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SHORT COMMUNICATION

Use of calcofluor white to detect β-glucan changes in *Phytophthora palmivora* oospores by fluorescence microscopy

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Abstract

Calcofluor white is a fluorochrome used for detecting β-glucans in cell walls of plant pathogenic fungi. The aim of this study was to detect β-glucans in oospores of the heterothallic *Phytophthora palmivora* by crossing two compatible A1 and A2 mating types on carrot agar plates with or without a supplement of aqueous French bean extract. Lack of calcofluor white induced fluorescence, in yellow to deep brown oospores, suggests a change in the type of β-glucans in the outer oospore-oogonium cell wall. This staining method is an easy, quick and visual way to monitor changes in β-glucans during oospore development.

Keywords Cellulose · Oogonium · Antheridium · Heterothallic

The plant pathogenic oomycete, *Phytophthora palmivora* (Butler), causes black pod disease of cocoa and is also capable of infecting a wide host plant range (Erwin and Ribeiro 1996; Guest 2007; Perrine-Walker 2020a, b). Apart from asexual sporangium/zoospore development as a means of rapid reproduction, this heterothallic species requires two compatible mating types (A1 and A2) for sexual reproduction (Ko 1978; 1988). In heterothallic species, the production of the oospores occurs through the fusion of the oogonium and antheridium (maternal and paternal gametangia, respectively) compared to homothallic ones which produce oogonia and antheridia in single cultures (Martin et al. 2012). Oospores serve as resting structures which act as inoculum for disease if viable between growing seasons (Judelson and Blanco 2005). Previous cytological and ultrastructure studies in gametangial development, oospore formation, germination and dormancy have contributed to the morphological identification of various heterothallic and homothallic species (Beakes and Bartnicki-Garcia 1989; Duncan 1988; Hüberli et al. 1997). Other morphological features of *Phytophthora* sexual organs are the antheridia being either paragynous or amphigynous and the ornamented oogonial walls (Martin et al. 2012). In this case, *P. palmivora* forms amphigynous antheridium where the oogonial hyphae grows through the antheridial hyphae forming a kind of collar that surrounds the antheridial stalk (Ho 1979) and non-ornamented oogonial walls (Martin et al. 2012).

β-glucans are polymers of β-D-glucose found in the cell walls of plants, fungi, yeast, and bacteria (Novak and Vetvicka 2008; Rebaque et al. 2021; Robinson and Bostock 2015). The most abundant form of β-glucans in the cell walls of plant fungal pathogens, β-1,3-glucans, have been shown to act as MAMPs (microbe-associated molecular patterns) and play a role in plant immune responses (Fesel and Zuccaro 2016; Klarzynski et al. 2000; Oliveira-Garcia and Deising 2013; Mélida et al 2018; Wanke et al. 2020). In addition, these β-1,3-glucans can be modified with β-1,6-linked glucose and in fungal cell walls, be covalently linked to another MAMP, chitin, a linear polysaccharide composed of β-1,4-linked N-acetylglucosamine residues (β-1,4-GlcNAc; Sánchez-Vallet et al. 2015; Rebaque et al. 2021; Wanke et al. 2021).

Phytophthora is known to have different types of β-glucans within its cell walls (Mélinda et al. 2013; Wang and Bartnicki-Garcia 1982). According to Mélinda et al. (2013), the cell wall analyses of two plant pathogenic species, *Phytophthora infestans* and *Phytophthora parasitica* were identified as Type I. Type I cell walls consisted 85.6% β-glucans where 32 to 35% was made up of cellulose (1,4-β linked glucose; β-1,4-Glc) and about 19.7% of β-1,3-glucans (Mélinda et al. 2013). In the case of *P. palmivora* cell walls,

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Tokunaga and Bartnicki-Garcia (1971) demonstrated that walls of cysts, hyphae and sporangia of *P. palmivora* consisted of β-glucans with 1,3-, 1,4- and 1,6- linkages. Later work by Lippmann et al. (1974) demonstrated that chemical composition of oospore-oogonium walls (oow) of *Phytophthora megasperma* var. *sojae* was made up of insoluble glucans (approximately up to 80%) where the majority was highly insoluble non-cellulosic glucan with β-1,3-linkages. Furthermore, less than 10% of the oow was cellulose (Lippmann et al. 1974).

In microscopy, calcofluor white (CFW) has been used to detect β-glucans in the cell walls of pathogenic fungi and yeast (Nicholas et al. 1994). It interacts with β-1,4-glucans such as chitin and cellulose (Nicholas et al. 1994) and other β-glucans such as callose (Hughes and McCully 1975; Maeda and Ishida 1967; Wood 1980). Previous work by Perrine-Walker et al. (2019), Perrine-Walker (2020b) used CFW to detect β-glucans in the cell walls of *Phytophthora cinnamomi* and *P. palmivora* hyphae and sporangia respectively. For this study, the aim was to use CFW to localise β-glucans during *P. palmivora* oospore development under fluorescence microscopy.

Two mating types of *P. palmivora* cultures were used: UQ3694 (A1) isolated from *Syagrus romanzoffiana* (cocos palm) and UQ3746 (A2) isolated from *Lupinus angustifolius* (NZ Blue Lupin). Pure cultures were maintained on Potato Dextrose Agar (PDA; CM0139B, Oxoid Ltd) and Carrot Agar (CA) (Erselius and Shaw 1982).

For mating, agar blocks (0.5 cm × 0.5 cm in size) from the edge of 7-d old *Phytophthora* mating type were places on opposite end of either fresh CA or CA supplemented with dwarf French bean extract plates (Fig. S1). The dwarf French bean extract was used according to the method of Duncan (1988) where 10 mL of the autoclaved extract was added to 100 mL of the CA molten agar media. *Phytophthora* sp. forms hyaline hyphae, sporangia and zoospores however oospores are coloured (yellow to brown) in agar and when grown in agar medium supplemented with dwarf French bean extract appear golden to deep brown (Duncan 1988). For the controls, same mating types were inoculated together. All cultures were incubated at 26 °C in the dark using BINDER BD 115 incubator (GmBH, Germany).

For light and fluorescence microscopy, images were captured using an Olympus BX51 microscope equipped with an Olympus digital colour and monochrome CMOS DP74 camera and the Olympus CellSens Standard software Version 2.2. Bright field was used for light microscopy and 1 × PBS (pH 7.4) or sterile water was used.

Under bright field microscopy, a *P. palmivora* oospore development map was generated over a 25 day-period. Duplicate plates were observed post 7, 14, 21 and 25 days. *P. palmivora* isolates UQ3694 (A1) and UQ3746 (A2) were grown in CA in the presence and absence of French bean extract following the method by Duncan (1988) for 25 days (Fig. 1). The total number of oospores at various stages of development observed were 133 and 175 on CA agar plates only and CA agar plates supplemented with French bean extract respectively. Oospore development appeared not to be affected in CA agar plates in the absence and presence of French bean extract (Fig. 1). In the absence and presence of French bean extract, no pigment or colour reaction were observed in the early stages of oospore formation i.e., from contact of A1 and A2 gametangial initials to oosphere formation, appearing hyaline (Fig. 1a–e) post 7, 14 and 21 days. No colour was observed after fertilization tube formation and during oosphere formation (Fig. 1f) in CA supplemented with French bean extract. In the late stages of oospore formation i.e., at oospore wall formation, a yellow to yellow–brown colour could be observed and appeared to be also associated with the oogonial envelope of putative aborted/germinated oospores in CA plates post 14 and 25 days (Fig. 1g, i). In the presence of French bean extract, an orange colour was observed in mature oospores (Fig. 1h) and was associated to the oogonial envelope of putative aborted/germinated oospores (Fig. 1k). In addition, empty oogonia were observed in CA with and without French bean extract post 7, 14 and 25 days and oogonial walls were hyaline (Fig. 1j).

For staining/fluorescence studies, triplicate plates were done for each testing condition and the experiments were replicated twice. The agar plates were removed from the incubator for microscopic observations after 25 days. Six to eight agar blocks (1 cm × 1 cm in size) within the mating zone i.e., containing oospores, were placed inverted on microscope slides as it was observed that oospores formed within or near the base of the agar medium. The total number of oospores which also included putative aborted/germinated oospores were recorded for each agar block. They ranged from 0 to 517 oospores per 1 cm^2 on CA agar block (n = 20 agar blocks) and 0 to 438 oospores per 1 cm^2 on CA supplemented with French Bean extract agar block (n = 19 agar blocks). Calcofluor white (CFW; Sigma-Aldrich PTY Ltd., no. 18909) was used following the manufacturer's protocol for fluorescence staining. One to two drops of CFW followed by 10% KOH solution were applied to the agar blocks before placing coverslips for viewing under UV fluorescence. To capture CFW-stained *P. palmivora* oospores in agar blocks, the U-MWU2 filter cube (excitation BP 330–385 nm) was used.

CFW fluorescence was observed in the oogonial wall and the antheridial wall at the oosphere formation stage (Fig. 2a, b). In empty oogonia with hyaline wall (n = 16), the remaining oogonial wall and the antheridial wall fluoresced with CFW (Fig. 2c, d). In yellow–brown oospores (n = 28) in carrot agar (with no French bean extract), CFW fluorescence was observed only in the antheridial wall at the

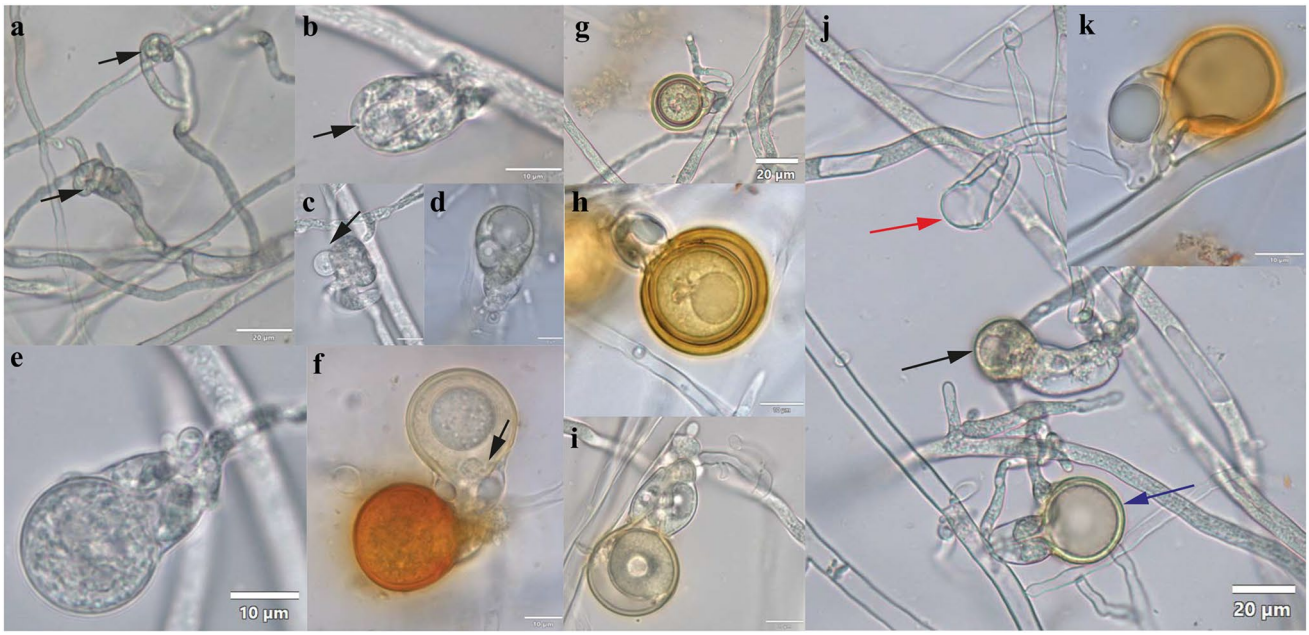

Fig. 1 Sexual morphogenesis in *Phytophthora palmivora* UQ3694 (A1) and UQ3746 (A2) under bright field microscopy. **a** contact post 14 days; **b** penetration or invagination of the proximal end of the antheridium by the oogonial initial post 7 days (black arrow); **c** oogonial expansion phase post 7 days; **d** later stage of **c** with two ooplasts post 21 days; **e** developing gametangium post 7 days; **f** oospore spore wall formation with the presence of a fertilization tube (black arrow) post 21 days; **g** mature, slightly aplerotic yellow oospore post 14 days; **h** mature, slightly aplerotic orange oospore with one central ooplast; **i** putative aborting or germinating oospore post 21 days; **j** putative aborted oogonia post 14 d. Note lack of yellow colour in the aborted gametangium in the oogonial expansion phase (red arrow), the elongated shape of the antheridium (black arrow) and a putative aborted empty yellow-walled oogonium (blue arrow); **k** aborted oogonium with orange coloured cell walls post 21 d. Images in **a–c**, **e**, **g** and **h** were captured on CA and in **d**, **f**, **h**, **i** and **k** on CA supplemented with French Bean extract medium plates respectively. Scale bars are 20 µm in **a**, **g** and **j** and 10 µm in **b–h** and **k**

base of the oogonia-oospore structure (Fig. 2e, f). In putative aborted/germinated oospores where the oogonial wall appeared yellow, there was weak or no CFW fluorescence in the oogonial wall but there was a strong fluorescence signal in the antheridial wall (Fig. 2g, h). Similar results were observed in CFW fluorescence when grown in carrot agar supplemented with French Bean extract (Fig. 2i, j). Oospores (n = 54) which accumulated colour due to the presence of French bean extract displayed localised CFW fluorescence only in the antheridial walls (Fig. 2i, j). Both compatible mating types A1 and A2 non-mating hyphal walls displayed CFW fluorescence (Fig. 2).

The use of CFW to detect β-glucans confirmed the presence of cellulose and other forms of β-glucans in the oogonium walls of immature or putative aborted/germinated oospores, the antheridium walls as well as non-mating hyphal walls. Interestingly, it appeared that prior to the oospore wall formation, CFW fluorescence was observed in the oogonial walls including oogonia which were empty. Mature oospores which appeared yellow–brown in carrot agar, lacked CFW fluorescence suggesting a change in the chemical composition of the oogonial walls surrounding such oospores. Similarly, orange-coloured oospores in CA plates supplemented with French Bean Extract in carrot agar lacked CFW fluorescence in the oogonial walls. In both cases, only the antheridium walls had a CFW fluorescence signal. Lack of CFW fluorescence in mature oospores suggests a reduction in cellulose/β-glucans content localized in the outer cell walls of the oospore-oogonium walls (oow) and the presence of cellulose/β-glucans within the antheridia. Work by Helbert et al. (1997) demonstrated the presence of cellulose in *Oomycota* and Grenville-Briggs et al. (2008) and McLeod et al. (2002) demonstrated the role of cellulose synthase genes, 1,3-β-glucanase and 1,3;1,4-β-glucanases genes in *P. infestans* mycelia, sporangia and zoospore/cysts in vitro as well as during infection of potatoes respectively. Nui et al. (2018) found seven proteins linked to glucan breakdown in oospores and non-mating hyphae of *P. infestans*. In vitro work by Antelo et al. (1998) and Wang and Bartnicki-Garcia (1976) demonstrated 1,3-β-glucan synthase activity in *P. sojae* and *Phytophthora cinnamomi* respectively. In addition, recent studies in *Phytophthora* spp. have identified a putative chitin synthase gene and it has been shown to be involved in asexual reproduction and pathogenesis (Cheng et al. 2019; Hinkel and Ospina-Giraldo 2017). A keyword search for chitin synthase and 1,3-β-glucan synthase identified, one putative chitin synthase gene (PHPALM_3836), four callose synthase genes

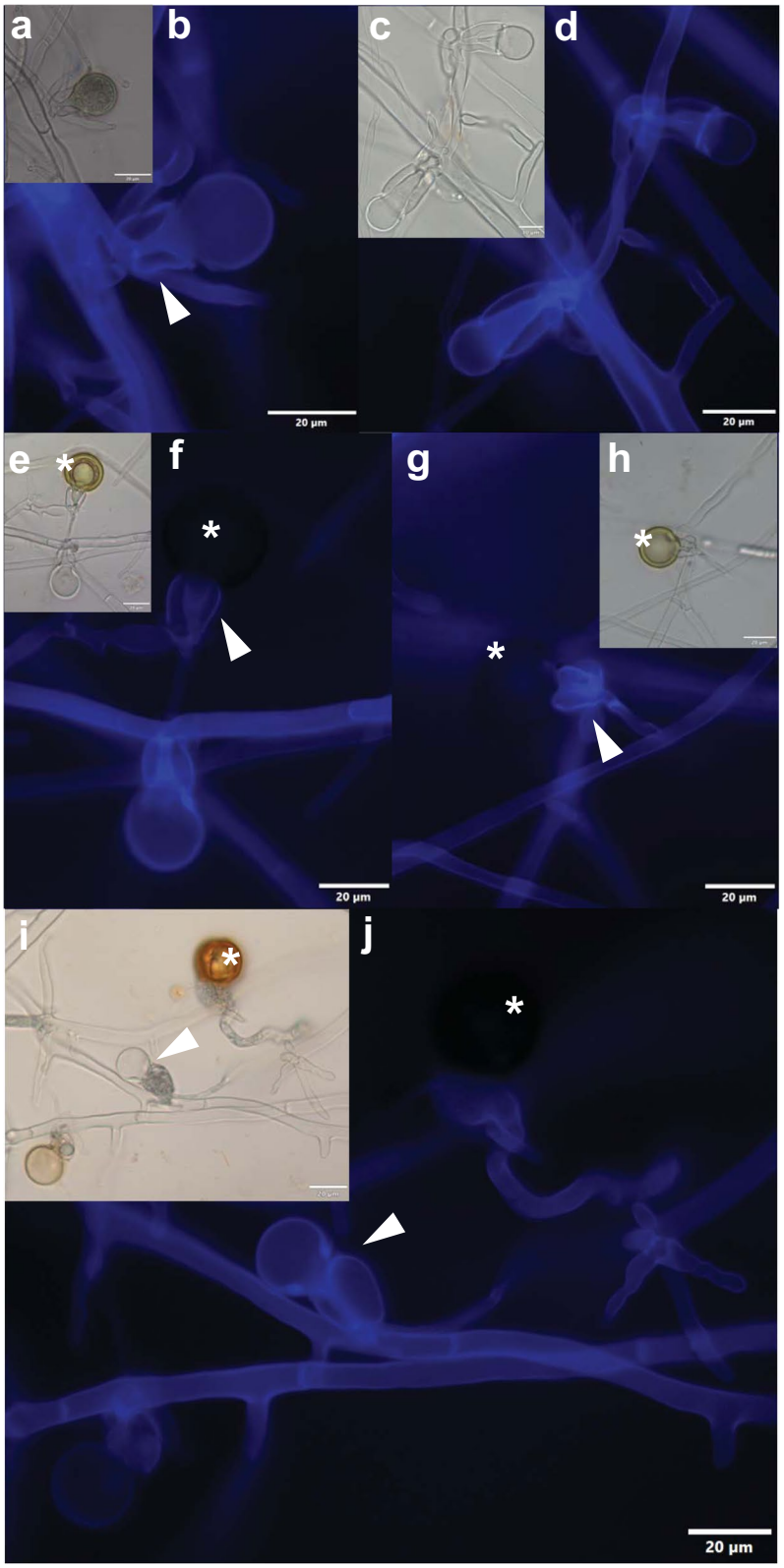

Fig. 2 CFW fluorescence during *P. palmivora* oospore development in carrot agar with or without French Bean extract post 25 d. **a** Early stage of oosphere formation; **b** same as **a** highlighting CFW fluorescence in the oogonial wall and the antheridial wall (white arrowhead); **c** empty oogonia with hyaline walls i.e., putative aborted oospores; **d** same as **c** under UV fluorescence; **e** Yellow–brown oospore (white asterisk) and an empty oogonium; **f** same as **e** under UV fluorescence. Note the lack of CFW fluorescence in the yellow–brown oospore (white asterisk) and the CFW fluorescence signal in the antheridial wall (white arrowhead); **g** putative aborted/germinated oospore with weak or no CFW fluorescence in the oogonial wall (white asterisk) and a strong fluorescence signal in the antheridial wall (white arrowhead); **h** same as **g** under bright field; **i** Oospore and oogonia at different stages under bright field; **j** CFW fluorescence profile of same oospore and oogonia shown in **i**. Note the lack of CFW fluorescence in orange-brown oospore (white asterisks) and CFW fluorescence in the oogonial and the antheridial walls of both putative aborted/germinated oospores. Images **a–h** were obtained on CA plates and images **i** and **j** were on CA plates supplemented French Bean extract. Images are representative of hyaline aborted oogonia (n = 16) and yellow-coloured oospores (n = 28) in CA plates and hyaline aborted oogonia (n = 9) and brown-coloured oospores (n = 54) in CA plates supplemented with French bean extract captured with the Olympus BX51 fluorescence microscope

(PHPALM_5465, PHPALM_1260, PHPALM_1878 and PHPALM_8937), one putative glycosyl transferase family 48 protein (PHPALM_29850) and one glycosyl transferase (PHPALM_10597) in *P. palmivora var. palmivora* str. sbr112.9 (GCA_002911725.1) ASM291172v1 database (https://protists.ensembl.org/index.html; Howe et al. 2020).

Changes in CFW fluorescence signals in oospores at different stages may be due to the changes in cellulose/β-glucans content within the oospore-oogonium walls (oow). The presence of different types of β-glucans in plant pathogenic *Phytophthora* and fungi is important due to its role in plant immunity (Fesel and Zuccaro 2016; Robinson and Bostock 2015; Mélida et al. 2018; Wawra et al. 2016). Future approaches using specific dyes such as Aniline Blue to detect β-1,3-glucans or the tagging of proteins linked to β-glucan synthesis and degradation with high resolution microscopy may contribute to our understanding of β-glucans in oospores and help in the control of plant disease.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42360-022-00510-y.

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Declarations

Conflict of interest The author declares that there is no conflict of interest for this submission.

Ethical approval This research does not contain any studies that include human participants or animals.

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