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RESEARCH NOTE

Use of cellophane to study the infection structures of PNG cocoa isolates of *Phytophthora palmivora* in vitro

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Abstract

Cocoa pod isolates of *Phytophthora palmivora* (Ppal) from different locations in Papua New Guinea (PNG) were grown in the presence of cellophane to determine their abilities to mechanically penetrate this barrier and to gain access to the growth media below. Four Ppal isolates, MAG14, MAG30, MAG50, NSP11 and the plant root pathogenic oomycete *P. cinnamomi* for comparison, were tested for their cellophane penetrating ability. All were able to penetrate the cellophane by forming appressoria and infection peg-like structures. All Ppal isolates formed frond-like structures which grew at various depths within the cellophane while *P. cinnamomi* formed hyphal aggregations and frond-like structures within the cellophane. This is the first in vitro study demonstrating how Ppal can modify its hyphal morphology to form lobed structures upon contact to the cellophane surface and branched aggregating hyphae within cellophane to gain access to growth media below.

Keywords Infection · Cellulose · Oomycete · Plant pathogen · Penetration assay

The plant pathogenic oomycete, *Phytophthora palmivora* (Ppal) causes rot diseases in many plants including cocoa (*Theobroma cacao* L.) (Acebo-Guerrero et al. 2012; Erwin and Ribeiro 1996). It infects various parts of the cocoa plant and when found in the cocoa pod causes black pod rot (Guest 2007). The latter has economic impact as the infected pods can no longer be sold contributing to the economic loss to many cocoa farmers around the world including those in Papua New Guinea (PNG) (Guest 2007). The oomycete's infection process begins when motile zoopores, released from cadacous sporangia, adhere to the plant surface, and form cysts which germinate after being triggered by environmental factors. The germ tubes enter plant tissue directly via openings such as stomata or via wounding and by the formation of an appressorium upon contact on the plant wall cell surface or the plant cuticle. An appressorium peg allows the oomycete to penetrate the plant cell wall and Ppal invaginates into the plant cell to form an haustorium during its biotrophic growth stage and switches to a necrotrophic lifestyle where plant disease symptoms can be observed.

Work by Saul Maora et al. (2017) collected 263 isolates of Ppal from diseased cocoa pods as well as isolates from soil, stem and leaf lesions, from various farms at five different locations in PNG or from culture collections. Genetic analysis revealed limited geographic, temporal or host specialization, suggesting continuous selection for pathogenicity from a genetic pool of Ppal (Saul Maora et al. 2017). To investigate further which genes contributed to the PNG Ppal pathogenicity in cocoa, we were interested in studying the infection process of these isolates. Due to limited access to cocoa pod material, I used an in vitro assay which has been used to study the infection of foliar plant pathogenic fungi such as *Magnaporthe grisea* (Bourett and Howard 1990), *Setosphaeria turcica* (Gu et al. 2014) and the oomycete, *Phytophthora parasitica* var. *nicotianae* (Gaulin et al. 2002). A cellophane membrane/sheet was used to study the development of infection structures by Ppal. The aim was to investigate the ability of PNG Ppal isolates to penetrate cellophane (regenerated cellulose) in vitro to mimic how *Phytophthora,* upon adhering to the cocoa pod surface, could penetrate a plant cell wall, which is composed of cellulose microfibrils

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cross-linked by heteropolysaccharides (Lampugnani et al. 2018).

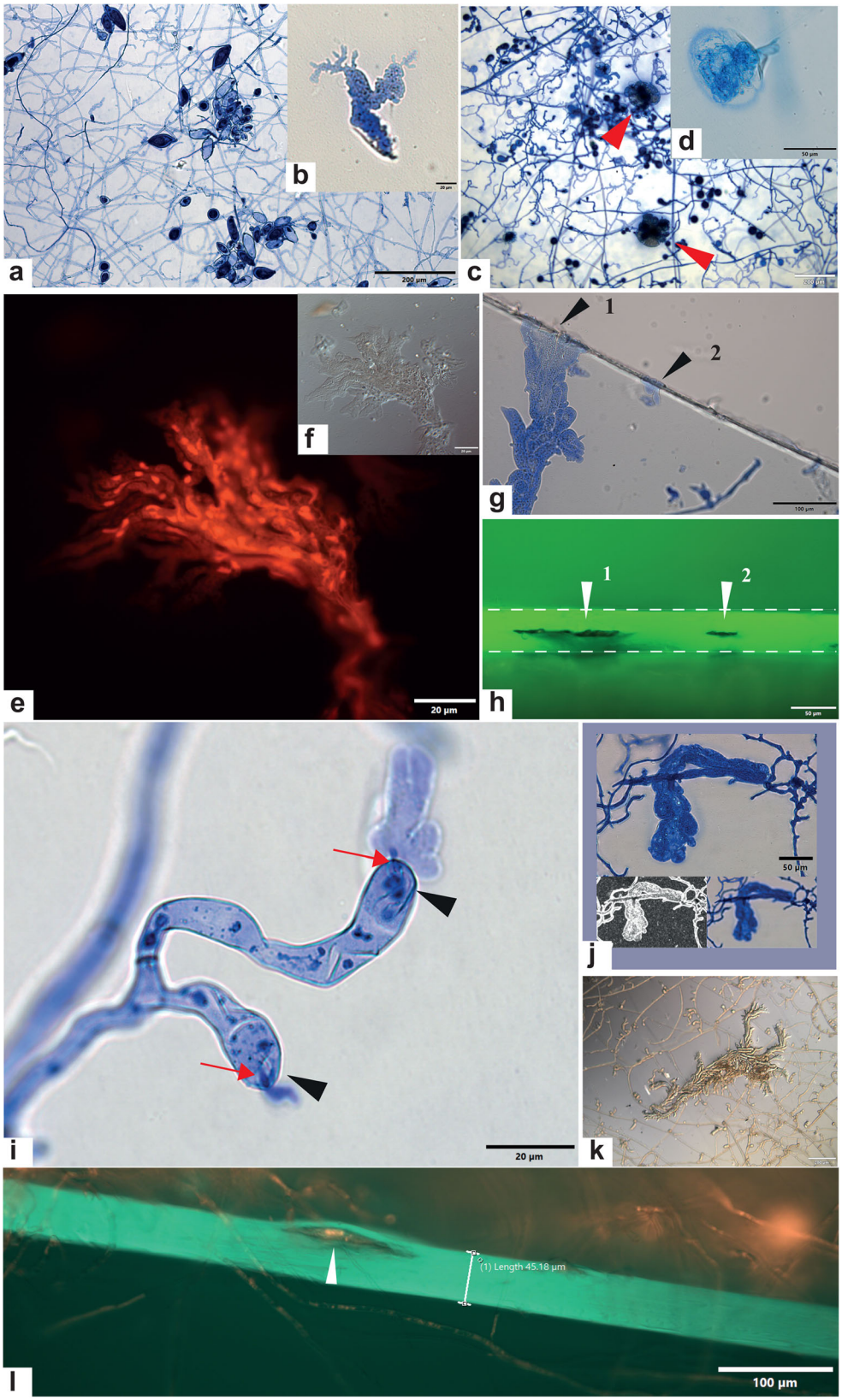
Cultures of four *Phytophthora* isolates from PNG were stored in water agar blocks in sterile distilled water in McCartney bottles at room temperature since 2005. Isolate MAG14, MAG30 and MAG50 were from diseased cocoa pods from plantations in Balama, Barum and Vidar respectively in the province of Madang, PNG. Isolate NSP11 was from a diseased cocoa pod in Panakei in the province of Bougainville, PNG. The isolate of *Phytophthora cinnamomi* Rands was kindly provided by the Royal Botanic Gardens and Domain Trust (Sydney, Australia). The water agar blocks from each culture were transferred to fresh carrot agar (CA) medium to determine their viability at 26 °C. Viable cultures were maintained by cutting 0.5 cm × 0.5 cm blocks of culture and subculturing on fresh CA, ¼ strength Cornmeal agar (CMA) or ¼ strength Potato Dextrose Agar (PDA) media. Petri dishes of culture were placed in the dark incubator at 26 °C. For the in vitro cellophane penetration assay, cellophane sheets about 45 μm in thickness were cut to fit 9 cm diameter petri dishes and were sterilised in water prior to use. Under aseptic conditions, one sterile cellophane sheet was placed on agar media and one block (0.5 cm × 0.5 cm in size) of 7-d old *Phytophthora* cultures was placed in the centre of the medium covered with a cellophane sheet. Petri dishes of culture were placed in the dark incubator at 26 °C. Triplicates in vitro cellophane penetration tests were performed on ¼ strength Cornmeal agar (CMA) or Potato Dextrose Agar (PDA) for each *Phytophthora* isolates.

To investigate the infection structures formed on the surface or within cellophane sheets, various stains were used. Drops of lactophenol blue solution (Sigma-Aldrich PTY Ltd., no. 61335) were directly applied on *Phytophthora* isolates grown on cellophane sheets in agar plates. Propidium iodide (PI; Sigma-Aldrich PTY Ltd., no. P4170) and calcofluor white (CFW; Sigma-Aldrich PTY Ltd., no. 18909) were used following the manufacturer's protocol for fluorescence staining to detect hyphal structures to pinpoint the location of *Phytophthora* isolates within the cellophane sheets. Three cut sections of cellophane with or without agar from each petri dish were placed on microscope slides with coverslips for viewing. For PI treatment, cellophane sheets were stained in PI (PI stock solution 1 mg/mL in sterile H_2O) for 15 min and washed in sterile water prior to viewing under the microscope. To determine growth within the cellophane by *Phytophthora*, cultures were carefully scraped off the surface of the cellophane and cut sections viewed under the microscope. Samples were either stained with lactophenol blue solution, PI or CFW.

For light and fluorescence microscopy, images were captured with an Olympus digital colour and monochrome CMOS DP74 camera with the Olympus CellSens Standard software Version 2.2. Differential interference contrast (DIC) or bright field was used. To capture CFW and PI stained *Phytophthora* isolates in cellophane, the U-MWU2 filter cube (excitation BP 330–385 nm) and U-MWIB2 wide band IF blue filter cube (excitation BP 460–490 nm /emission 510 nm interference filter/ dichromatic mirror 505 nm) were used respectively. The U-MGFPHQ filter cube (excitation BP 460–480 nm/ emission BA 495–540 nm/ dichromatic mirror 405 nm) was used on lactophenol blue solution stained cut sections. Under both U- MGFPHQ and U-MWIB2 filter cube settings, cellophane autofluorescence was observed appearing green in colour. To capture images from samples that extended beyond the depth of focus, the Extended Focus Image (EFI) option was used (under manual setting). This allowed the use of the fine focus adjustment to combine many images in the z-axis to provide a single combined output which can be used for visualization within the cellophane sheet. The number of stacks taken for such images was recorded.

Post 7 d growth, all PNG Ppal isolates from cocoa pods formed hyphae, chlamydospores and sporangia on the surface on the cellophane (Fig. 1a). For *P. cinnamomi*, collaloid hyphae, hyphal swellings and/or chlamydospores were observed on the cellophane surface (Fig. 1c; Fig. S1a). Observations under the microscope of the cellophane sheet found that all PNG Ppal isolates from cocoa pods and *P. cinnamomi* were able to penetrate the 45 μm thick cellophane sheet on ¼ strength CMA or ¼ strength PDA media (Fig. 1b–e – l; Fig. S1a-h). Appressorium-like and penetration peg-like structures were observed upon contact to the surface of the cellophane (Fig. 1i; Fig. S1a). This is interesting as the hydrophobic surface of leaves induces the formation of appressoria in *M. grisea* but when grown on the

Fig. 1 Growth and hyphal branching of PNG cocoa Ppal isolates and *P. cinnamomi* on cellophane post 7 d. **a,** Growth of Ppal isolate MAG14 on cellophane. **b,** Formation of lobed and frond-like hyphae by isolate MAG14 remaining on the cellophane sheet after removing the overlaying growth on the cellophane surface. **c,** Growth of *P. cinnamomi* on cellophane. **d,** Close up of the lobed or dense hyphal aggregations of *P. cinnamomi* on the surface and within the cellophane under DIC. **e,** Formation of lobed/frond-like hyphal structures of isolate MAG50 with the cellophane sheet stained with PI. **f,** Same as (e) under DIC. **g,** Formation of frond-like hyphae by Ppal isolate MAG30 within the cellophane. **h,** Transverse section of (g) highlighting the position within the cellophane (between the dashed white lines). **i,** Formation of the appressoria-like (black arrowheads), penetration pegs (red arrows) and lobed structures on the surface of cellophane by Ppal isolate MAG30 (black arrowheads). **j,** EFI profile of Ppal isolate MAG30 composed of 17 segments at 1 μm increment. **k,** Frond-like hyphae of isolate MAG30 within cellophane under DIC. **l,** Intra-cellophane growth of NSP11 (white arrowhead) after staining with PI highlighting the depth of the cellophane layer (45.18 μm). Images a, b, c, d, g, h, i and j were stained with lactophenol blue solution. Images (e) and (l) were stained with PI. All observations were on ¼ strength CMA medium. In images (h) and (l), cellophane autofluorescence (in green) under U-MGFPHQ and U-MWIB2 filter cubes respectively



hydrophilic surfaces of rice roots, hyphododia formed prior to penetration (Sesma and Osbourn 2004). However, Bircher and Hohl (1997) demonstrated that Ppal appressoria formation occurred at low levels on the hydrophilic surface of cellophane but increased in frequency on scratched surfaces of cellophane. Topographical signalling

such as scratches/grooves on the cellophane surface (Fig. S1i-j) could have induced Ppal appressorium-like formation on the surface of the cellophane. Furthermore, hyphal swellings (appressoria) were observed in *P. cinnamomi* prior to intracellular penetration in eucalypt roots (Tippett et al. 1976). Below such structures, lobed hyphal structures extended from them which appeared to be within the cellophane (Fig. 1i; Fig. S1a). To confirm if cocoa Ppal isolates and *P. cinnamomi* were able to grow within the cellophane, cut sections of cellophane had surface growth removed and were viewed under the microscope. *P. cinnamomi* and Ppal isolates formed lobed (Fig. 1c) or frond-like (Fig. 1 e-l; Fig. S1d-g) hyphal structures within the cellophane layer which spanned over several focal planes (Fig. 1j; Fig. S1d-g). *P. cinnamomi* formed dense hyphal aggregations on or within the cellophane (Fig. 1c, d; Fig. S1d). Cocoa Ppal isolates and *P. cinnamomi* were able to gain access to the agar media below the cellophane sheet where hyphal branching structures occurred within the cellophane post 7 d growth (Fig. 1l, Fig. S1d -h).

Such hyphal branching structures observed in cocoa Ppal isolates are similar to those that have been observed with *Phytophthora gonapodyides* (Trinci 1984) and *P. parasitica* var. *nicotianae* (Gaulin et al. 2002) grown on cellophane while *P. cinnamomi*'s dense hyphal aggregations are similar to those observed in *P. cinnamomi* on the surface of naturally infected roots of *Banksia grandis* and in 4-week-old V8A cultures (Jung et al. 2013). Gaulin et al. (2002) demonstrated that the cell wall of *P. parasitica* var. *nicotianae* contained a CBEL (cellulose binding elicitor of defense in plants and lectin-like) protein (Gaulin et al. 2002). Furthermore, transformed *P. parasitica* var. *nicotianae* strains suppressed in CBEL expression were unable to adhere to a cellophane membrane, to differentiate into lobed structures upon contact with the cellophane and to form branched aggregating hyphae on cellophane (Gaulin et al. 2002). Interestingly, lectins and one CBEL were identified as putative extracellular proteins within the secretome of Ppal (strain LILI) (Evangelisti et al. 2017). Recent genome and transcriptome Ppal infection studies in cocoa pod husks have also identified several putative CBELs (Ali et al., 2017; Morales- Cruz et al. 2020) suggesting that CBELs in PNG Ppal cocoa isolates could play a role in the pathogenicity in cocoa. CBEL was one of seven putative pathogenicity genes identified by RNA-Seq analysis of pre-infection structures in *P. cinnamomi* (Reitmann et al. 2017). The use of EFI confirmed intra-cellophane growth by Ppal and *P. cinnamomi* spanned several micrometres in depth explaining how some isolates could penetrate the cellophane sheet to reach the agar media. Many phytopathogenic and saprotrophic fungi such as *Fusarium graminearum* and *Arthrobotrys oligospora* have been shown to modify their hyphae for intra-cellophane growth forming branched aggregating hyphae (Demoor et al., 2019) similarly to that observed by Ppal cocoa isolates in this study.

In conclusion, Ppal formed lobed structures upon contact with the cellophane and branched aggregating hyphae in the in vitro cellophane penetration test.

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Author's contributions The author contributed to the study conception and design. Material preparation, data collection and analysis were performed by F. P-W.

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