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Morphometric and Energy Dispersive X-Ray Analysis of Polyphosphate Distribution in the VAM Fungus *Glomus versiforme* Associated with Leek

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Abstract

Allium porrum L. (leek) seedlings were colonized by the vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus versiforme* (Daniels and Trappe) Berch, and the roots harvested after 7 days. Using morphometry, all stages in the colonization of roots were examined for the amount of polyphosphate stored as granules. All VAM structures, including external hyphae, appressoria, inter- and intracellular hyphae, arbuscules and vesicles contained poly-P granules. Intercellular hyphae had significantly fewer poly-P granules than all other structures except intracellular hyphae. There was no significant difference in number of poly-P granules among intracellular hyphae, arbuscules, external hyphae, appressoria or penetrating hyphae. The X-ray spectrum of poly-P granules in all structures showed major P and Ca peaks as determined by energy dispersive spectroscopy (EDS). The ratio of cation:P was close to 1:1 in poly-P granules analyzed in all VAM structures. There was no significant difference in size of poly-P granules among intercellular hyphae, intracellular hyphae and arbuscules.

Keywords: polyphosphate, *Glomus versiforme*, leek, VAM, X-ray analysis

1. Introduction

The majority of physiological studies with vesicular-arbuscular mycorrhizas (VAM) involve some aspect of phosphorus nutrition (Powell and Bagyaraj,

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1984). It has been documented that excess phosphorus is concentrated as polyphosphate (poly-P) granules in the hyphae and translocated, presumably via cytoplasmic streaming, in this form (Cox et al., 1980). Recently, however, it has been pointed out that the precise mode of phosphorus transport in hyphae of VAM fungi is in doubt (Jennings, 1987).

Poly-P granules have been demonstrated in VAM hyphae by histochemical methods (Ling-Lee et al., 1975; Cox et al., 1975; Strullu et al., 1981), energy dispersive X-ray analysis (Schoknecht and Hattingh, 1976; White and Brown, 1979), wavelength-dispersive X-ray analysis (Cox et al., 1980; Strullu et al., 1981), and high voltage electron microscopy (Cox et al., 1975, 1980). In addition, estimates of poly-P in VAMs have been made by extraction and gel electrophoresis (Callow et al., 1978).

There has been some interest in determining the relationship between the presence of poly-P granules and the stage of fungal colonization of roots (White and Brown, 1979; Strullu et al., 1981), but with the exception of the study by Cox et al. (1980), no attempt has been made to quantify this in a systematic way.

The objectives of this study were to determine, by morphometry, the distribution of poly-P granules in all stages of colonization of *Allium porrum* (leek) roots by the VAM fungus *Glomus versiforme* and to determine the relative cation and phosphorus content of poly-P granules for each stage of the VAM.

2. Materials and Methods

Leek (*Allium porrum* L. cv. Titan) seeds, obtained from Stokes Seeds Ltd., St. Catharines, Ontario, Canada were surface sterilized with 10% aqueous commercial bleach for 10 min, soaked overnight in distilled water and transferred to a bed of moist sand covered with moist paper towelling for 5 days. The germinated seeds were then transplanted into a flat of sterile Turface® (Montmorillonite Clay, International Minerals and Chemical Corporation, Mundelin, IL 60060, USA) for 14 days, followed by transplanting into 1 year old pot cultures containing leek plants and the mycorrhizal fungus *Glomus versiforme* (Daniels and Trappe) Berch (Brundrett et al., 1985). Seedling roots were harvested after 7 days and rinsed with water.

Roots were submersed in fixative (2.5% glutaraldehyde in 0.07 M HEPES buffer, pH 6.8), cut into 2 mm segments and allowed to fix at room temperature for 3 hr. Root pieces were then rinsed four times in HEPES buffer and twice in distilled water, followed by dehydration in a graded acetone series. Following several changes of absolute acetone, root pieces were embedded in Spurr's resin (Spurr, 1969) and polymerized at 65°C for 36 hr.

Resin-embedded roots were sectioned on a Reichert OmU3 ultramicrotome. Sections (approximately 150 nm thick) were picked up on copper grids and allowed to dry, then examined in a JEOL 100CX scanning transmission electron microscope (STEM) operated in the scanning transmission mode. Sections were not stained prior to examination. The STEM was equipped with a horizontal entry energy dispersive spectrometer (EDS) interfaced to a Tracor Northern TN 2000 microanalyser. X-ray microanalyses were performed at the eucentric point in the microscope stage at a take off angle of 30° under standardized lens conditions for a live time of 100 s, with a static spot. Such analyses were carried out on polyphosphate granules found in the following structures: external hyphae, appressoria, penetrating hyphae, hyphal coils, intercellular hyphae, intracellular hyphae, arbuscules and vesicles. When one of these structures was identified, that field of view was photographed onto 120 mm film, and X-ray microanalysis was performed on each of 3 randomly chosen polyphosphate granules within the photographed structure.

The films were developed and the resulting negatives were projected onto a 50.8 cm^2 digitizing tablet (Model DTR-2020 Datatizer, GTCO Corp, MD, USA). The X-Y coordinates from the cursor used to trace the perimeter of the structure being examined (vesicle, arbuscule or external hyphae, for example) were analysed with a microcomputer to give total area of the structure in question. The number of polyphosphate granules within that structure was then counted. In this way, the average number of polyphosphate granules per unit area for each type of structure was determined. Sample size varied from structure to structure, this being dependent on their prevalence in the tissue examined. For morphometric analysis of size of poly-P granules, elevennegatives were chosen that had intercellular hyphae, intracellular hyphae and arbuscules present. These were projected onto white paper and the outline of poly-P granules drawn. These drawings were subsequently measured using the same microcomputer system described above. Results were analyzed using a one way ANOVA with random block design (blocks were negatives).

3. Results

Poly-P granules were identifiable in STEM analysis of unstained, relatively thick sections by their shape and electron opacity (Fig. 1). EDS analysis confirmed that these granules were phosphorus-rich and they were found in the fungus at all stages of colonization. Spectra for granules analyzed from an external hypha (Fig. 2), an intercellular hypha (Fig. 3) and a vesicle (Fig. 4) are representative of spectra obtained from all stages of colonization. Each granule analyzed had distinctive and major phosphorus, calcium and chlorine

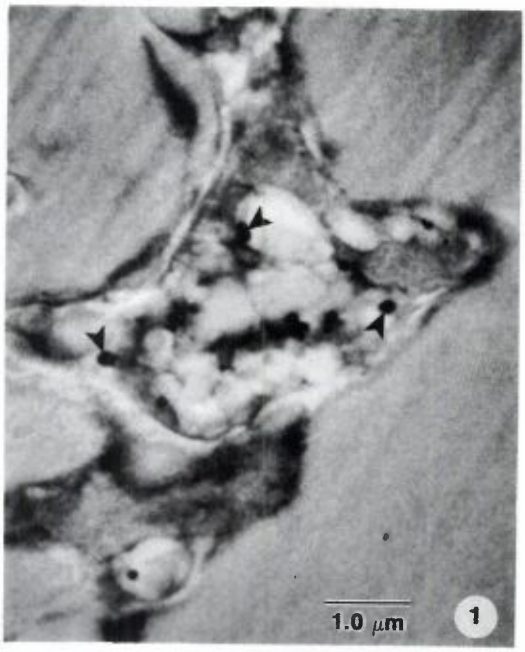
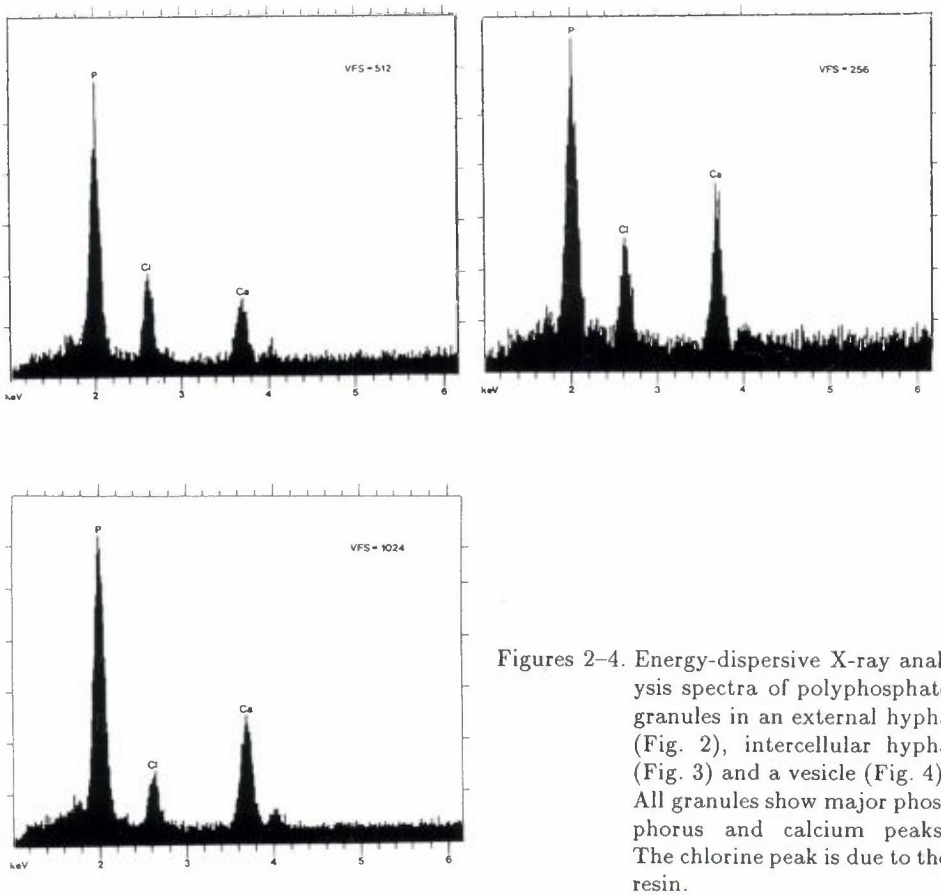


Figure 1. Section through a fine arbuscular hypha showing several polyphosphate granules (arrowheads) within vacuoles

peaks, the latter being due to the resin as indicated by a 'background' reading of blank resin. Small peaks for magnesium and potassium were occasionally seen. The total cation (Ca^{++}, Mg^{++}, K^{+}) and phosphorus (P) content per poly-P granule was determined by calculating peak:background ratios for each element. There was no significant difference in cation or P content/granule among all structures analyzed (Table 1).

Morphometric analysis to determine the quantity of poly-P for each stage of colonization showed that intercellular hyphae had a comparable number of poly-P granules to intracellular hyphae but significantly fewer poly-P granules than arbuscules, external hyphae, appressoria and penetrating hyphae (Table 2). There was no significant difference among intracellular hyphae, external hyphae, appressoria, penetrating hyphae and arbuscules as to numbers of poly-P granules (Table 2). Although poly-P granules were present in vesicles, these were not included in the analysis because so few were located in the material analyzed.

There was no significant difference in mean size of poly-P granules among intercellular hyphae, intracellular hyphae and arbuscules (Table 3).



Figures 2–4. Energy-dispersive X-ray analysis spectra of polyphosphate granules in an external hypha (Fig. 2), intercellular hypha (Fig. 3) and a vesicle (Fig. 4). All granules show major phosphorus and calcium peaks. The chlorine peak is due to the resin.

4. Discussion

This is the first report of poly-P granules in all stages of a VAM association, and the first quantification of poly-P in the same stages. Previously, poly-P granules have been localized in intercellular hyphae, arbuscules and vesicles in a *Glycine max* (L.) Merr./*Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trap. mycorrhiza and a subjective estimate of their abundance in these structures

Table 1. Phosphorus and cation levels in polyphosphate granules during colonization of leek roots by *G. versiforme*

Mean VAM structure	# of granules analyzed	Mean Peak: Background (P)	Mean Peak: Background (Ca, Mg, K)
intercellular hyphae	244	1.894 a*	1.598 a*
intracellular hyphae	171	2.116 a	2.265 a
arbuscules	102	2.420 a	2.516 a
external hyphae	30	1.416 a	1.435 a
appressoria	33	1.977 a	1.823 a
penetrating hyphae	30	2.055 a	2.051 a
vesicles	6	2.741 a	2.733 a

* Values within columns followed by the same letter are not significantly different ($P < 0.05$) using Duncan's new multiple range test.

Table 2. Distribution of polyphosphate granules during colonization of leek roots by *G. versiforme*

VAM structure	# of samples	Mean # of granules/μm^2 cytoplasm
intercellular hyphae	104	1.06 a*
intracellular hyphae	68	1.34 ab
arbuscules	76	1.61 b
external hyphae	10	1.64 b
appressoria	11	1.65 b
penetrating hyphae	11	1.73 b

* Values followed by the same letter are not significantly different ($P < 0.05$) using Duncan's new multiple range test. Values transformed for analysis. Actual values given in table.

Table 3. Size of polyphosphate granules in various structures within leek roots colonized by *G. versiforme*

VAM structure	# of samples	Mean size of granules (μm)
intercellular hyphae	85	0.033027 a
intracellular hyphae	85	0.025554 a
arbuscules	85	0.024814 a

* Values followed by the same letter are not significantly different ($P < 0.05$) using Tukey's studentized range (HSD) test.

given (White and Brown, 1979), in the trunk and fine branches of arbuscules in mycorrhizal *Taxus baccata* L. roots (Strullu et al., 1981; Strullu et al., 1982), in intercellular hyphae, intracellular hyphae and arbuscules of *Allium cepa* – *Glomus mosseae* mycorrhizae (Cox et al., 1980), in arbuscules of *Allium cepa* – *Glomus fasciculatus* mycorrhizae (Schoknecht and Hattingh, 1976), and in inter- and intracellular hyphae and vesicles in mycorrhizal *Liquidambar styraciflua* roots (Ling-Lee et al., 1975). It is obvious from the present and previous studies that VAM fungi are capable of storing poly-P from the beginning of the colonization process, i.e. in external hyphae attached to the root, appressoria and penetrating hyphae, through to the formation of arbuscules and vesicles. In the present study, the fewest poly-P granules were found in intercellular hyphae, likely due to the fact that, in leek roots, these hyphae are rapidly extending the infection units at 7-days post colonization (Brundrett et al., 1985) and therefore would not be expected to store much poly-P. An earlier report of intercellular hyphae and vesicles storing the largest amount of poly-P in a soybean mycorrhiza (White and Brown, 1979) does not contradict our findings since these authors noted that highly vacuolated intercellular hyphae were involved. This suggests that the soybean mycorrhizas examined were at a later stage in colonization although the authors did not report the age of the samples processed.

In the leek – *G. versiforme* mycorrhizas examined at 7 days after inoculation, very few collapsed arbuscules or vesicles were present, indicative of the early stage of colonization (Brundrett et al., 1985). It is of interest that young arbuscules examined had as many poly-P granules as external stages and if the current dogma regarding transport of poly-P granules by cytoplasmic streaming is correct (Cox et al., 1980), then this result is not that surprising. Obviously, however, if the plant is to utilize the P present in poly-P granules, then the granules must be altered metabolically for uptake of phosphorus across root/cell plasma membranes. Previous workers suggest that this takes place as arbuscules mature (White and Brown, 1979), perhaps in the fine branches (Cox et al., 1975). Consistent with this, Ling-Lee et al. (1975)failed to find poly-P granules in arbuscules which appeared to be devoid of all cell contents, and Strullu et al. (1981) report the absence of poly-P granules in collapsed arbuscules.

The results of energy-dispersive X-ray microanalysis showing that the elemental composition of poly-P granules is primarily P and Ca^{++}, agree with previous results on conventionally fixed and embedded VAM (White and Brown, 1979; Strullu et al., 1981). One must be cognizant, however, of the very real probability that the high Ca^{++} content may be artifactual due to displacement of K^{+} by Ca^{++} during tissue processing using the usual methods (see Orlovich

et al., 1989). Analysis of poly-P granules in freeze-substituted tissue could verify this.

There is no difference between P content and combined cation content of poly-P granules among all structures analyzed, suggesting that there is probably one form of poly-P in this symbiotic association. The ratio of cation/P in poly-P granules was close to 1:1 in all structures (see Table 1). This is similar to the values given for Ca^{++} only in comparison to P in poly-P granules of *Taxus baccata* VAM (Strullu et al., 1981). Although there is certainly a size range of poly-P granules within each VAM structure, morphometric analysis showed no significant difference in mean size. It should be of interest to determine the effect of varying the phosphorus level during the colonization process on the quantity, size, and distribution of poly-P granules in a VAM association.

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