Conidial morphology, structure and development in *Valdensinia heterodoxa*

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As a pathogen and possibly biological control agent, the morphological development process of the conidia of *Valdensinia heterodoxa* is studied under a controlled environment. Conidial production in culture can be controlled by exposure to light. No conidia are produced at 20°C in the dark on weak oatmeal agar, but after placing in an growth chamber with a 12°C/19°C (night/day) diurnal temperature cycle, mature conidia form from initial cells within 24 h and discharge by 48 h. The initial cell is obovate with a thick peridium. Preceding conidial differentiation, the generative cell breaks through the peridium, forming a central axillary plate cell on an obovate conidiophore cell. The axillary cell develops radiating conical protrusions, which initially curve upward and then develop turgid, plicated evaginations on their upper and flank sides. The radiating protrusions elongate into arms that ultimately bend downwards toward the surface of medium. There are normally four or five arms per conidium, but some have three to six arms. The axillary plate cell also forms numerous radiating clavate cells in the central area surrounded by the arms, eventually forming a hemispherical conidial centre. With continuing maturation, involving inflation of the clavate apical cells and the plicated evaginations on the arms, the arms straighten and contract to the centre of the conidiophore, pushing downward on the substrate. A further pressure-increase eventually ruptures the joints between conidiophores and conidia, resulting in forcible discharge of the conidia. Conidia are star-shaped prior to dispersal, but become tear-drop-shaped when discharged due to the depressed arms. Conidial size varies with culture media and substrate. Single conidia cause infection on detached leaves of salal (*Gaultheria shallon*). Viscous granules on the conidial arms and apex help with adhesion to host plants and also play an important role in the infection process.

**Keywords:** *Valdensinia heterodoxa*; conidia development; morphology; viscous granules; infection

**Introduction**

*Valdensinia heterodoxa* Peyronel (Sclerotiniaceae; anamorph *Valdensia heterodoxa* Peyronel) is a parasitic fungus found in both North America and Europe. It attacks or damages at least 33 host plant species within 15 families in Europe, and more than 14 different plant species in North America, including the ericaceous plants *Gaultheria shallon* Pursh. (salal) and *Vaccinium myrtillus* L. (Peyronel 1927, 1953; Redhead and Perrin 1972a,b; Redhead 1974, 1979; Norvell and Redhead 1994; Mel’nik 1995). The fungus was originally found on blueberries in Italy (Peyronel 1923). In eastern Canada, early reports involved infections on *Vaccinium* spp, including *V. ovalifolium* J.E. Sm., *V. angustifolium* Ait, *V. myrtillus* Michx., as well as *Diervilla lonicera* Mill. (Redhead 1979; Parmelee 1986; Norvell and Redhead 1994). In the western regions of Canada and the United States, it attacks salal and other ericaceous plants, such as *V. alaskense* T.J. Howell and *V. Membranaceum* Dougl. ex Torr. (Redhead 1974; Norvell and Redhead 1994). Plants in other families may be incidentally damaged by conidia originating on ericaceous hosts (Redhead and Perrin 1972a). As a plant pathogen and potential biocontrol agent, *Valdensinia heterodoxa* has received increasing attention in forest vegetation management and ecosystem studies. In Europe, serious epiphytotics of *V. heterodoxa* on bilberry (*Vaccinium myrtillus*) are being reported. There is a reported linkage between increased nitrogen availability in forest and increased incidence of the disease caused by this fungus, significantly affecting the composition of the understory plant community and thus the forest ecosystem (Aamlid 2000; Strengbom et al. 2002, 2006; Witzell et al. 2005). In Canada, *V. heterodoxa* infects wild blueberries in Quebec and Newfoundland (Redhead 1979; Parmelee 1988; Norvell and Redhead 1994) and can seriously affect commercial stocks of lowbush blueberries in Nova Scotia (Hildebrand and Rendro 2007). In British Columbia, *V. heterodoxa* has been studied as a biocontrol agent for the control of salal (Magnussen et al. 2004; Vogelsgang and Shamoun 2002, 2004; Wilkin et al. 2005; Zhao and Shamoun 2006). Further characterization of *V. heterodoxa* is needed to understand the epidemiology and etiology of the diseases.

Early studies showed that the teleomorph of *Valdensia heterodoxa* is *Valdensinia heterodoxa*, and that its sexual spores develop in apothecia arising from sclerotia on infected veins of overwintered leaves which were infected the preceding summer (Redhead 1974). Infections result in
large brown spots and may result in premature leaf loss (Norvell and Redhead 1994). The conidia of this fungus are stellate, multicellular propagules that can discharge from substrates, such as salal leaves, to a height of 20 cm (Redhead and Perrin 1972a). Conidial discharge is involved in dispersal over short distances and is probably the main way that the disease spreads during the summer (Redhead and Perrin 1972a). The conidial structure, production process and dispersal mechanisms were described by Peyronel (1923) from infections on bilberry. Most salient features of the conidia were described in the earlier studies, but some of the development and structure of the stellate conidial morphology has yet to be described in detail. The purpose of this paper is to describe the conidial morphology process and possibly their functions in disease development. This information is necessary to assess the effect of V. heterodoxa on the host plants, its ecological niche in forest vegetation communities and its potential as a biological control agent.

Materials and methods

Materials

This study examined Valdensinia heterodoxa isolate PFC2761, isolated from (Gaultheria shallon) salal on Vancouver Island (Vogelgsang and Shamoun 2002). A stock culture of the fungus was maintained on 3.9% potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI, USA) at 4°C. Weak oatmeal agar (WOA) (Zhao and Shamoun 2006) was used as the culture medium. Rice and wheat bran (household grade; Canada Safeway Limited) were used as solid substrates for the production of conidia. Salal seedlings used for host inoculations were 3–4 months old and maintained in a greenhouse (20–25°C and 35–75% relative humidity) at the Pacific Forestry Center, Victoria, Canada. Seedlings were grown in pots containing a soil of peat/vermiculite/sand (3:1:1, v/v) with low rate Osmocote 18-6-12 fertilizer. A light microscope (Nikon SMZ1000), dissection microscope (Nikon OPTIPHOT-2), and camera (Nikon COOL PIX 995) were used for observation and picture taking. A sterile surgical scalpel with a No. 11 blade was used to harvest conidia from the WOA culture medium. The growth chamber (Conviron, Model E-15; Controlled Environments, Winnipeg, MB, Canada) was maintained using a 12/19°C (night/day) diurnal cycle with a 12 h day\(^{-1}\) photoperiod (180–200 Em\(^{-2}\) s\(^{-1}\)).

Methods

Conidial development and structure

A 5mm diameter disc from a PDA starter culture was placed at the centre of each WOA plate, incubated in darkness at 19°C for 8 day, and then transferred to the growth chamber for incubation. Twenty four plates were checked every 12 h to observe and photograph conidia formation and discharge at the start of each photoperiod.

To prepare for the inoculation of solid substrates, 20 g of wheat bran were placed in each of six 250 ml flasks with 10 ml water and autoclaved twice at 121°C for 30 min. This was repeated with 20 g of rice in six flasks. Each flask was inoculated with 10 mycelial discs (5 mm diam) from WOA, and incubated in darkness at 19°C for 10 days. The cultures were dried under sterile conditions at 23°C for 2 days, and stored in darkness at 4°C for later use. To obtain mature, uncontaminated conidia for the inoculation of salal leaves, 1 g of each inoculated substrate was evenly spread onto wet filter paper in separate Petri dish and placed in the growth chamber.

Size of conidia

To measure the size of the mature conidia, discharged conidia from wheat bran and rice cultures (12 conidia each) were harvested from the media with a dissecting surgical blade under a dissection microscope. The dimension of the entire conidium was measured under a microscope, photographs were then taken, and later the dimensions of the heads (width and length), the arm length and the apex length were measured from calibrated pictures. The process of conidia generation and sampling was repeated once.

Disease etiology on salal leaves

A control-treatment test was carried out to evaluate the disease etiology of conidia on salal leaves. In the control group, a mature conidium was picked up from wheat bran and without treatment, placed on the lower surface of a salal leaf from a 3-month-old plant. In the treatment group, a mature conidium was picked up and dipped into distilled sterile water for 1 min and then the conidium was placed on the lower surface of a detached salal leaf. The processes of conidium harvesting and inoculation were performed under a dissection microscope by using a dissecting surgical blade. The inoculated leaves were checked under a dissecting microscope to make sure the head portions of the conidia were attached to the leaves. The inoculated leaves were placed in Petri plates on wet filter paper, sealed with Parafilm and incubated in a growth chamber. They were checked 3 days later. Each group had 20 replicates, and the test was repeated once.

Data analysis

The data for the duplicated tests were pooled after an equal variance (homogeneity) test indicated that it was valid to do so. Analyses were performed using the SigmaStat (Version 2.03; Systat Software Inc., Richmond, CA, USA) statistical software package. Collected data were analyzed via
ANOVA (one-way) to determine the effect of substrate on conidial size, and the treatment of conidia on disease etiology.

**Result**

**Conidial development and structure**

After 8 days of incubation in darkness at 19°C, colonies on WOA covered over two-thirds of each plate and no mature or primordial conidia were observed. Following the transfer of the plates to growth chamber with light regimes, conidiogenesis was observed on the substrate surfaces after 24 h (Figures 1 and 2). Immature conidia take the shape of a starfish with three to six arms laying flat on the surface of the media (Figures 6 and 7). After 48 h, discharged conidia were observed on the covers of Petri dishes, with the head attached to the lids, and the arms slightly open (Figure 17). Some of the mature conidia did not discharge despite having down-turned arms, seemingly because the arms became tangled with the mycelium. During the subsequent incubation (6–10 days), conidia continued to be produced and discharged.

Conidial initials appearing on culture surfaces were obovate (Figures 1 and 2). A second swelling that ultimately serves as an axillary central cell develops acropetally by growth through the cell wall of the generative conidium base, which is the equivalent of a conidiogeneous cell (Figures 3–5). The ruptured residue of the peridium of the conidium initial remains around the obovate conidiophore cell (Figure 5). Three to six (normally 4–5) short conical protrusions develop, evenly and radi ally around the central cell. The boundaries between central cell and protrusions are clear (Figures 3 and 4).

As the conidia grow, the arms curve upward and become transversely plicate (Figures 4 and 5). Eventually, the plications become more pronounced and numerous and geniculate, granulate nonplicate acute appendices differentiate, and an umbellate cluster of acropetalous cells arise forming the adhesive head (Figures 6, 7 and 10). With further development, all plicae and their geniculations on each arm swell ultimately causing each arm to contract downward, producing pressure against the surface of the substrate (Figure 7). The pressure increased with maturing of the conidium and straightening of the arms until the connection between the conidiophore and the conidium ruptured, the released force from the turgid arms push the conidium against the media and propel the conidium into the air. On WOA, about 10% of mature conidia were aerially dispersed, because most of the conidia became tangled with aerial mycelia, or conical apices stabbed into the medium. Some of the conidia did not launch; instead, they pivoted anticlockwise or clockwise on their arms before the arms folded evenly on each other (Figures 8 and 9).

Discharged conidia with contracted arms, became lacrymiform (Figure 20). When conidia adhered to Petri dish covers, or when they were dipped in water, their arms were slightly opened (Figure 11). With a lacrymiform shape, a mature conidium can be described in three parts: head, arms and the apices on the arms (Figure 21). The head of the conidium, which is differentiated from the central cell (Figure 3), is a somewhat hemispherical structure comprised of 130–180 clavate end cells (Figures 11, 19, 20 and 22) that arise from 40–60 original protuberances that immediately become branched (Redhead and Perrin 1972a). These clavate cells germinate rapidly and produce germ tubes when the conidium is mature and detached from the conidiophore (Figures 16, 17, 20 and 22). Mycelia from germinated conidia can become melanized (Figure 23). The arm of a conidium has an arched, cylindrical skeleton (Figures 14 and 18) with plicate dorsal covering cells that appear rectangular in surface view, while the geniculate cells on the lateral sides are evaginate (Figures 11 and 18). Unlike the clavate cells composing the adhesive head, cells on the arms do not germinate. The arm apices, which are rigid and conical, have no external cells. When dried, each arm apex becomes hooked (Figure 13).

The surface of conidia is covered by a layer of mucus. On the head and arms, the mucus is made of thin particles; while on the apex, the particles are large and take the shape of granules (Figures 11 and 12). When a conidium is dipped in water, the thick mucus readily dissolves to form a white, cloudy area around the conidium (Figures 15 and 16).

**Size of conidia**

Conidial dimensions varied with the type of culture medium (Table 1). On WOA, the length (from the end of head to the end of arm apex, shown in Figure 21) of discharged conidia was 238–389 μm. On solid substrates, the average length of conidia from wheat bran (374 μm) was significantly larger than those from rice (283 μm). The ratios of conidial length, head length and head width to the apex length were similar on both substrate – 3.3, 1.1 and 1.8 on wheat bran, and 3.6, 1.1 and 2.0 on rice, respectively. Hence, the apex, which is a little shorter than the head, is about 30% of the length of the entire conidium, and the arm is about 70%.

**Disease etiology on salal leaves**

In the control group, 37 of 40 conidia (93%) in the two tests caused infection on detached salal leaves. The lesions varied from 2 to 7 mm in diameter after 3 days. In the treatment group, only four of the 40 conidia (10%) caused infection on salal leaves. The difference between the two groups was significant on infection.
Figures 1–12. (1) Initial conidial cell (arrow a, from above). Scale bar = 40 μm. (2) Initial conidial cell (arrow a, lateral view). Scale bar = 40 μm. (3) Four conical protrusions (arrow a) forming from the initial conidial cell (arrow b, from above). Scale bar = 40 μm. (4) The developing arms curving upward (above). Note the boundaries (arrow a) between protrusions and central axillary cell. Scale bar = 60 μm. (5) The developing arms curving upward (lateral view). Note the obovate conidiophore cell (arrow a) and the cracked peridium (arrow b). Scale bar = 25 μm. (6) A maturing conidium. Note the formation of placae (arrow a), appendices (arrow b) and center plate (arrow c). Scale bar = 100 μm. (7) A maturing conidium. Note that the distal end of each arm appears to depress the surface of the agar substrate (arrow a). Scale bar = 120 μm. (8) Mature conidium with folded arms anticlockwise-twisted (from above). Scale bar = 65 μm. (9) Mature conidium with folded arms anticlockwise-twisted (from below). Scale bar = 60 μm. (10) Maturing conidial arms. Note the cylindrical cells on dorsal and lateral sides (arrow a). Scale bar = 40 μm. (11) A dispersed conidium. Note that mucus and granules cover the placae (arrow a) and other parts of the conidium (arrow b, c). Scale bar = 30 μm. (12) Granules coating (arrow a) the apex of a conidial arm. Scale bar = 40 μm.
Discussion

The morphological characteristics of *Valdensinia heterodoxa* conidia and their discharge mechanism are unique.

We have confirmed earlier descriptions of the conidial structure on host plants using laboratory cultures, and we have uncovered some previously unknown morphological
and functional aspects of the conidia previously described by Peyronel (1923) and Redhead and Perrin (1972a).

Conidia of *Valdensinia heterodoxa* form quickly once the cultures are exposed to light and have some special features not previously reported. On host plants, the time between formation and dispersal of conidia is about 48 h (Redhead and Perrin 1972a). On culture media, kept under the condition of diurnal 12/19°C (night/day) temperature cycle, dispersible conidia were formed from conidio-geneous cells in 24 h or less. Erected on the surface of the WOA media, conidia begin development with a obovate cell, which is covered by a peridium that leaves a cracked residue around the conidiophore when the erected cell further differentiated into a conidium (Figure 5). The existence of a peridium was not reported in early studies (Peyronel 1923; Redhead and Perrin 1972a). It can be seen that the initially differentiated central axial cell and protrusions are different in colour and that there were distinguishing boundaries between them (Figures 3 and 4). This suggests that a septum might exist when these protrusion initially form, but septa were not described by earlier authors. On mature conidia, the clear outline of the skeleton of each arm surrounding the central plate (Figure 14) shows that each arm is independent and separate from central plate. The connection between the arms and central axillary cell had not been detailed in the previous studies. Further research to clarify this is necessary and important in understanding the discharge mechanism of *V. heterodoxa*.

Cells on conidia originated from different initial structures and, therefore, had different shapes and functions.

Table 1. Size of collected conidia from wheat bran and rice media.

|                     | Wheat bran | Rice  |
|---------------------|------------|-------|
| Conidial length range (μm) | 336–423    | 250–338 |
| Conidial length (μm) | 374.5 ± 7.7 | 283.3 ± 7.1 |
| Apex length (μm)    | 113.6 ± 2.3 | 78.1 ± 2.0 |
| Head length (μm)    | 119.7 ± 3.4 | 86.0 ± 2.5 |
| Head width (μm)     | 199.8 ± 7.8 | 152.9 ± 5.5 |

Figures 20–23. (20) A mature dispersed conidium with a lacrymiform morphology. Note the germination of cells from conidial head (arrow a). Scale bar = 40 μm. (21) Structure of a dispersed conidium: head, arms and the apices. HW = head width; CL = conidia length; HL = head length; AL = apex length. Scale bar = 25 μm. (22) Germinating of clavate cells (arrow a) at the head of a dispersed conidium. Scale bar = 40 μm. (23) A melanized conidium (arrow b) after germination on wet filter paper, with melanized hyphae (arrow a) growing from the conidium. Scale bar = 350 μm.
The cells on the head differentiated from the early central axial cell as protuberances that branch to form clavate end cells packed together so as to resemble a compound insect-like eye. When mature, the distending of these appressed cells forms a hemispherical head, forcing the arms to contract towards the centre. With suitable temperature and humidity, they germinate, forming germ tubes that subsequently become septate and form hyphae. Arms are initiated as conical protrusions around the initial central axial cell, and cells on the arms have two shapes. On the dorsal side, evaginated cells are transverse and plicated, but on the lateral sides, the juxtaposed cells are cylindrical. These distensible cells presumably help straighten, then recurve, and strengthen the arms during the launching process. We did not observe any germination of cells originating from the arm.

Redhead and Perrin (1972a) reported that there are sparsely distributed granules on the tips of the arms. We found that the whole conidium was covered by granules, but granules on other parts of conidium were very fine. Peyronel (1923) described the covering as “muco piuttosto abbondante” (rather thick mucus), and described the head of a conidium as a “viscous plate”. The granules on the head are viscous and they readily dissolve in water to form a white, cloudy area around the conidium (Figure 15 and 16), especially around the head. Also, in the process of harvesting conidia, we found that the heads of conidia were more viscous than the arms, because it was easier for the head to adhere on the tip of scalpel blade than that of arms of conidium. Even though there are granules on the arm apices, we did not observe any viscous effect from them. Conidia flushed of their mucus lost most of their ability to infect salal leaves. It is possible that the mucus may perform a dual function; it may serve as an adhesive and it may also contain enzymes important to the infection processes. Probably, the flushing of the viscous materials and enzymes by the water dramatically reduced the virulence of conidia in the treatment group. Further work needs to be done to confirm this.

We observed that most mature conidia in culture did not disperse because the apices adhered to the agar surface or were tangled in mycelia. In a previous study, we found that conidial discharge rates are affected by the substrates (Zhao and Shamoun 2006). Solid substrates have higher dispersal ratios than those on agar media; the surface texture of solid substrate affects the dispersal ratio, too. Normally discharged conidia have a lacrymiform shape, which aerodynamically should make the conidium travel further when discharged. Some mature conidia from weak oat meal had twisted arms; the arm apices were evenly arranged and made the conidia to take the shape of paper windmills. The mechanism of the spiral twist is not clear, but we suspect that pressure imbalances within each arm may cause a spiral twist. The pressure imbalance could be caused by disproportionate thicknesses in the skeleton, or one of the apices becoming stuck into the agar media or tangled with mycelia while arms contract together. Alternatively, slight twisting while pushing down may be the norm and actually assists with sudden release from the conidiophore.

Conidia of V. heterodoxa readily germinate once dispersed. Normally, for other fungi, conidial germination is inhibited by the presence of the parental mycelium (Weber and Hess 1976). Unlike most other hyphomycetous, once detached, the conidia of V. heterodoxa can germinate, even on the parental mycelium. With regard to the undischarged conidia with twisted arms, the conidiophore was most likely not detached from the conidia under the central axial cell and this may explain why we did not observe any germination from them. However, Redhead and Perrin (1972a) observed conidia that developed embedded in agar that germinated into fuzzy balls of mycelium.

The heads of conidia are almost hemispherical and, when produced in culture, are composed of 130–180 thin-walled, club-shaped cells. In the Redhead and Perrin (1972a) description, the conidia first developed into 40–60 globose tubercles on the head, and then each developed into three to five thin-walled, clavate protuberances which formed a compact layer of the head. The number of club-shaped cells on head is in a similar range with what we have observed.

Further research is imperative to elucidate this unique dispersal and discharge mechanisms in V. heterodoxa, its infection process, ecological role in forest vegetation management and potential use as a biological control agent for management of salal in conifer regeneration sites of coastal British Columbia.

Acknowledgements
We would like to thank Island Timberlands and the Canadian Forest Service, Pacific Forestry Center for funding this project. The authors thank Drs Brenda Callan, Richard Winder and Marianne Elliott for reviewing the manuscript. The authors deeply appreciate Dr. Scott Redhead’s constructive suggestions on this manuscript.

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