Extended survival of *Puccinia graminis* f. sp. *tritici* urediniospores: implications for biosecurity and on-farm management

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Abstract

Puccinia graminis f. sp. *tritici,* the causal organism of stem rust, is of global importance across wheat growing countries. However, some epidemics commence without the obvious presence of 'alternate' or 'green bridge' hosts, suggesting urediniospores can survive in the absence of suitable host plants for many weeks. Testing a range of inert material types, including metals, plastics, fabrics and woods, highlighted a significant effect of material type and temperature on urediniospore viability (P<0.001), with urediniospores remaining attached and viable on these materials (aluminium, paper, rubber, all fabric and all woods) for up to 365 days at 23°C/4°C

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day/night. However, at 36°C/14°C day/night, urediniospore viability was retained for a maximum of 300 days on denim and jute. . Further, at 45ºC/15ºC day/night, urediniospores remained viable to a maximum of 180 days on cotton and on jute. The frequency of recovery of attached urediniospores was also dependent upon the material type, with significant differences between materials in their abilities to retain urediniospores after washing (P<0.001). Urediniospores recovered even after 300 or 365 days from the lower two temperature regimes successfully initiated infections of wheat seedlings. Results confirmed the potential importance of inert materials as long-term carriers of viable stem rust of wheat (*P. graminis* f. sp. *tritici*) urediniospores, highlighting risks of spread of new pathotypes and strains across wheat growing regions, the significant biosecurity implications for contaminated carrier materials, and its likely survival across seasons without a host.

Introduction

Stem rust of wheat, caused by *Puccinia graminis* f. sp. *tritici* (*Pgt*) is of major economic importance worldwide, with severe epidemic outbreaks reported in South Africa, China, East Africa, Europe, Canada and South America (Roelfs and Bushnell, 1985; Saari and Prescott 1985; Kolmer, 2001; Kolmer *et al*., 2007;Morgounov *et al*., 2012). In the United States of America *Pgt* caused major wheat stem rust epidemics from 1900-1954 (Roelfs, 1978; Kolmer *et al*., 2007) with an average yield loss of 19.3% in South Dakota, 25.4% in Minnesota and 28.4% in North Dakota (Singh *et al*., 2015). Under suitable environmental conditions, yield losses to stem rust can be 70% or more reaching up to 100% under extreme disease conditions (Saari and Prescott 1985; Beard *et al*., 2004). In Australia, it has constituted a significant threat to cereal production since early 1900s (Brennan & Murray, 1988; Loughman *et al*., 2005; McIntosh, 2007; Park, 2007) and is considered the most important wheat disease in Australia (Zwer *et al*., 1992).

Severe rust epidemics of 1889, 1899, 1947 1950 and 1973 in Australia have resulted not only in significant production losses (up to 100%) but also impacted on farmer welfare (Rees, 1972; Rees & Syme, 1981; Park, 2007). There, warm, moist conditions promote the development of stem rust and increase its severity (Beard *et al*., 2004).

Control measures for stem rust are based on genetic resistance, eradication of alternate hosts, application of fungicides and preventive agricultural practices. An annual protection cost of AUD$124 million has been estimated for control measures against stem rust in Australia (Brennan & Murray, 1988).Stem rust has been controlled during the last century through resistant varieties using resistance genes (e.g., *Sr31)* and, outside of Australia, by eradication of the alternate barberry host (Morgounov *et al*., 2012). Although eradication of the alternate host eliminated sexual recombination in *Pgt*, urediniospores were still dispersed and transported long distances as a primary source of inoculum (Kolmer *et al*., 2007). While foliar fungicide sprays can be useful in the early stages of the infection, the extent of yield loss depends on the resistance of the wheat variety (Beard *et al*., 2004). Despite of the widespread development and use of resistant varieties, fungicides still contribute 41% of the total costs for managing overall rust (Murray & Brennan, 2009a,b). This is particularly in the wheat stem rust-prone summer rainfall areas of northern New South Wales and Queensland where prior to the introduction of such varieties the likelihood of significant crop losses was about one year in four (McIntosh & Brown, 1997).

In 1998 the identification of a new race of *Pgt*, Ug99, in Uganda, saw a renewed threat to wheat growing countries (Pretorius *et al*., 2000). Ug99 or its variants are virulent on many currently resistant varieties and can cause 80-90% crop losses under favourable conditions (http://www.fao.org/agriculture/crops/rust/stem/rust-report/stem-ug99racettksk/en/). Since then, *Ug99* and related races have been detected in Kenya, Ethiopia, Sudan, Yemen, Iran, Zimbabwe, Mozambique, South Africa and Tanzania (http://www.fao.org/agriculture/crops/rust/stem/en) and it is predicted to migrate further. Similarly, a new race TKTTF emerged in Turkey in 1990 and has subsequently been detected in Iran in 2010, Ethiopia and Lebanon in 2012 and Egypt in 2013 (Singh *et al*., 2015). Should these *Pgt* races become established in Australia stem rust would likely re-emerge as the single most major disease of wheat.

Pgt is a macrocyclic, heteroecious fungus that generally requires both primary (wheat or grasses) and alternate (*Berberis* or *Mahonia* spp.) host plants to complete its lifecycle via sexual teliospores (Schumann *et al.*, 2000). In Australia, teliospores are non-functional in disease epidemics as no alternate hosts are found (Watson and Luig, 1958; Park, 2007). Hence the pathogen only survives through generation and dispersal of asexual urediniospores and/or parasitic survival of mycelium on remnant living susceptible cereal hosts (e.g., wheat and

triticale in particular, but also barley and some grasses) (Park, 1996, 1997). These can provide a 'green bridge' for stem rust carryover from one season to another, with subsequent wind spread of urediniospores over large distances (Anonymous, 2017). For this reason, rust epidemics in Australia are often worse following wet summers that have supported the widespread growth of volunteers (Hollaway, 2016). In contrast, it is challenging to explain the occurrence of some rust outbreaks in the Mediterranean-type climatic region of south-west Western Australia. This region is characterised by hot dry summers and cool wet winters and it has long been suspected the authors that urediniospores of various cereal and other rusts can survive and remain infective over considerable periods, particularly under dry conditions without rainfall (MJ Barbetti, unpubl.).

Although rust urediniospores in general are vulnerable to environmental factors, such as temperature, moisture and ultraviolet light (Zadoks, 1961), *Pgt* urediniospores are relatively tolerant of a range of light and temperature conditions, especially when relative humidity is low, (Singh *et al.,* 2002*;* Hernandez Nopsa & Pfender, 2014). It is widely claimed that rust urediniospores do not survive on seed, stubble or soil (Hollaway, 2016), only surviving in the field for several weeks, to germinate and infect susceptible hosts (Singh *et al.*, 2002). However, urediniospores of bean rust (*Uromyces phaseoli*) survive overwinter in residues in fields in North Dakota (Gross & Venette, 2001), potentially providing initial inoculum for the next season's bean crops. Similarly, Twizeyimana & Hartman (2010) showed that viable soybean rust (*Phakopsora pachyrhizi*) urediniospores harvested from infected soybean leaves could be maintained at 23-24°C at 55- 60% RH for up to 18 days, while freshly harvested urediniospores that were first desiccated remained viable for up to 30 days. At 40 to 50°C, *P. pachyrhizi* urediniospore survival is temperature sensitive, for example, urediniospores are killed after 4-6 hours of exposure at 40-50°C but at 25°C survival was 15 hours (Twizeyimana & Hartman, 2010). Another example is subterranean clover rust, *Uromyces trifolii-repentis*, which has been found to readily survive on infested dead subterranean clover residues from one growing season to the next in Western Australia (MJ Barbetti, unpubl.). It is likely that the same occurs for *Pgt* and this has significant implications 'rolling' stem rust epidemics from one year into the next in the absence of infected host materials.

There may be possible movement of *Pgt* pathotypes across regional, state and international borders via air and sea cargo or directly through other human activities (McNeill *et al*., 2011; Savage *et al*., 2012). While pathogens may be introduced with increasing international trade and tourism (Wellings, 2007; Holliday *et al*., 2013), there is a wide range of potential 'carrier materials' that could facilitate entry and spread of exotic fungal spores (Barua *et al.*, 2017b). Some of extensively used materials such as fabrics, metals, paper, rubber tyre, leather and wood etc. in general can be the potential carrier of viable fungal spores (Hughes *et al.*, 2010; Osyczka et al. 2012).While urediniospores are the main source of inoculum of *Pgt* and can be dispersed long-distance up to 2000 km by wind (Luig, 1985), they are also subject to human-mediated transport (Brown & Hovmøller, 2002; Aylor, 2003; Yamaoka, 2014) as occurs with a wide range of fungal species. At Honolulu International Airport, 65 fungal species from 39 genera were isolated from shoes of travellers arriving from San Francisco (Baker, 1966). Two significant instances of human aided introduction of rust diseases are barley stripe rust into Columbia in 1975 (Roelfs & Bushnell, 1985) and wheat stripe rust (human, goods, or machinery urediniospore contamination) into Australia in 1979 (Wellings *et al*., 1987; Wellings, 2007). Further, in the early 1980's, high numbers of plant pathogenic fungal spores, including *P. coronata* (Sheridan & Nendick, 1988) were collected from clothing and baggage of passengers arriving by air in New Zealand (Sheridan and Nendick, 1988; Sheridan, 1989). In 1982, these examples included an estimated 70,000 viable rust urediniospores brought into New Zealand on travellers' clothing and baggage (Sheridan, 1989). Sheridan (1989) also monitored the movement of fungal spores attached to human bodies and clothing while conducting disease surveys of cereal crops and how human bodies and clothing carried many viable pathogenic fungi, including urediniospores of rusts such as *Puccinia coronata* and *Pgt*, *P. hordei*. Lana *et al.* (2012) reported low numbers of *Puccinia psidii* spores on wood products (timber and pulp) and suggested that these low numbers were due to adverse environmental conditions in the wood storage areas and during overseas transport that did not foster spore survival. However in 2004, the Australian Plant Quarantine Service (AQIS) detected *P. psidii* urediniospores on kiln–dried *Eucalyptus* timber imports from Brazil, plastic wrapping and the external surfaces of shipping containers and used molecular analysis to confirm maintenance of their viability throughout the two month sea journey (Grgurinovic *et al.,* 2006).

To address the biosecurity concerns outlined above, studies were undertaken to assess the long–term survival of *Pgt* urediniospores on various material surfaces. We highlight long-term survival of *Pgt* urediniospores on a range of different material types and across different temperatures and discuss implications.

Materials and methods

Under controlled environmental conditions, investigations were undertaken to determine the long term viability of *Pgt* urediniospores, in the absence of a host and at three different temperature regimes, on different urediniospore carrier materials.

Pgt inoculum

Pgt urediniospores (pathotyped as 34-1,2,7 rather than the more recent 34-1,2,7 +*Sr38* pathotype; Cuddy & Park, 2013) were provided by the Department of Agriculture and Food Western Australia . The urediniospores had been collected from infected wheat plants using a handheld vacuum collector and then dried over silica gel prior to storing at -80^{0}C until needed. Desiccated urediniospores were used for following reasons. First, fresh urediniospores were not consistently available across the time period of these repeated studies. Second, their use avoided any possible confounding effects of variation from successive urediniospore collections made across different wheat varieties, temperatures, ages of urediniospores, and their exposure to different relative humidities. In addition, urediniospores undergo natural desiccation from mid to late spring onwards under the rain-free Mediterranean period, a situation that may not change until autumn/early winter the following year. Viability of urediniospores was confirmed on potato dextrose agar (PDA) media (~95% relative humidity) and found to be ≥80% germination. Prior to inoculation, urediniospores were suspended in 0.001% Tween 20® and adjusted to a concentration of 10^6 urediniospores.ml^{-1} measured using a haemocytometer counting chamber (Superior® Marienfeld, Germany). Tween 20® was used to obtain full retention of inoculum to test surfaces and the low concentration used was pretested to show it did not affect viability or germination of *Pgt* urediniospores

nor that of other pathogens such as *Leptosphaeria maculans* (Phoma stem canker) and *Kabatiella caulivora* (northern anthracnose).

Selection of carrier materials

A total of 21 different carrier materials were selected to determine their effectiveness as potential spore carriers and any direct effects of the materials upon urediniospore survival. These were selected as test materials as they were common materials used in everyday life, found around farms and/or associated in one way or another in the transport of wheat, or commonly used by travellers or associated with freight transport. These were: *metals* - aluminium, brass, corrugated steel, galvanised iron sheet, steel, rusted steel, zinc; *fabrics* - cotton, denim, fleece, silk, fibre polyester; *woods* - *Eucalyptus marginata* (Jarrah), *Pinus radiata* (pine), *Eucalyptus regnans* (Tasmanian oak); and *'others'* - glass, leather, jute, paper, plastic and rubber tyre. All test materials were washed under running tap water for 5 minutes to remove any environmental contaminants and air-dried before use. Timber materials were sawn-milled, with no bark attached, and no commercial preservation treatments had been applied.

Effect of temperature and time on viability of *P. graminis* f. sp. *tritici* urediniospores

Inoculations of carrier materials.

The materials were cut into 0.5 cm squares, autoclaved and randomly placed into rows in sterile 48-well cell culture plates (Greiner® sterile).The material in each well was inoculated individually with 10 µl (10^6 urediniospores ml^{-1}) of inoculum and allowed to dry in a laminar flow for 2-3 h. All sides of the inoculated culture plates were sealed with Parafilm wrap and placed under one of the three controlled environmental conditions: 23 ± 1ºC day/8 ± 1ºC night, 36 ± 1ºC day/14 ± 1ºC night, or 45 ± 1ºC day/15 ± 1ºC night, that were based the 10 year climate data of 2005-2014 to represent the highest, lowest and intermediate average maximum/minimum temperature combinations for winter, summer and autumn/spring, respectively, in Western Australia. The photoperiod was 14 h and 10 h for day/night, with available light source LED cool white and incandescent light

bulbs with an intensity of 250 µmol.m^{-2}s^{-1}, 260 µmol.m^{-2}s^{-1}, or 320 µmol.m^{-2}s^{-1}, respectively (Quantum Flux MQ100, Apogee). Relative humidity (RH) in controlled environmental rooms ranged from 25-37% during the day and 68-84% during the night and was comparative in regions where *Pgt* epidemics can commence, e.g. the Western Australian grain-belt at Merredin, (mean RH at 9.00am across 1911-2010 October-March ranges 53 to 63%, http://www.bom.gov.au/climate/averages/tables/cw_010093.shtml). There were six replicates in different 48-well culture plates for each inoculated carrier material and each non-inoculated control material, with every sampling time and each temperature treatment arranged in a fully randomized design to measure the effect of temperature and time on urediniospore viability. The experiment was run over 365 days sampling to assess the effect of temperature on viability of urediniospores and the ability of the materials to retain or release viable urediniospores starting daily from day 1 until day 7, then weekly till day 30, after which sampling was undertaken at regular 30 day intervals concluding at day 365. The entire experiment was repeated twice (total of three identical experiments).

Effect of material type on viability of *Pgt* urediniospores

Recovery of *Pgt* urediniospores from inoculated carrier materials and determination of viable number of urediniospores.

Urediniospores were recovered from the carrier materials as described by Barua *et al*. (2017a, b). Briefly, 800 µl of 0. 1% Tween 20® was added directly to the treatment plates and then plates were placed on a rotary shaker for 40 min at 700 rpm. Preliminary studies showed that a concentration of 0.1% Tween 20® in deionised water was optimal for washing urediniospores from the materials without damaging them (data not shown). After washing, the carrier materials were removed from the residual spore suspension and prepared for microscopy studies. The urediniospore suspension was used to determine numbers of urediniospore recovered. Then, urediniospore viability was assessed using a recently developed, rapid and miniaturized system using Alamar Blue (resazurin dye; 7-hydroxy-3H-phenoxazin-3-one 10-oxide) where viable fungal spore metabolic activity converts dark blue resazurin into pink resorufin that provides a reliable indicator of the presence of viable spores (Barua *et al*., 2017a). In brief, the Alamar Blue bioassay was initially optimized as a spore viability carrier for *Pgt*

and optimum time for maximum metabolic activity was determined as 3 h. *Pgt* urediniospore suspensions ranging in concentration from 10^7 to 10 urediniospres.ml^{-1} by tenfold serial dilution were set up in 96-well assay plates and used as comparison standards. Six replicate wells were used for each concentration and were set up using 100 µl of fresh urediniospore dilution for the standards with 20 µl of Alamar Blue reagent. In the same way residual urediniospore solutions after washing were set with 100 µl of residual solution/wash suspension containing urediniospores with 20 µl of Alamar Blue reagent. Negative controls were also similarly set up with no urediniospores, but only deionised water, to determine the extent of any background absorbance. The assay plates were covered with aluminium foil (to exclude light) and then incubated on a rotary platform shaker (Innova™ 2100, New Brunswick Scientific) at 150 rpm at 22ºC for 1 min. Plates were then incubated at 37ºC for 3 h. The reaction was terminated by adding 50 µl of 3% sodium dodecyl sulphate (SDS) before measuring the absorbance. Plates were then placed back on the rotary shaker at the same speed for 30 sec to ensure a uniformly mixed end product before measuring absorbance at 600 nm and 570 nm using a spectrophotometer (Thermo Scientific Multiskan® Spectrum). The average of the background absorbance value at 600 nm for control wells was subtracted from all absorbance values of experimental wells at 570 nm.

A standard curve of 570-600 nm absorbance versus urediniospore concentration was plotted to calculate the viable number of rust urediniospores in residual solutions after washing. The absorbance readings were directly proportional to germination. The total number of viable urediniospores was determined for each replicate using the regression equation from the standard curve, and the mean calculated from the six replicates.

Determination of germination rate of the urediniospores recovered from carrier materials after time and temperature treatments.

After washing at each time interval, the residual spore suspensions recovered from the carrier materials were examined. A 20 µl of the recovered residual spore suspension was spread over PDA agar plates (≥95% relative humidity) and incubated at 22°C (8 h photoperiod) to observe the germination of the urediniospores. There were three single plate replications for each sample and 100 spores randomly selected to assess the percentage of germinated urediniospores using an Olympus (BX51) microscope.

Ability of recovered *Pgt* urediniospores to infect wheat seedlings after temperature treatments

Four-week-old wheat seedlings (cv. Wyalkatchem) were inoculated with rust urediniospores recovered from materials after 12 months from 23ºC/8ºC. Leaves of seedlings were inoculated with 20 µl of recovered residual spore suspension at a concentration of 10^3 urediniospores ml^{-1}. Three spot-inoculations were made randomly on each leaf across six replicate pots of wheat plants. Relative humidity was maintained at 85-90% for 24 h in a dew chamber and subsequently maintained in a glasshouse at approximately 18± 1°C. Six replicate pots of non-inoculated wheat plants as controls were also maintained similarly. Inoculated leaves from the test and non-inoculated control plants were collected from days 1 to 30 for microscopy studies. Similarly, wheat seedlings were inoculated with urediniospores recovered from 36ºC/14ºC day/night after 10 months and from 45 ºC/15ºC night after 7 months.

To examine differences in the ability of urediniospores from each treatment to cause infection, inoculated leaves were harvested after 12 days post-inoculation. Harvested leaves both showing and not showing visible symptoms were subjected to decolourization by immersing in a solution containing glacial acetic acid and ethanol at 1:1 ratio for 1 d (Carrillo *et al*., 2013) and then in acetic acid/ethanol/water (2:2:1) in plastic vials maintained at 23°C for 4-5 d (Uloth *et al*., 2015). Decoloured samples were washed with two changes of deionized water and stained with 1% cotton blue in lactophenol for 3 min. Whole wet mounts of stained leaves on microscope slides were then examined using an Olympus (BX51) microscope mounted with an Olympus DP71 camera system.

Experimental design and data analyses

Carrier material treatments were arranged in a complete randomized design with numbers of replications as indicated above. All experiments were carried out at controlled laboratory and glasshouse environments. All experiments were repeated twice (i.e., three identical experiments for each study) and the relationship between the initial and repeat experiments assessed using a paired *t*-test using GenStat (16th edition, GenStat Procedure Library Release PL23.2). Where there were no significant differences between experiments ($P>0.05$), data sets from the two most-similar experiments were pooled, re-analysed and presented as a single data set. All numbers

of viable *Pgt* urediniospores were expressed as a percentage of total number of urediniospores inoculated. Single factor ANOVAs were conducted using GenStat to determine the effects of temperature on the viability of the *Pgt* urediniospores at each assessment time across the 365 d period and across test materials (see Table 1 for significances and LSDs). Subsequently, multiple factor ANOVA including temperature, test material and time were undertaken (see Table 1 for significances and LSDs) as there were no significant changes to individual time point, temperature, or test material outcomes within the multiple factor ANOVA compared with single factor ANOVAs. Fisher's least significant differences were used to show significant differences.

Results

Effect of temperature and time on viability of *Pgt* urediniospores

The extent of viability of urediniospores depended upon the carrier material and the temperature over the 365-day time period. The viability decreased over time. At 23ºC/8ºC day/night, urediniospores remained viable for 365 days on materials except for brass, corrugated steel, zinc and plastic (Table 1). At 36ºC/14ºC day/night, viability was retained for up to 300 days on denim and jute. At 45ºC/15ºC day/night, urediniospores remained viable to a maximum of 180 days on cotton and on jute..

Maximum time for retention of urediniospore viability on each material at three temperature regimes.

Metals: At 23ºC/8ºC day/night, the viability of urediniospores decreased by 50 -70% on day 1 (after 24 h). The spore viability further decreased by 98-99% with viable urediniospores recovered up to 365 days from aluminium (1%), 300 days from brass (1%), and 120 days from corrugated steel (2%), 330 days from galvanised sheet (1%), 210 days from steel (1%) and rusted steel (1%) and 180 days from zinc (1%). At 36ºC/14ºC day/night, viability of urediniospores decreased by 80 -70% on day 1 (after 24 h) with a further decrease in viability by 96-99% with viable urediniospores were recovered up to 5 days from aluminium (2%), 3 days from brass (1%), and 4 days from corrugated steel (4%), 5 days from galvanised sheet (1%), 30 days from steel (1%),

4 days rusted steel (1%) and 3 days from zinc (1%) with no further viability observed. At 45ºC/15ºC day/night, viability of urediniospores decreased by 90 -99% on day 1 (after 24 h) with viable urediniospores were recovered up to 4 days from aluminium (1%), 2 days from brass (4%), and 3 days from corrugated steel (2%), from galvanised sheet (1%) and from rusted steel (1%), 6 days from steel (1%), 4 days rusted steel (1%) and 3 days from zinc (1%) with no more viability (Table 1).

Woods: At 23ºC/8ºC day/night, viability of urediniospores decreased by 34-54% on day 1 (after 24 h) which further decreased to 92-99% with viable urediniospores recovered from all three wood materials for up to 365 days; viz. jarrah wood (1%), pine wood (8%) and Tasmanian oak (3%) with no further viability observed. At 36ºC/14ºC day/night, viability of urediniospores decreased by 77-90% on day 1 (after 24 h) with viable urediniospores recovered up to 60 days jarrah wood (1%), 120 days from pine wood (1%) and 21 days Tasmanian oak (1%). At 45ºC/15ºC day/night, viability of urediniospores decreased by 82-93% on day 1 (after 24 h) further decreased by 99% with viable urediniospores recovered up to 7 days on jarrah wood (1%) and on Tasmanian oak (1%) and up to 21 days on pine wood (1%) (Table 1).

Fabrics: At 23ºC/8ºC day/night, viability of urediniospores decreased by 26-36% on day 1 (after 24 h) which further decreased to 90-99% with viable urediniospores recovered from all the fabric materials up to 365 days; viz. cotton (10%), denim (4%), fleece (1%), silk (3%) and fibre polyester (1%). At 36ºC/14ºC day/night, viability of urediniospores decreased by 48-81% after day 1 with further decrease to 99% with viable urediniospores recovered up to 240 days on cotton (1%), 300 days on denim (1%), 150 days on fleece (1%), 180 days on silk (1%) and 180 days on fibre polyester (1%). At 45ºC/15ºC day/night, viability decreased by 99% with viable urediniospores were recovered up to 180 days from cotton (1%), 150 days from denim (1%), 60 days from fleece (1%), 90 days from silk (1%) and up to 120 days from fibre polyester (1%) (Table 1).

'Others': At 23ºC/8ºC day/night, viability of urediniospores decreased by 33-60% on day 1 (after 24 h) which further decreased to 90-99% with viable urediniospores were recovered up to 365 days from glass (1%), jute (8%), leather (10%), paper (8%) and from rubber tyre (1%); only up to 300 days from plastic (1%). At 36ºC/14ºC day/night, viability of urediniospores decreased by 98-99% with recovery of viable up to 3 days on glass (1%), 300 days on jute (1%), 60 days on leather (1%), 120 days on paper (1%), 5 days on plastic (2%), and

60 days on rubber tyre (1%). At 45ºC/15ºC day/night, viability of the urediniospores decreased by 99% with viable urediniospores were recovered up to 2 days from glass (1%), 180 days from jute (1%), 14 days from leather (1%), 60 days from paper (1%) and 30 days from rubber tyre (1%) (Table 1).

Effect of material type on viability of *Pgt* urediniospores

The percentage of viable urediniospores isolated from different carrier materials varied with type of material. Different groups of materials i.e. group 1 (metals), group 2 (woods) group 3 (fabrics) and group 4 ('others') had different capacity to retain viable urediniospores and this was also dependent upon the temperature the materials were maintained at. The maximum and minimum average percentage of viable urediniospores recovered from the carrier materials varied with the temperature and type of material. The average maximum viable urediniospores recovered at three different temperature regimes after 365 days were as follows. At 23ºC/8ºC day/night, the maximum viable urediniospores were recovered from pine wood (44%), at36ºC/14ºC day/night from denim (18%) and at 45ºC/15ºC day/night from cotton (8%). There was a significant (P<0.001) negative correlation between time and temperature in terms of viability of urediniospores. At 23ºC/8ºC day/night, the maximum average percentages of viable urediniospores recovered after 365 days across each carrier material group were 21% from aluminium (group 1, metals), 44% from pine wood (group 2, wood), 37% from silk (group 3, fabrics) and 36% from leather (group 4, 'others'. At 36ºC/14ºC day/night, the average maximum percentages of viable urediniospores recovered after 365 days across each carrier material group were 6% from steel (group 1, metals), 4% from pine wood (group 2, woods), 18% from denim (group 3, fabrics) and 8% from jute (group 4, 'others'). At 45ºC/15ºC day/night, the maximum percentages of viable urediniospores recovered after 365 days across each carrier material group were 2% from steel (group 1, metals), 2% from pine wood (group 2, woods), 8% from cotton (group 3, fabrics) and 6% from paper (group 4, 'others').

The percentage of viable urediniospores recovered from materials within the same group type also varied with temperature. For example, while the maximum percentage of viable urediniospores recovered from metals at 23ºC/8ºC day/night was from aluminium (21%), while the maximum viable urediniospores recovered from steel at 36ºC/14ºC day/night and at 45ºC/15ºC day/night were 6% and 2%, respectively. Among the woods group of

carrier materials, maximum viable urediniospores recovered from pine wood depended upon temperature regime; viz. 44%, 4% and 2% across 23ºC/8ºC, 36ºC/14ºC and 45ºC/15ºC day/night, respectively. The maximum viable percentage urediniospores obtained from fabrics varied with the carrier material and temperature, from silk (38%) at 23ºC/8ºC day/night, from denim (18%) at 36ºC/14ºC day/night and from cotton (8%) at 45ºC/15ºC day/night. Across the 'others' group of test carrier materials, maximum viable urediniospores recovered from leather (36%) was at 23ºC/8ºC day/night, from jute (8%) it was at 36ºC/14ºC day/night, and from paper (6%) it was at 45ºC/15ºC day/night (Table 1).

Validation of assay: determination of germination rate of urediniospores recovered from carrier materials after time and temperature treatments

The germination of the spores varied over time and also with temperature. There was a significant (P<0.001) positive correlation between the percentage urediniospores germinated and percentage of viable urediniospores that reduced resazurin to resorufin in Alamar blue assay at temperature 23ºC/8ºC day/night over the time period of 365 days (R^2 = 0.97). Similar correlation was observed at 36ºC/14ºC day/night (R^2 = 0.99) and at 45ºC/15ºC day/night (R^2 = 0.95). At least 5% of the urediniospores recovered after 365 days from 23ºC/8ºC day/night, 1% from 36ºC/14ºC day/night after 270 days and 0.5% from 45ºC/15ºC day/night after 270 days successfully germinated on PDA.

Confirmation of infection of wheat seedlings from recovered *Pgt* urediniospores and detection of *Pgt* from inoculated wheat plants

Puccinia graminis f. sp. *tritici* urediniospores that been recovered from 23ºC/8ºC day/night or 36ºC/14ºC day/night caused infection symptoms on wheat following their inoculation. Early stages of infection were confirmed by appearance of light brown discolouration of the leaf tissues at points of inoculation by four days' post-inoculation. Subsequently, small brown rust lesions 0.5 mm to 2 mm developed at all inoculation sites within 7 to 10 days' post-inoculation; and by 11-12 days' post-inoculation, extension of lesions beyond the site of

inoculation was evident. However, urediniospores recovered from 45ºC/15ºC day/night, despite being viable (0.5% germination), did not produce infection symptoms.

Discussion

The current study opens up new possible explanations of extended survival *per se* that can initiate subsequent rust epidemics. This study demonstrates a significant effect of material type and temperature on *Pgt* urediniospore viability, for the first time across a range of material types, including metals, plastics, fabrics and woods, with urediniospores remaining viable on tested carrier materials for up to 365 days (aluminium, cotton, denim, fleece, fibre polyester, silk, jarrah wood, Tasmanian oak, jute, leather, paper and rubber) at 23°C/4°C up to 300 days (denim and jute) at 36ºC/14ºC, and up to 180 days (cotton and jute) at 45ºC/15ºC. While the number of viable urediniospores decreased over time and with increasing temperature, there is clearly greater potential for *Pgt* urediniospores to survive for much longer periods in the absence of a host than only up to several weeks previously considered feasible. However, there were suggestions that stem rust urediniospores could be more durable, and clearly have some resistance to atmospheric conditions providing their moisture content is moderate (20-30%, with long-distance transport and infection possible across the North American Great Plains (Roelfs 1985), from Australia to New Zealand (Luig 1985) and occasionally from East Africa to Australia (Watson & de Sousa, 1983). Importantly, in the current study, urediniospores recovered even after 12 months from the lower two temperature regimes (23°C/4°C and 36ºC/14ºC) successfully infected of wheat seedlings. However the urediniospores recovered from the higher temperature (45ºC/15ºC) were unable to infect the wheat seedlings although a very small percentage of spores (1%) successfully germinated on PDA.

The current study, showing the long period for which urediniospores can remain viable on a range of carrier materials and under a wide range of temperature conditions, highlights the importance of deposition and 'attachment' of urediniospores on various carrier materials that further enhances dispersal potential, both locally and large-scale as a consequence of a range of inadvertent human activities. Urediniospores of stem rust are relatively resistant to light and temperatures then other rusts at a relatively low humidity of 30% (Aydoğdu & boyraz, 2012). While urediniospores of soybean rust (*Phakopsora pachyrhizi*), both non-desiccated and

desiccated could remain viable up to 18 days and up to 30 days at 23-24°C at 55- 60% RH respectively could remain viable up to 4-6 hours at 40-50°C (Twizeyimana & Hartman 2010). Urediniospores of *P. psidii* from Eucalyptus spp. maintained viability up to 10 days at 35 or 40◦C with 35–55% relative humidity (Lana *et al*., 2012). It is noteworthy, that in the current study, high temperatures up to 45°C did not kill all urediniospores. This implies that some urediniospores, could survive and remain viable from one season to the next, even under relatively hot and dry environmental conditions frequently occurring between cropping seasons in Western Australia. Further, the frequency of recovery of urediniospores was dependent upon the carrier material type. These findings have major implications both for carryover across sequential cropping seasons to initiate stem rust epidemics, and for domestic and international movement of freight and commodities that clearly can effectively carry and retain viable urediniospores for up to 365 days.

The challenge in managing initiation and spread of *Pgt* epidemics is that urediniospores are primarily wind dispersed both locally and large scale (Sache, 2000). In particular, long-distance transport of urediniospores is especially important for distributing new genetically variants of rusts over lager areas (Rees, 1972), especially as the estimated mean rate of spread of *Pgt* approximates 35 km d^{-1} (Aylor, 2003). The dispersal patterns associated with the commencement and spread of rust epidemics is not only determined by the interaction between urediniospore availability and release timing, but also wind patterns (Savage *et al*., 2010). Wind plays an important role in urediniospore dispersal capacity, and it also dehydrates urediniospores, thereby promoting their long-term survival, as dried urediniospores survive longer than those retained in moist environments (Chen, 2005). In Israel, under a similar Mediterranean-type environment to south western Australia, it is observed that *Puccinia dracunculina* does not complete its sexual cycle there and urediniospores overwintering in dry leaves serve as the source of initial inoculum for the following season (Cohen *et al.*, 2013). Similarly, *Pgt* epidemics can be generated from minute amounts of viable inoculum; for example, studies in the Netherlands have shown that even a single uredinium/hectare surviving the winter, a level of inoculum which is below detectable thresholds, was sufficient to generate a subsequent spring rust epidemic (Singh *et al.,* 2002). A study by Mackey et al. (1991) demonstrated that while initial inoculum pressure influences the rust development, a significant epidemic could develop in highly susceptible varieties of barley from a relatively small amount of inoculum. Hence, even just a few *Pgt* urediniospores attached to wheat residues and/or inert carrier materials could play an important

role in the spread and subsequent initiation of infections and initiate subsequent epidemics from these apparently inconspicuous initial levels of inoculum.

The current study highlights a clear interaction between the type of carrier material and the quantity of urediniospores retained, with woods able to retain the most viable urediniospores, followed by fabrics and metals. Not only did retained urediniospores remain viable for up to a year on these materials, we confirmed their subsequent ability, even after 365 days, to infect wheat plants under favourable conditions. However, it is noteworthy that recovered urediniospores from carrier materials at 23ºC/8ºC and 36ºC/14ºC caused infection while those recovered from 45ºC/15ºC, no longer retained ability to successfully produce infections, the latter possibly for reasons outlined above. Although rusts are biotrophic pathogen (Duplessis *et al*., 2011), the pathogen urediniospores can easily be spread by wind or through infected plant material as well as contaminated clothing, footwear, baggage, plastic wrapping and the external surfaces of shipping containers etc. (Sheridan, 1989; Grgurinovic *et al.,* 2006).

In conclusion, the current study demonstrates that not only do *Pgt* urediniospores remain viable for a much longer period and at higher temperatures when attached to or embedded in non-living materials than has been historically accepted, but that at least some of these urediniospores still retain the ability to infect wheat. The outcomes of the current study demonstrate the potential importance of inert materials as long-term carriers of viable *Pgt* urediniospores, highlighting both the risks of spread of new pathotypes and strains into wheat growing regions as well as significant biosecurity implications in general for contaminated carrier materials previously considered as low-risk. We believe these studies will not only prompt new evaluation into possible sources of carryover of stem and other rusts in Australia, particularly in relation to their survival and spread from infested stubbles, but a wider reconsideration of risks associated with transmission of fungal spores being transmitted from one place to another involving human activities. Re-evaluation is especially needed for materials such as metals, plastics, fabrics and woods that have been historically considered of low biosecurity risk in regards to the movement of fungal plant pathogens.

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Table 1. Mean number of viable urediniospores (%) of *Puccinia graminis* f. sp. *tritici* isolated from tested metals, fabrics, woods and other miscellaneous materials ('others') over time period of 365 days incubation at each of the three different temperature regimes; 23 ± 1°C day/8 ± 1°C night, 36 ± 1°C day/14 ± 1°C night and 45 ± 1°C day/15 ± 1°C night.

Materials		Metals							Fabrics							Woods			Miscellaneous			
Temperature	Time (days)	Aluminium	Brass	Corrugated	Galvanised	Rusted steel	Steel	Zinc	Cotton	Denim	Fleece	Fibre polyester	Silk	Jarrah	Pine	Tasmanian Oak	Jute	Glass	Leather	Paper	Plastic	Rubber
	1	51	31	31	51	32	35	46	54	65	44	70	74	46	66	47	53	42	67	41	42	54
	2	46	31	29	45	27	26	38	53	60	43	70	74	45	65	46	52	42	62	40	40	53
	3	46	30	21	44	23	23	38	51	43	41	69	73	45	65	46	51	40	61	39	39	51
	4	42	42	21	41	22	21	36	51	43	41	67	71	45	65	46	51	40	68	39	38	50
	5	41	41	20	41	20	20	22	50	41	41	65	71	44	65	46	49	36	65	40	33	50
	6	41	41	20	33	19	20	15	49	40	38	64	61	43	61	46	49	31	62	35	29	47
	7	39	39	20	32	19	20	13	49	40	38	63	52	42	59	45	47	29	61	30	27	44
	14	39	39	19	32	19	19	11	49	37	26	61	47	42	57	44	37	24	57	27	25	43
23 ± 1°C day/8 ± 1°C night	21	21	21	16	17	18	19	11	48	36	19	56	46	42	56	44	34	15	42	27	17	43
	30	17	17	12	14	16	16	6	47	35	18	53	43	38	56	40	29	15	39	26	15	38
	60	14	14	9	13	13	11	3	46	32	13	51	32	29	55	35	24	14	35	25	13	22
	90	12	10	9	11	10	10	1	44	14	5	35	22	22	52	34	15	12	26	24	12	15
	120	12	10	2	10	9	8	1	42	12	3	22	20	13	42	29	14	9	22	19	11	12
	150	11	9	0	4	7	6	1	39	9	2	10	20	12	36	28	14	4	18	17	4	9
	180	7	7	0	2	2	2	1	30	5	1	3	19	11	29	24	13	2	11	14	2	3
	210	4	3	0	1	1	1	0	26	4	1	2	16	11	26	20	12	1	11	14	1	1
	240	1	1	0	1	0	0	0	15	4	1	1	16	11	20	20	10	1	10	11	1	1
	270	1	1	0	1	0	0	0	10	4	1	2	14	10	19	16	10	1	11	10	1	1
	300	1	1	0	1	0	0	0	10	4	1	1	11	9	12	10	8	1	10	10	1	1
	330	1	0	0	1	0	0	0	10	4	1	1	8	2	10	8	9	1	10	9	0	1
	365	1	0	0	0	0	0	0	10	4	1	1	3	1	8	3	8	1	10	8	0	1
Average		21	18	11	19	12	12	12	37	26	18	36	38	27	44	32	28	17	36	24	17	26
	1	11	6	11	1	10	20	9	32	52	29	20	19	15	23	11	20	1	14	26	11	20
	2	9	4	10	1	9	20	5	29	46	25	19	18	11	15	8	20	1	14	20	10	20
	3	7	1	9	1	3	19	1	29	35	22	20	18	10	12	2	19	1	13	19	9	16
	4	7	0	4	1	2	18	0	29	35	18	18	18	5	11	1	16	0	13	15	10	11
	5	2	0	0	1	0	18	0	29	34	14	16	18	4	9	1	12	0	8	12	2	11
	6	0	0	0	0	0	10	0	26	30	12	11	17	3	5	1	11	0	7	10	0	10
	7	0	0	0	0	0	9	0	21	31	9	10	13	1	4	1	10	0	7	9	0	6
	14	0	0	0	0	0	9	0	19	30	9	10	10	1	1	1	10	0	4	8	0	1
36 ± 1°C day/14 ± 1°C night	21	0	0	0	0	0	3	0	17	23	7	9	9	1	1	1	9	0	2	5	0	1
	30	0	0	0	0	0	1	0	12	22	7	4	8	1	1	0	9	0	1	4	0	1
	60	0	0	0	0	0	0	0	11	16	6	2	7	1	1	0	8	0	1	2	0	1
	90	0	0	0	0	0	0	0	11	9	4	1	5	0	1	0	7	0	0	1	0	0
	120	0	0	0	0	0	0	0	8	8	1	1	3	0	1	0	2	0	0	1	0	0
	150	0	0	0	0	0	0	0	6	4	1	1	2	0	0	0	2	0	0	0	0	0
	180	0	0	0	0	0	0	0	3	2	0	1	1	0	0	0	2	0	0	0	0	0
	210	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0
	240	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0
	270	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
	300	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
	330	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	365	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Average		**2**	**1**	**2**	**0**	**1**	**6**	**1**	**14**	**18**	**8**	**7**	**8**	**2**	**4**	**1**	**8**	**0**	**4**	**6**	**2**	**5**
	1	11	5	9	1	10	11	8	25	22	22	19	19	13	18	7	20	1	13	22	19	16
	2	8	4	7	1	8	10	5	23	22	20	18	16	10	12	1	19	1	10	20	7	16
	3	4	0	2	1	1	8	1	20	21	19	15	14	8	10	1	15	0	9	19	2	13
	4	1	0	0	0	0	6	0	19	10	14	14	11	4	3	1	15	0	8	14	0	9
	5	0	0	0	0	0	3	0	16	9	12	11	10	3	2	1	8	0	1	12	0	7
	6	0	0	0	0	0	1	0	11	9	10	10	9	3	1	1	8	0	1	10	0	5
	7	0	0	0	0	0	0	0	10	9	9	10	7	1	1	1	6	0	1	9	0	4
	14	0	0	0	0	0	0	0	10	8	7	8	4	0	1	0	6	0	1	7	0	1
45 ± 1°C day/15 ± 1°C night	**21**	0	0	0	0	0	0	0	10	7	4	7	2	0	1	0	6	0	0	4	0	1
	30	0	0	0	0	0	0	0	10	5	2	1	1	0	0	0	4	0	0	2	0	1
	60	0	0	0	0	0	0	0	9	3	1	1	1	0	0	0	2	0	0	1	0	0
	90	0	0	0	0	0	0	0	5	1	0	1	1	0	0	0	2	0	0	0	0	0
	120	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	1	0	0	0	0	0
	150	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0
	180	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
	210	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	240	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	270	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	330	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	365	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Average		**1**	**0**	**1**	**0**	**1**	**2**	**1**	**8**	**6**	**6**	**6**	**4**	**2**	**2**	**1**	**5**	**0**	**2**	**6**	**1**	**3**

	Material	Temperature	Time	Material x Temperature	Material x Time	Temperature x Time	Material x Temperature x Time
Significance	*P*<0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001
l.s.d (P<0.05 =)	0.1924	0.0727	0.1924	0.3332	0.8816	0.3332	1.5269