Occurrence of Vesicular-Arbuscular Mycorrhizal Fungi in Alberta, Canada

S. M. Boyetchko* and J. P. Tewari
Department of Plant Science, University of Alberta, Edmonton, Alberta, Canada T6G 2P5

Z. Naturforsch. **48c**, 923–929 (1993); received April 6, 1993

Vesicular-Arbuscular Mycorrhizae, Taxonomy, Calcium, X-Ray Microanalysis

Three VA mycorrhizal fungal species were isolated from soils in Alberta, Canada and examined by scanning electron microscopy and energy-dispersive X-ray microanalysis. Mature spores of *Glomus aggregatum* developed an outer hyaline wall which contained lower levels of calcium than the middle wall. Examination of *G. pansihalos* spores revealed a lower level of calcium in the outer evanescent wall as compared to the ornamented wall. When spores of *Entrophospora infrequens* were examined, the wall of the vesicle was found to contain similar levels of calcium as the ornamented wall of the spore. The significance of the results concerning the presence of calcium in mycorrhizal spore walls is discussed, as is the occurrence of the mycorrhizal species.

Introduction

Interest in vesicular-arbuscular (VA) mycorrhizal fungi in Canada has increased tremendously over the last decade. Taxonomic identification is a must for isolate typification before any meaningful experimental work can be undertaken. The American Phytopathological Society identified this area as a research priority. Although the number of species of VA mycorrhizal fungi discovered world-wide is increasing, very little effort has been initiated to document those species indigenous to Canadian soils. Six species of *Glomus* Tul. & Tul. from Québec were first described by Thaxter [1]. Other collections of VA mycorrhizal fungi from eastern Canada have been reported [2–6]. A limited number of studies reporting the geographic distribution of the Endogonaceae have been conducted in the western Canadian provinces of British Columbia [7, 8], Alberta [9–12], and Saskatchewan [13]. Zak *et al.* [9] reported the presence of *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe, *G. aggregatum* Schenck & Smith emend. Koske, *G. tenue* (Greenall) Hall, and *Entrophospora infrequens* (Hall) Ames & Schneider in amended mine spoils in Alberta. A new species, *G. dimorphicum* Boyetchko & Tewari, was reported to be associated with barley in Alberta [12].

* Current address of first author: Agriculture Canada, Research Station, P. O. Box 440, Regina, Saskatchewan, Canada S4P 3A2.

Reprint requests to Prof. J. P. Tewari.

Verlag der Zeitschrift für Naturforschung,
D-72072 Tübingen
0939–5075/93/1100–0923 $ 01.30/0

Several reports have documented the association of VA mycorrhizal fungi with agricultural crops in western Canada [10, 12–15]. However, authors do not always specify which VA mycorrhizal fungal species was used in each study [14, 15]. Since some hosts vary in their VA mycorrhizal root colonization [16], it is important to be cognisant of the particular VA mycorrhizal fungal species being used in an experiment. Therefore, if further research, particularly field trials, on VA mycorrhizal fungi is to be continued in western Canada, and results compared with other reports, further collections and identification of species indigenous to the region is necessary.

The purpose of this study was to collect and identify some species of VA mycorrhizal fungi associated with field crops in Alberta. Although this was not an exhaustive taxonomic/ecological study, the information presented extends the known geographic distribution of four VA mycorrhizal fungal species. This study also provides some new information on the chemical and physical characteristics of the VA mycorrhizal fungal spore walls. Special emphasis was placed on studying the relative levels of calcium in different cell wall layers, as this aspect has so far received very little attention in fungi. The importance of calcium as a component of the cell wall will be discussed.

Materials and Methods

Collection of soil samples

Soil samples were collected from various sites in Alberta where barley, red clover, and alfalfa were grown, brought back to the laboratory, and stored

at 4 °C until samples could be processed. The VA mycorrhizal fungi obtained from these soils were multiplied in pot culture by placing approximately 250 g of the field soil below the seed of alfalfa (*Medicago sativum cv.* Beaver) in a potting medium consisting of authoclaved sand and loam (3:1). The pots were fertilized with Hoagland's solution, minus phosphorus, once a week, in addition to regular watering. Greenhouse temperatures were 25–27 °C during the day and maintained at 20 °C during the night. The photoperiod was 16 h with a maximum light intensity of 500 μE/m²/s. Pot cultures were harvested after 8 to 10 months. Spores were collected by wet-sieving and decanting the soil according to the methods of Gerdemann and Nicolson [17]. In some cases, a sucrose gradient was employed to collect the VA mycorrhizal spores (Dr. J. Morton, personal communication to Dr. J. P. Tewari).

Light and scanning electron microscopy

VA mycorrhizal fungal spores were examined under a stereoscopic microscope and picked up, individually, with a dissecting needle. Spores were observed by light microscopy by mounting them in either lactophenol or in polyvinyl alcohol (PVA).

Spores prepared for scanning electron microscopy (SEM) were placed onto filter-paper, air-dried, and vapor-fixed with 2% osmium tetroxide in water overnight. Spores were then mounted onto stubs, coated with gold, and examined in a Cambridge Steroscan 150 SEM. In some cases, VA mycorrhizal fungal spores were fractured with a dissecting needle to obtain cross-sectional views of the spore walls. Energy dispersive X-ray microanalyses were carried out in the same instrument using a Kevex Micro-X 7000 analytical spectrometer. When an electron beam is held in one spot of a sample, the X-rays emitted from that site can be analyzed. By this technique, elements between sodium and uranium in atomic weight can be detected [18]. The angle of incidence of the electron is normalized and the zone of analysis for detection of an element in the sample is approximately 1 to 2 μm. Information provided on the X-ray spectra includes the VFS which refers to vertical full scale (*i.e.* the number of counts on the graph to the top of the graph). Semi-quantitative comparisons of elements in the sample can be made by using the K-ratio, which is normalized within each sample.

Results and Discussion

Spores of *G. aggregatum* were isolated from soil and roots associated with alfalfa *(Medicago sativum)* in Devon, Alberta (Lat. 53° 22′ N, Long. 113° 44′ W). The spores formed in loose clusters (sporocarps) with as many as 100 or more spores per cluster (Fig. 1). The spore wall morphology was similar to that described for *G. aggregatum* by Koske [19]. Mature spores were pale yellow to yellow-brown and had outer hyaline, middle laminate and thin membranous wall layers. The spores of this isolate consistently developed the outer, hyaline wall layer (Fig. 2), which is known to occur occasionally in *G. aggregatum* [19]. The laminate wall layer was 1–3 μm thick and the membranous wall layer was approximately 0.5 μm thick. The inner membranous wall layer had a greenish tint when viewed by light microscopy. X-ray microanalysis revealed a concentration of calcium in the middle wall layer (Table I, Fig. 7 b). The outer hyaline wall layer (Fig. 7 a) had relatively less calcium than the middle wall layer (Table I, Fig. 7 b).

The spores of *G. pansihalos* were collected from the same soil sample as *G. aggregatum* in the Devon area. The spores have been observed mostly as single spores but occasionally formed in groups of 2 to 5 spores. Color of spores were yellow-brown to dark-brown, were usually subglobose, ranging in size from 150–180 × 120–160 μm. The spores developed three walls: an outermost wall which was 3–6 μm thick and expanded in PVA, a middle laminate wall, 3–6 μm thick, whose outer surface was composed of hemispherical warts, and a thin, inner

Table I. Comparison of calcium levels in spore walls by semi-quantitative analysis[a].

Specimen		Calcium (K-ratio)
Glomus aggregatum	outer hyaline wall	0.257
	laminate wall	0.624
Glomus pansihalos	outer wall	0.377
	ornamented wall	0.504
Entrophospora infrequens	ornamented wall	0.517
	vesicular envelope	0.543

[a] Semi-quantitative analysis was obtained by X-ray microanalysis. The normalized intensity ratio (K-ratio) was used for comparison between samples.

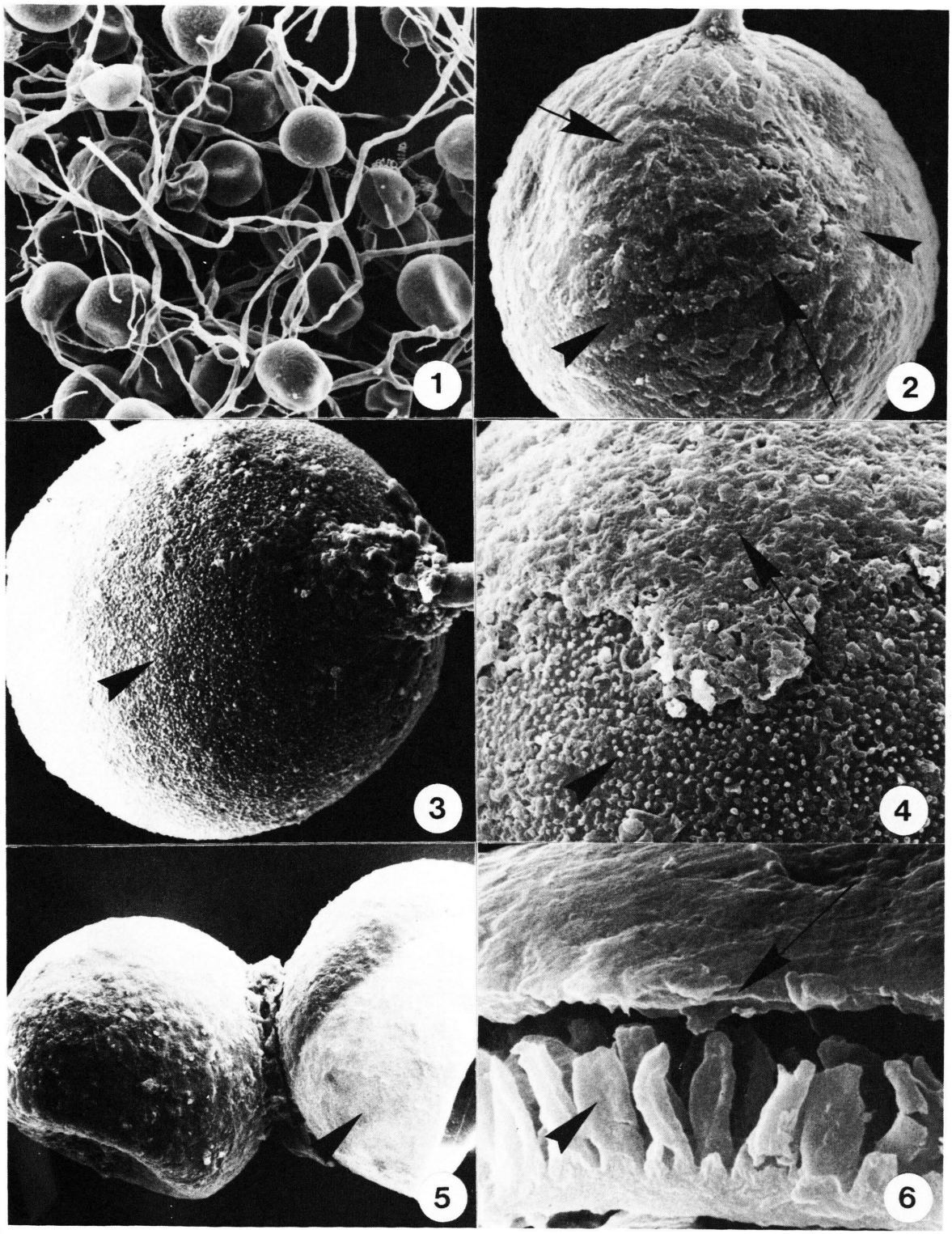
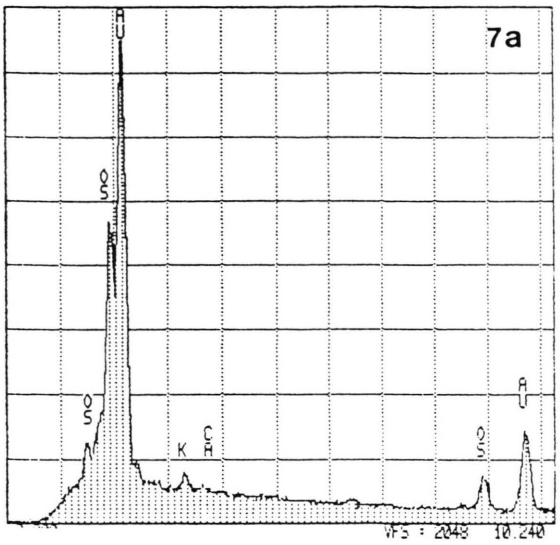


Fig. 1–6. SEM photographs of spores of VA mycorrhizal fungi.
Fig. 1. Sporocarp of *Glomus aggregatum* containing several globose to subglobose spores (×150). Fig. 2. Spore from sporocarp of *G. aggregatum*. Various areas of the wall surface show the hyaline, outer wall which is roughened and may eventually slough-off (arrows) and the smooth surface of the middle wall (arrowheads) (×1200). Fig. 3. Mature spore of *G. pansihalos* showing laminate wall covered with hemispherical warts (arrowhead) (×550). Fig. 4. Enlarged view of outer expanding wall (arrow) and laminate wall covered with hemispherical warts (arrowhead) of the spore of *G. pansihalos* (×7000). Fig. 5. Typical spore of *Entrophospora infrequens* with vesicle (sporiferous saccule) remaining attached (arrowhead) (×400). Fig. 6. Cross-sectional view of wall from vesicular stalk (arrow) surrounding the highly ornamented wall of the spore of *E. infrequens* (arrowhead) (×6000).



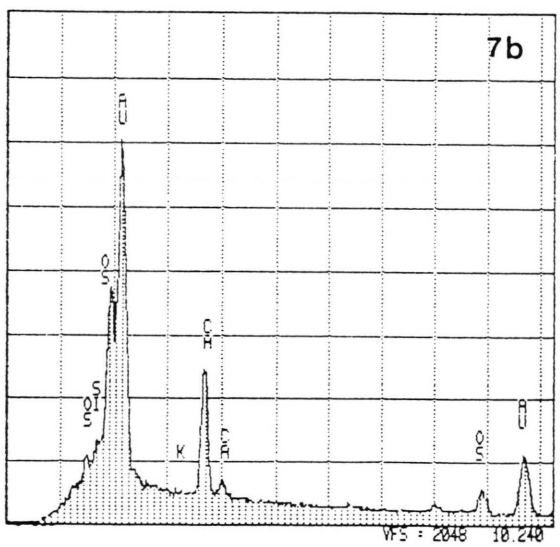
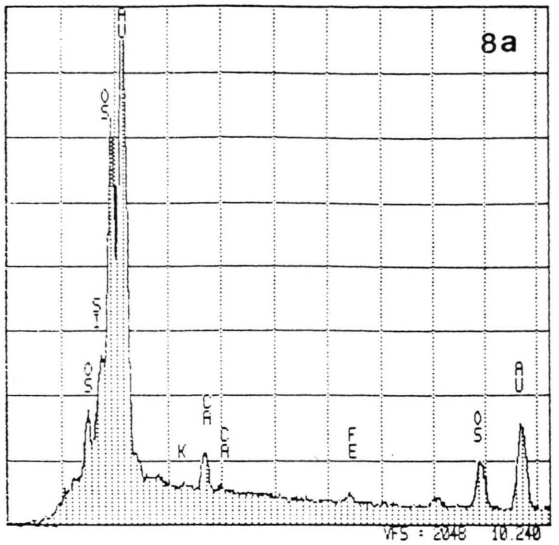


Fig. 7. X-ray spectra of spore walls of *Glomus aggregatum*. X-ray spectrum of the outer, hyaline wall (7 a). Note the low concentration of calcium in this wall. X-ray spectrum of second laminate wall (7 b) revealed a greater amount of calcium than in the outer wall.



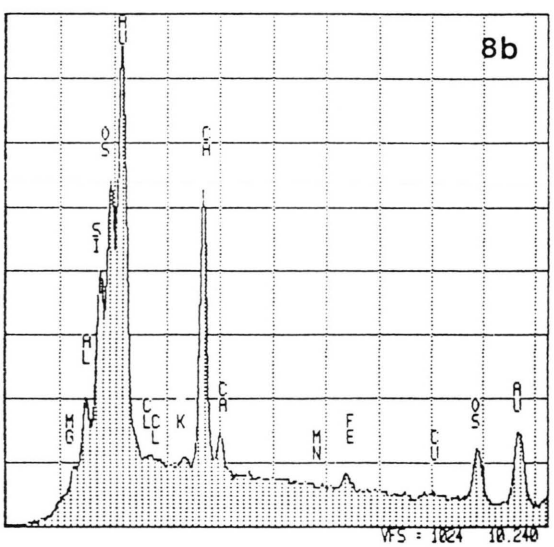


Fig. 8. X-ray spectra of spore wall of *Glomus pansihalos*. X-ray spectrum of outer expanding wall (8 a). Some calcium is detected in this wall. X-ray spectrum of laminate wall (8 b) revealing a greater amount of calcium in this wall as opposed to the outer wall.

wall which was 1 – 2 μm thick (Fig. 3, 4). The sporogenous hypha was cylindrical and composed of 1 – 3 walls. X-ray microanalysis showed the presence of calcium in the outer expanding wall and the middle wall (Table I, Fig. 8). However, the middle, laminate wall had relatively higher levels of calcium (Fig. 8 b) than the outer wall (Fig. 8 a).

The spores of *E. infrequens* were associated with red clover roots at Breton (Lat. 53° 10′ N, Long. 114° 30′ W). Attempts to establish pure pot cultures of this species have failed, as was the case with Ames and Schneider [20]. However, *E. infrequens* occurred as a common contaminant in pot cultures of other VA mycorrhizal fungal species. The spores of *E. infrequens* develop from a vesicle which empties as the spore develops (Fig. 5). Spores were globose to subglobose, ranging in size from 110 – 130 × 100 – 130 μm while the vesicle was

globose to subglobose ranging in size from 110–130 × 110–130 μm. The spores were enclosed by the hyaline wall of the vesicular stalk and possessed an ornamented wall, 2–8 μm thick, consisting of vacuolated spines (Fig. 6). X-ray microanalysis revealed a concentration of calcium in the wall of the vesicle as well as the ornamented wall (Table I, Fig. 9). The amount of calcium present in both walls was found to be similar.

This is not the first report of *G. aggregatum* from Alberta. It has also been collected from peat deposits near Camrose [11] and from mine spoils near Fort McMurray [9]. It appears to be one of the most frequently encountered VA mycorrhizal fungal species in Alberta to date, in addition to *G. mosseae* [21].

Definitive identification of *G. aggregatum* was difficult because these spores are similar to those of *G. fasciculatum* (Thaxter) Gerd. & Trappe emend. Walker and Koske. Detailed examination of the spores and comparison with emended descriptions of *G. aggregatum* [19] and *G. fasciculatum* [22] indicated that the spores matched those described for *G. aggregatum*. The outer, hyaline wall we observed in some spores of *G. aggregatum* was also observed in another Alberta isolate by Zak *et al.* [9]. The innermost wall appears to be similar to that described by Walker [23]. This wall had a greenish color when viewed by bright-field illumination and, thus, matches the description by Koske [19]. Failure of the innermost wall to stain dark red-brown with Melzer's reagent, as seen with *G. fasciculatum* [22] confirmed that this species was *G. aggregatum* [19].

Entrophospora infrequens was also recovered from field soil, and has been reported from mine spoils in Alberta [9]. Although successful pure pot cultures of this species could not be established, it did occur readily as a contaminant in some pot cultures of *G. dimorphicum*, which are being maintained in the Department of Plant Science greenhouses. Contamination of *G. mosseae* pot cultures with *E. infrequens* has also been reported [20]. It was noted that *E. infrequens* did not parasitize the spores of *G. mosseae*, and this has also been the case in our study.

The isolation of spores of *G. pansihalos* from Devon is the first report of this species from Alberta. This species was readily recognized by the outer, hyaline wall which expands in the presence of lactic acid, and by the middle, laminate wall whose surface was covered with hemispherical warts [24]. *Glomus pansihalos* has been reported from southern Ontario soils, associated with fern roots [25], as well as from sand dunes in California, New Jersey, and Michigan [24].

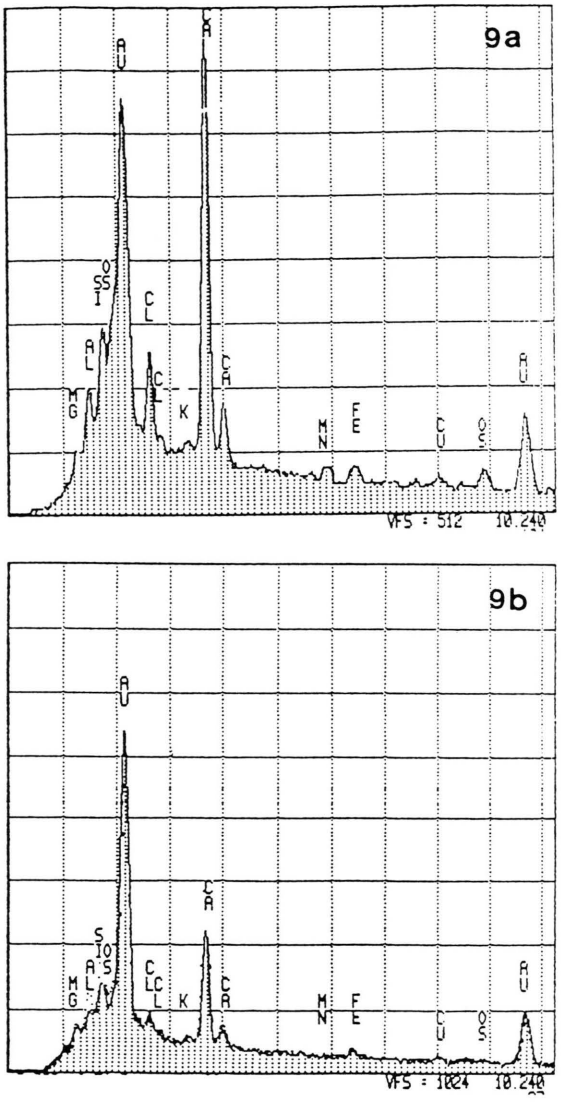


Fig. 9. X-ray spectra of spore wall of *Entrophospora infrequens*. X-ray spectrum of wall of vesicle (9a). Calcium was detected in the wall. Note that the VFS = 512. X-ray spectrum of ornamented wall (9b) shows that this wall contains similar levels of calcium as the vesicular wall. Note that the VFS = 1024. Therefore, when comparing the 2 spectra, the peak obtained for calcium in 9a will be approximately the same as that seen in 9b.

Energy dispersive X-ray microanalysis was a technique employed to study the characteristics of the spore walls of VA mycorrhizal fungi isolated from field soil. This method has been previously used by Boyetchko and Tewari [12] to examine the outer and middle wall layers of *G. dimorphicum* spores. Examination of VA mycorrhizal fungal spore walls revealed that the spore walls of VA mycorrhizal fungi contain relatively high levels of calcium. Semi-quantitative analysis showed that, for some species, one wall may contain more calcium than another wall, for the same spores. The outer hyaline wall of *G. aggregatum* had lower amounts of calcium than the middle wall. Similarly, the ornamented wall of *G. pansihalos* spores contained more calcium than the outer wall. It has also been reported that the outer evanescent wall of *G. dimorphicum* had higher levels of calcium than the middle laminate wall [12].

The importance of calcium in the cell walls of VA mycorrhizal fungi is not clear. Calcium oxalate crystals have been reported in the walls of fungi belonging to the Mucorales [26–28]. Crystals were observed to be firmly embedded in the cell walls of zygophores and sporangiophores of *Mucor mucedo* Brefeld [27]. Pitt and Ugalde [29] speculated that calcium oxalate crystals in fungal cell walls may be an end-product of fungal metabolism and may, therefore, be secreted through the plasma membrane. Whitney and Arnott [28] suggested that the crystals may detoxify calcium ions in fungi. They also proposed that the random, interlocking network of calcium oxalate crystals found in the sporangiophore wall of *Gilbertella persicaria* (Eddy) Hesseltine may provide rigidity to the fungal wall. In lower fungi, such as *Phytophthora cactorum,* oospore production decreases under calcium-deficient conditions [30]. Fletcher [31] similarly showed that low concentrations of calcium in growth medium resulted in high incidence of oogonium and oosphere abortion of *Saprolegnia diclina.* It is possible that the presence of this element in the spore walls of VA mycorrhizal fungi signifies the importance of this element in growth and sporulation. High levels of calcium are also known to induce resistance against many pathogens [32, 33]. Formation of papillae, a thickening of host cell wall and a dynamic host response to pathogens, has been reported in VA mycorrhizal fungi [34]. Papilla may contain large levels of calcium [32] which may play a role in inducing resistance of mycorrhizal fungal spores against parasites occurring in the soil.

Acknowledgements

Award of a graduate fellowship to S. M. Boyetchko from the Canadian Wheat Board is gratefully acknowledged. Mr. George Braybrook is also gratefully acknowledged for his help in the SEM work.

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