Mineral Ion-containing Vacuolar Inclusion Bodies Associated with Ca^{2+} Deficiency in Oogonia of *Saprolegnia diclina* and *S. terrestris*

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The frequency of occurrence of electron-dense vacuolar inclusions in oogonia of *Saprolegnia diclina* and *S. terrestris* was significantly greater under Ca^{2+} -deficient culture conditions than under Ca^{2+} -sufficient conditions. Bodies similar to inclusions seen in sections of oogonia were present in debris from living cultures ground up in de-ionized water. Energy dispersive X-ray analysis of inclusions in sections showed the presence of Mg, P, Ca, and Mn in both species and Na, S and Zn in *S. diclina*. The bodies from ground cultures contained Mg, P, Mn and Zn in both species and Na in *S. terrestris*.

INTRODUCTION

In *Saprolegnia diclina*, Ca^{2+} deficiency results in abnormal volume densities and abnormal behaviour of some organelles in developing oogonia (Fletcher, 1979*a*) and in a large increase in the abortion rates of both oogonia and oospores (McCann & Stuart, 1973; Fletcher, 1979*a*). In *Saprolegnia terrestris*, Ca^{2+} deficiency results in the formation of some multinucleate oospores due to failure of supernumerary nuclei to degenerate, in the formation of unusually large numbers of oospores in some oogonia, and in some increase in abortion rates (Fletcher, 1979*b*). Fletcher (1979*a*) reported the presence of crystal-like inclusions in vacuoles of developing oogonia as part of the abnormality syndrome in *S. diclina* grown in Ca^{2+} -deficient culture medium. Further observations on these inclusions, and observations on similar inclusions in oogonia of *S. terrestris*, are reported here.

METHODS

Organisms and culture. Stock cultures of *Saprolegnia diclina* Humphrey and *S. terrestris* Cookson ex Seymour were maintained at 20 °C on a culture medium containing (g l⁻¹ in distilled, de-ionized water) glucose, 1.66; sodium glutamate, 0.666; DL-methionine, 0.016; KH₂PO₄, 0.046; K₂HPO₄, 0.056; MgCl₂.6H₂O, 0.33; MnCl₂.4H₂O, 0.02; CaCl₂.6H₂O, 0.006; FeCl₃.6H₂O, 0.004; ZnSO₄.7H₂O, 0.01 and solidified with 1.5% (w/v) agar (Oxoid no. 1). Before addition to culture media, stock solutions of sodium glutamate (133.2 g l⁻¹, BDH laboratory grade) were purified of contaminant metal ions by passage through a 251 × 13 mm column of cation-exchange resin (Amberlite IRC-50(H), BDH) which had been purified by the method described by Hirs *et al.* (1953) for Amberlite IRC-50 (XE-64) resin, converted to the sodium form by stirring with 2.8 M-NaOH (33 ml per 100 g resin) for 3 h and repeatedly washed with distilled water until the wash water pH was about 10. All glassware used for preparation of culture media and for growth of experimental cultures was rinsed five times with distilled water before use. Experimental cultures were grown at 20 °C in 70 mm diameter glass Petri dishes in a liquid medium of similar nutrient composition to the stock culture medium but diluted to one-quarter strength and of variously modified Ca^{2+} concentration. Ca^{2+} -sufficient cultures were grown for 5 to 10 d with either 6.85 µM-CaCl₂ (*S. diclina*) or 109.6 µM-CaCl₂ (*S. terrestris*). Ca^{2+} -deficient cultures were either grown with 1.71 µM-CaCl₂ for 2 d followed by transfer to CaCl_2 -free culture medium for 5 to 10 d (*S. diclina*), or grown continuously with 1.71 µM-CaCl₂ for 5 to 10 d (*S. terrestris*).

Abbreviation: EDX analysis, energy-dispersive X-ray analysis.

Electron microscopy. For electron microscopy of sectioned material, experimental cultures were subjected to one of the following treatments: (1) fixed for 20 min in a mixture of aqueous 2% (w/v) formaldehyde and 2% (v/v) glutaraldehyde, rinsed three times with water, post-fixed for 1 h with aqueous 1% (w/v) osmium tetroxide, rinsed three times with water (all at room temperature and buffered at pH 7.2 with 0.05 M-sodium cacodylate/HCl), and dehydrated in an acetone water series; (2) fixed in the formaldehyde and glutaraldehyde mixture only and dehydrated as in (1); or (3) dehydrated as in (1) without fixation. Material prepared by procedure (1) was bulk stained by retention for 1 to 4 h in 50% (v/v) acetone saturated with uranyl acetate during dehydration. Dehydrated material was embedded in either Araldite or Epon. Sections were examined either unstained or after staining for 15 min with lead citrate (Reynolds, 1963). Energy dispersive X-ray (EDX) analysis was carried out in a Jeol JEM 100C electron microscope equipped with STEM and with EDAX International analysis accessories, using a probe diameter of 1 nm and counting times of 100 to 600 s depending on the count rate obtained. For the analysis of sectioned material, unstained, thick (250 nm) sections of embedded material fixed by procedure (2) were mounted on carbon-stabilized Formvar support films on either beryllium or copper grids. For examination and EDX analysis of non-chemically treated material, live Ca^{2+} -deficient cultures were ground in de-ionized water with a pestle and mortar and drops of the resultant slurry dried on to carbon-stabilized Formvar support films on copper grids. Significance of X-ray spectrum peaks was determined as described by Chandler (1977).

RESULTS

In thick (500 nm) sections of oogonia, electron-dense vacuolar inclusions varied in profile from ellipsoidal to needle-like (Fig. 1*a*). In material from Ca^{2+} -sufficient cultures, inclusions were found in two out of eleven oogonia examined for *S. diclina* and in two out of nine for *S. terrestris*, while in material from Ca^{2+} -deficient cultures, inclusions were found in 14 out of 16 oogonia for *S. diclina* and in four out of five for *S. terrestris*; assuming the presence or absence of inclusions to be a binomially distributed variable, these differences between Ca^{2+} -sufficient and Ca^{2+} -deficient cultures are significant at $P < 0.001$ for both species. In material from Ca^{2+} -deficient cultures, inclusions were occasionally seen in vacuoles of antheridia and hyphae but more frequently seen in vacuoles of oogonia. Inclusions were present and appeared equally electron dense in sections of unfixed and unstained oogonia and in sections of fixed oogonia treated with both osmium tetroxide and uranyl acetate. Staining sections with lead citrate removed the inclusions leaving holes in the embedding plastic. Bodies similar to the inclusion bodies present in sections were found in debris from Ca^{2+} -deficient cultures ground in water (Fig. 1*b, c*). EDX analysis of the bodies in sections and from ground material revealed the presence of Na, Mg, P, S, Ca, Mn and Zn, respectively, in some or all of the bodies (Table 1).

DISCUSSION

An increased incidence of inclusion bodies in vacuoles of oogonia of *S. diclina* and *S. terrestris* appears to be significantly associated with the growth of cultures in Ca^{2+} -deficient culture media and, consequently, with the developmental abnormalities induced by Ca^{2+} deficiency reported previously (McCann & Stuart, 1973; Fletcher, 1979*a, b*). The presence of inclusion bodies in sections of unfixed oogonia and of similar bodies in debris of cultures ground in water suggests that the inclusions are not artefacts and are present as solid bodies in living oogonia – differences in preparation procedure could account for differences in the elements identified between bodies from sectioned and ground material, respectively. The removal of inclusions from sections by lead citrate stain shows that resin monomers did not penetrate inclusion bodies and suggests a small inter-molecular spacing of inclusion body components, while the intrinsically high electron density of the bodies suggests a preponderance of elements heavier than carbon, hydrogen and oxygen. It is therefore possible that inclusion bodies are primarily inorganic in composition.

Inclusion bodies possibly chemically similar to those reported here have been found in mitochondria and in small cytoplasmic vacuoles of *Didymium squamulosum* (Gustafson & Thurston, 1974); these contained Ca and P and were removed from sections by 0.1 M-NaOH. Dense bodies in oogonia of *Saprolegnia furcata* have been shown to contain P and S (Gay, 1972); dense bodies are normal cytoplasmic components of *Saprolegnia* spp., distinct from the vacuolar

Fig. 1. Electron-dense bodies from Ca^{2+} -deficient cultures of two *Saprolegnia* species. (a) Inclusion bodies in the central vacuole of a thick-sectioned (500 nm) oogonium of *S. diclina*. The arrows indicate bodies apparently included in the section entire. Fixed with formaldehyde and glutaraldehyde, post-fixed with osmium tetroxide, and bulk stained with uranyl acetate. The bar marker represents 5 μm . (b, c) Bodies from debris of cultures of *S. diclina* (b) and *S. terrestris* (c) ground in de-ionized water. The bar markers represent 0.1 μm .

Table 1. Elements identified by EDX analysis in electron-dense bodies from *S. diclina* and *S. terrestris*

Five bodies were analysed per species per preparation procedure. None of the elements listed was identified in areas immediately adjacent to the bodies analysed. S, vacuolar inclusion bodies in sectioned oogonia. G, bodies in whole mounts of live cultures ground in water. +, Peaks significant in all analyses; (+), peaks significant in some analyses; —, no significant peaks obtained.

Species	Na	Mg	P	S	Ca	Mn	Zn
<i>S. diclina</i>							
S	+	+	+	+	+	+	+
G	—	+	+	—	—	+	(+)
<i>S. terrestris</i>							
S	—	+	+	—	+	+	—
G	+	+	+	—	—	+	+

inclusions reported here, and are not removed from sections by lead citrate stain in either *S. diclina* (Fletcher, 1979a) or *S. terrestris* (Howard & Moore, 1970; Fletcher, unpublished). Polyphosphate bodies containing P, K, Mg, Ca, Fe, Cl and S occur in *Aureobasidium pullulans* (Crang, 1980), gamma particles containing P, Ca, K, Cl and S (together with DNA and RNA) in *Blastocladiella emersonii* (Hutchinson *et al.*, 1977) and gamma-like bodies containing P and Ca in *Rozella allomyces* (Wool & Held, 1976); morphologically, all these bodies appear more similar to the dense bodies of *Saprolegnia* spp. than to the vacuolar inclusions reported here. Amongst non-mycelial protists, lipid-containing, refractive, intracellular granules have been shown to contain various elements, including Ca, Mg and P in *Tetrahymena pyriformis* (Coleman *et al.*, 1973b) and Ca, Mg, K and P in *Amoeba proteus* (Coleman *et al.*, 1973a). Inorganic, intracellular concretions containing a variety of elements have been found in several animal genera (Simkiss, 1979).

The observations on *S. diclina* and *S. terrestris* reported here suggest that mineral elements that are constituents of the culture medium are sequestered in vacuolar inclusion bodies and that

this sequestration is increased under conditions of Ca^{2+} deficiency. It is possible that increased sequestration of biologically important elements such as Mg, Mn and P, all identified both in inclusions in sections and in bodies from ground cultures in both species, might at least contribute to developmental abnormalities occurring under Ca^{2+} -deficient conditions. If Ca is a normal component of inclusion bodies that is lost when cultures are ground in water, increased sequestration of Ca under conditions that are already Ca^{2+} -deficient could tend to aggravate the effects of the Ca^{2+} deficiency.

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