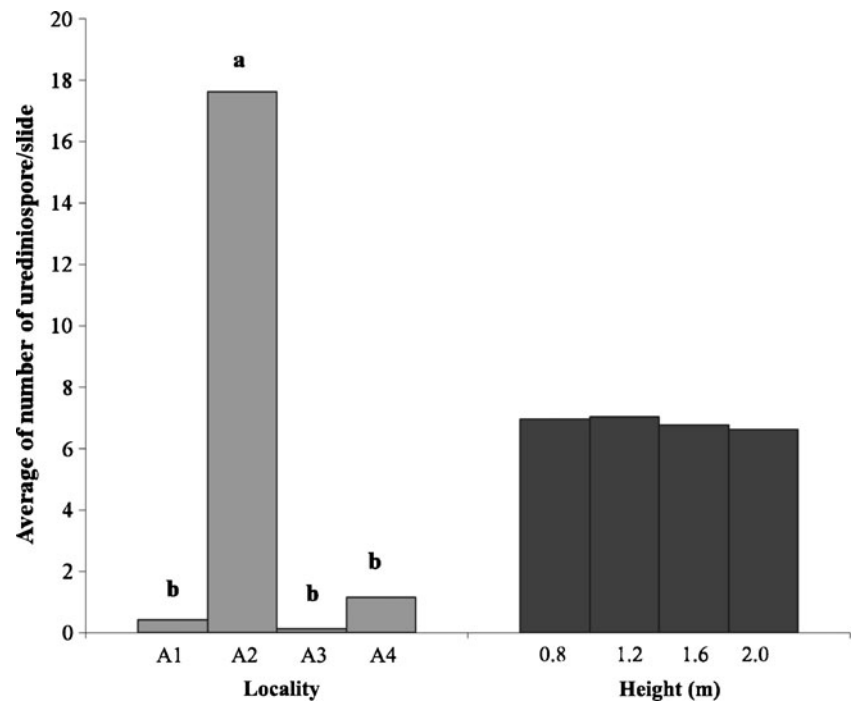
No environmental variable analyzed was correlated with spore capture at location A1. At locations A2, A3 and A4, relative humidity and precipitation were positively correlated

with spore capture. Temperature and solar radiation did not influence spore capture at the locations evaluated (Table 1).

Spore survival

Significant interactions were found between temperature, relative humidity and storage time ($p < 0.0001$) (Table 2). Both the FDA and LT methods proved efficient for evaluating *P. psidii* spore survival (Table 2), although FDA is

Fig. 4 Average of number of urediniospores/slide captured (A) in four different locations (A1, A2, A3 and A4, respectively), and (B) at four heights (0.8, 1.2, 1.6 and 2.0 m). Means followed by the same letter in each variable do not differ by Kruskal-Wallis ($p < 0.05$) test. The heights do not differ by Kruskal-Wallis ($p < 0.05$) test. The slide is 26×76 mm ($1,976$ mm²). A1—a saw mill; A2—a eucalypt plantation containing 5 month old infected plants; A3—a pulp mill; and A4—a harbour used for export of eucalypt lamber and pulp



more precise and less time consuming. Because the regression analysis of the data (Table 2) was not significant, we plotted the percent survival over time (Figs. 6 and 7). Higher survival of both spore types occurred at lower temperature and lower relative humidity. Spore survival significantly decreased with the increasing of storage time, regardless of the temperature, relative humidity and evaluation method. Highest survival was observed for spores stored at 15°C and 35% relative humidity. Both spore types survived for a maximum of 90 days, regardless of the storage and detection methods (Table 2, Figs. 6 and 7).

Discussion

In the present study, no differences were found in urediniospore capture at different heights, between 0.8 and 2m.

Zauza et al. (2010) found a significant negative correlation between *P. psidii* rust occurrence and height in a susceptible *E. grandis* clone in Viçosa Minas Gerais, Brazil, due to the reduced leaf wetness period and spore numbers that reached the leaf surface at greater heights; above 3 m the plants tended to escape infection.

No significant correlation was found between temperature and spore capture, but the urediniospore capture correlated positively with relative humidity at three locations and with rainfall at two locations. Greater urediniospore capture occurred from October 2006 to February 2007 at the locations where traps were installed. Zauza (2007) and Zauza et al. (2010) observed lower spore capture between December and June, a period of higher rainfall that may have washed spores from the atmosphere. Young plants, temperatures between 18 and 25°C and prolonged periods of leaf wetness are favorable for *P. psidii* infection in the field (Alfenas et al.

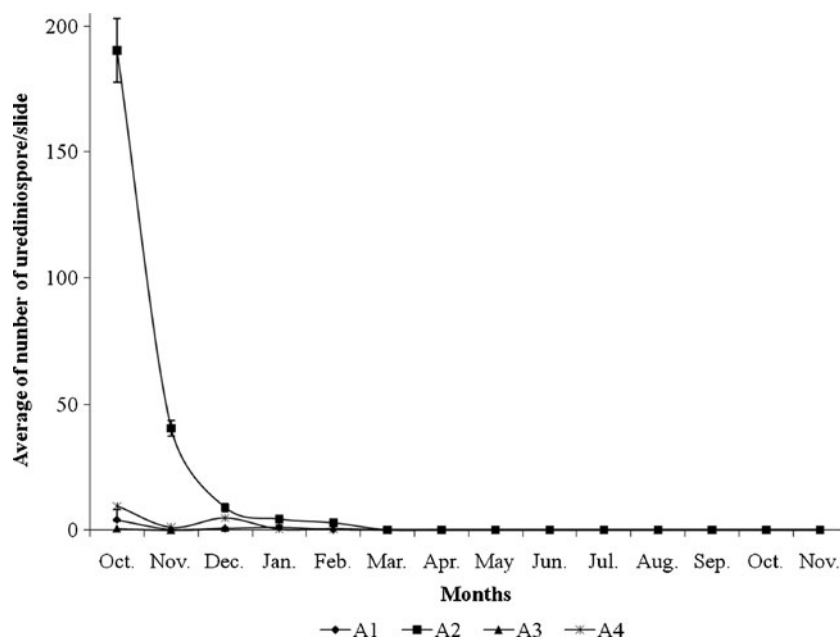
Table 1 Pearson correlation coefficients between number of urediniospores of *Puccinia psidii* spore capture and environmental conditions at four locations (A1 to A4)

Climatic variable	Unit	Mean	Locations			
			A1	A2	A3	A4
Global radiation	(MJm ⁻² d ⁻¹)	17.80	0.02*	-0.30*	-0.23*	-0.16*
Minimum temperature	(°C)	22.96	0.25*	0.10*	0.41*	0.39*
Average temperature	(°C)	23.41	0.24*	0.07*	0.40*	0.38*
Maximum temperature	(°C)	23.89	0.24*	0.05*	0.40*	0.38*
Minimum relative humidity	(%)	77.81	0.30*	0.73**	0.50**	0.53**
Average relative humidity	(%)	80.54	0.30*	0.71**	0.45**	0.53**
Maximum relative humidity	(%)	82.73	0.29*	0.69**	0.49**	0.52**
Rainfall	(mm)	98.65	0.13*	0.64**	0.18*	0.60**

* Not significant ($p > 0.05$)

** significant ($p < 0.05$)

Fig. 5 Average of numbers of urediniospores captured from October, 2006 to November, 2007 in four areas. Vertical bars represent standard errors of the mean. The slide used is 26× 76 mm (1,976 mm²)



2009). In general, when plants reach phenological stage B (Ferreira, 1989), and are about 3 to 4 m tall they escape infection (Zauza et al. 2010) and the quantity of infected plants that may disperse spores is thus reduced (Ferreira 1989). In the present study, no spores were captured after the first 5 months, which coincided with the onset of less favorable conditions for plant infection.

The FDA method of viable spore detection is less time consuming and more reliable than the LT method. FDA is used commonly to distinguish between viable and unviable cells of different pathogens. Viable cells turn blue as a result of hydrolysis of FDA by acetylsterase that produces a fluorescent compound readily observed under fluorescence microscopy (Brunius 1980; Dhingra and Sinclair 1995; Jones and Senft 1985; Ross et al. 1989). FDA also indicated a higher initial percent viability, up to 70% for both urediniospores and teliospores, whereas the highest germination on LT was only 15% for urediniospores and 25% for teliospores. These results indicate that the method of FDA is more appropriated than the LT method to evaluate spore survival of *P. psidii*, since live but dormant spores may not germinate readily. An increase in relative humidity and temperature resulted in reduced viability of urediniospores and teliospores, similar to results reported by Suzuki and Silveira (2003). Temperatures above 25°C reduced germination, colonization and sporulation of *P. psidii*, which partly explains the lower incidence of rust during warmer periods (Ruiz et al. 1989). Low *P. psidii* spore germination may be linked to the presence of auto-inhibitors activated under unfavorable conditions. The same authors observed that spore germination may be re-established when more favorable conditions are restored. This may help explain the lower percent urediniospore and teliospore germination

when the storage temperature was above 30°C and relative humidity was above 55%. In addition, under these conditions the saprophytic fungus *Aspergillus* sp. began to proliferate after 20 days of storage. Salustiano et al. (2008), using special storage conditions, including deep freezer (−80°C), liquid nitrogen (−196°C), and dissector (5°C)

Table 2 *Puccinia psidii* storage survival time at different temperatures and relative humidity evaluated by spore germination on *Syzygium jambos* (urediniospore) or *E. urophylla* × *E. grandis* (teliospore) leaf tissue (LT) and by flourescein diacetate staining (FDA)

Temperature (°C)	Relative humidity (%)	Survival of spores (days)			
		Urediniospore		Teliospore	
		FDA	LT	FDA	LT
15	35	50	90	60	90
	55	50	50	60	90
	70	30	20	30	50
	87	40	10	20	40
25	35	30	20	20	20
	55	30	20	20	20
	70	5	10	10	10
	87	5	20	0	5
30	35	10	10	10	30
	55	5	10	5	10
	70	5	10	5	10
	87	5	10	0	5
35	35	10	10	5	5
	55	10	5	0	5
	70	0	10	0	0
	87	0	5	0	0

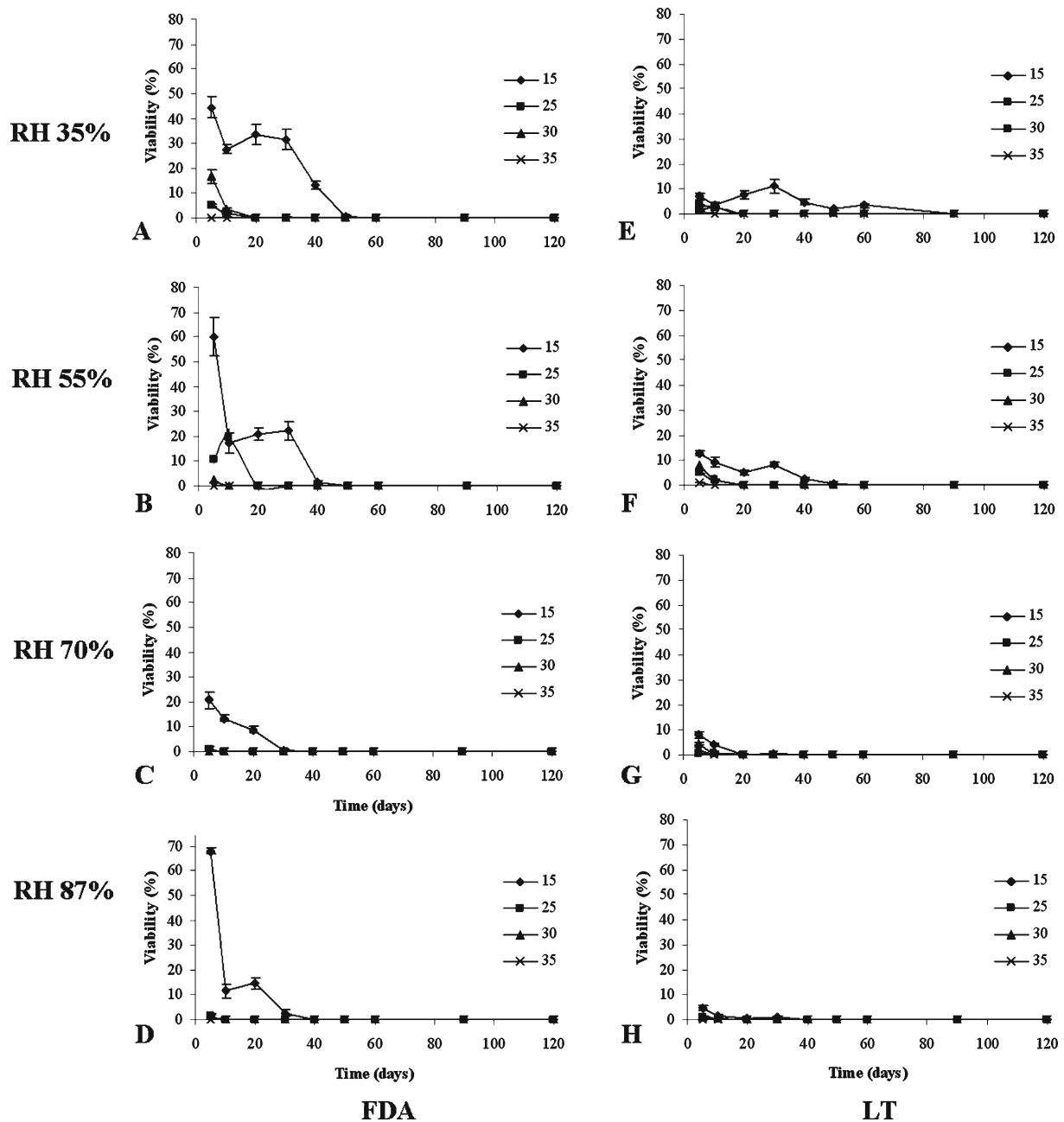


Fig. 6 Survival of *Puccinia psidii* urediniospores stored at different temperatures and relative humidities (RH) using two different evaluation methods. **a, b, c** and **d**—fluorescein diacetate staining (FDA). **e, f, g**

and **h**—germination on *Syzygium jambos* leaf tissue (LT). Vertical bars represent standard errors of the mean

found that urediniospores were kept alive and infective for up to 150 days. However, when they were maintained at 25°C as herborized specimens or in test tubes, they did not survive longer than 90 days. Under these conditions, spore germination dropped from about 23% at the beginning of the experiment for 1–2% at 30 days of storage (Salustiano et al. 2008). In our study, we tested different methods of storage of spores, trying to simulate the environmental conditions most likely to be found in the ship's hold, and both

urediniospores and teliospores did not survive longer than 90 days.

Commercial timber is cut and dried in driers that control temperature (40 to 90°C), relative humidity and air flow (Jankowsky 2000) and are lethal to pathogens. Similarly, spores do not survive the pulping and bleaching processes used in wood pulp production. Pulp is packaged and stored for export in covered warehouses. Even if spores land on package surfaces, they will hardly be able to survive the

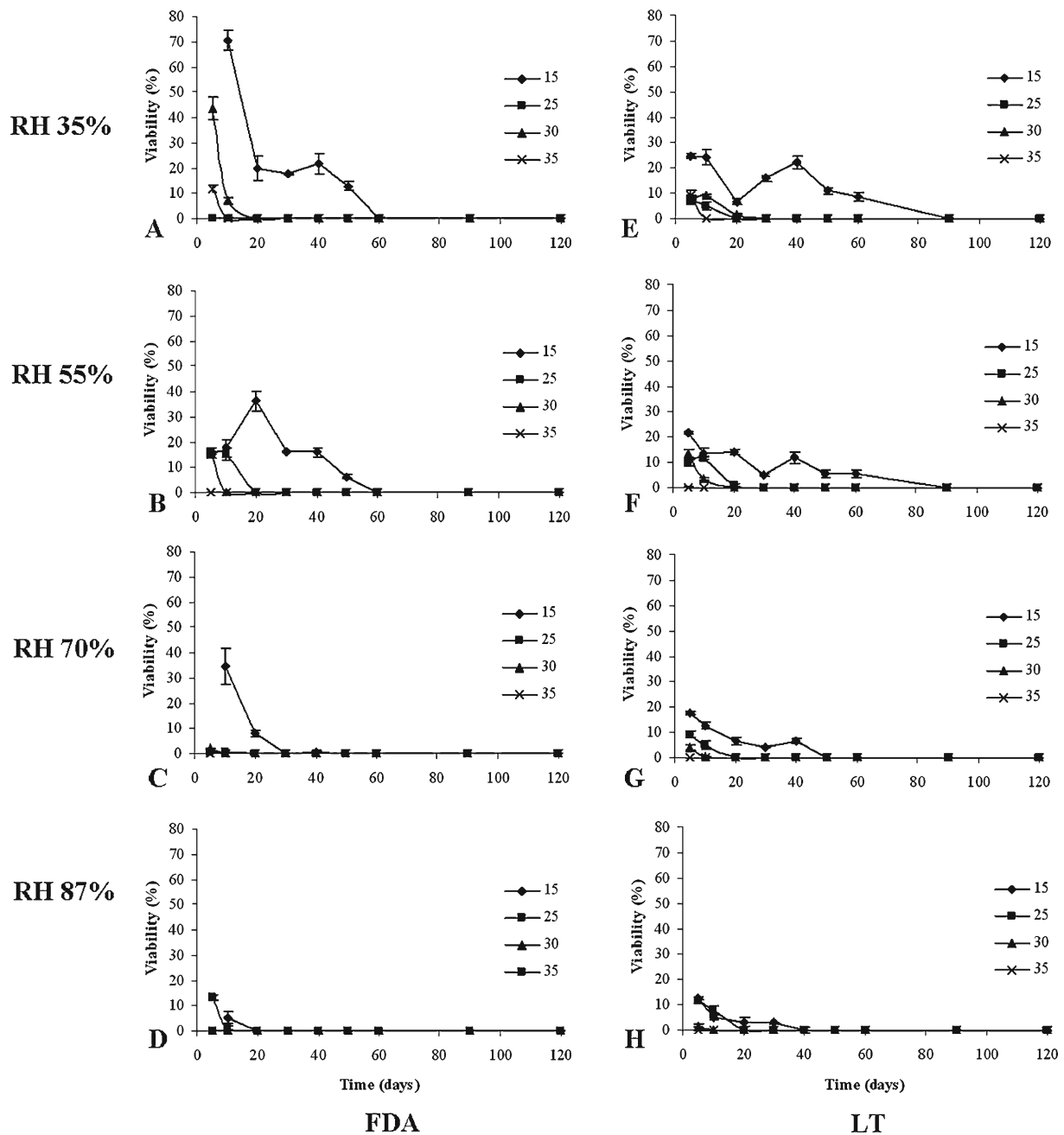


Fig. 7 The survival of *Puccinia psidii* teliospores stored at different temperatures and relative humidities (RH), for two different evaluation methods. **a, b, c** and **d**—fluorescein diacetate staining (FDA). **e, f, g**

and **h**—germination on eucalypt leaf tissue (LT). Vertical bars represent standard errors of the mean

temperature and humidity fluctuations commonly encountered during shipment to the product's final destination. Our results do not support the statement of Grgurinovic et al. (2006) on the presence of viable spores of *P. psidii* found as contaminant on *Eucalyptus* timber imported from Brazil to Australia.

Few studies have examined the possibility of pathogen dispersal via wood and wood products (Wingfield et al. 2001). Based on the results of the present study we conclude

that the risk of spread of rust spores carried by timber and pulp wood products exports is practically nonexistent because of the low inoculum quantity that reaches these products and the unfavorable environmental conditions typically encountered during their handling and transport. Storing wood products at temperatures above 25°C and relative humidity above 55% for 20 to 30 days is unfavorable for *P. psidii* spore survival. Furthermore, storage of wood products in closed warehouses far from pathogen sources will

avoid spread of disease to regions that import these products.

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