The Lichenologist **37**(5): 445–462 (2005)
doi:10.1017/S0024282905014982 *Printed in the United Kingdom*

Isidia ontogeny and its effect on the CO_2 gas exchanges of the epiphytic lichen *Pseudevernia furfuracea* (L.) Zopf

Mauro TRETIACH, Paola CRISAFULLI, Elena PITTAO, Simona RININO, Enrica ROCCOTIELLO and Paolo MODENESI

Abstract: The development of isidia in thalli of *Pseudevernia furfuracea* from the Carnic Alps (North-eastern Italy), and the effects of these structures on CO_2 gas exchanges were investigated. The ontogenetic events were studied by comparison of sections stained with different histochemical tests and SEM observations. A high cell turnover rate in both symbiotic partners is the first sign of isidium development, followed by an increased aplanosporogenesis of algae and growth of neighbouring medullary hyphae which become oriented upwards. Large nuclei and an intense cytoplasm activity characterize the mycobiont cells. The surface of very young isidia shows an irregular structure of spherical to ovoid protruding tips of perpendicular cortical hyphae, that are later organised in a pseudomeristematic area similar to that observed in the apex of growing lobes. CO_2 gas exchange measurements carried out in the laboratory confirmed the high metabolic activity of isidia. At optimal water content and favourable light conditions, isolated isidia had rates of gross photosynthesis and dark respiration that were twice those of non-isidiate lobes. Isolated isidia also had a very low CO_2 saturation point, probably because of their favourable surface/volume ratio, and a high light saturation, probably linked to their high content of photosynthetic pigments. The different roles played by isidia in the biology of *Pseudevernia furfuracea*, and particularly their rejuvenating effect on aged lobes, are discussed, and the presence of thalloconidia is briefly mentioned.

Key words: chlorobiont, hydration, mycobiont, morphogenesis, ontogeny, photosynthesis, respiration, thalloconidia, vegetative propagule

Introduction

One of the most striking features of the lichen symbiosis is the production of a wide spectrum of symbiotic vegetative diaspores that permit the simultaneous dispersal of both symbionts (Honegger 1998, 2001). In this way the lichen symbiosis by-passes the problem that any germinating sexual ascospore encounters, i.e. to find a compatible photobiont partner to re-establish the symbiotic phenotype (Poelt 1993; Walser *et al.* 2001).

Soredia and isidia are the most frequent type of diaspores produced by lichens (Büdel & Scheidegger 1996). Corticated, variable in size and shape, isidia are particularly frequent in foliose and fruticose species of *Collemataceae*, *Parmeliaceae*, *Peltigeraceae*, *Pyxinaceae*, *Stictaceae*, and *Umbilicariaceae* (Bowler & Rundel 1975), being rarer in crustose species, although isidia occur regularly in members of, for example, *Pertusariaceae* (Dibben 1980). Soredia have been extensively studied from many points of view (Bailey & James 1979; Armstrong 1987, 1990, 1991; Raineri & Modenesi 1988; Stocker-Worgotter & Türk 1989, 1990; Tretiach & Carpanelli 1992; Marshall 1996; Lorentsson & Mattsson 1999; Zoller *et al.* 2000; Nimis & Martellos 2003), but, in contrast, our knowledge concerning isidia is relatively poor. The most recent studies have been mainly focused on their dispersal, establishment and survival (Gilbert 1988; Scheidegger *et al.* 1995*a*; Zoller *et al.* 2000),

M. Tretiach (corresponding author), P. Crisafulli and E. Pittao: Dipartimento di Biologia, Università di Trieste, Via L. Giorgieri 10, I-34127 Trieste, Italy.
S. Rinino, E. Roccotiello and P. Modenesi: DIP. TE.RIS., Università di Genova, Corso Dogali 1/c, I-16136 Genova, Italy.

or on development of the juvenile thallus (Honegger 1987, 1993; Zoller *et al.* 2000). The development of a dense cover of isidia, however, causes many interesting modifications at the thallus level. The large increase in surface area, for instance, modifies thallus water absorption (Rikkinen 1997) and water holding capacity (Jahns 1984), while the darker colour typical of isidial outgrowths probably influences the thermal operating-environment (Kershaw 1983; Coxson *et al.* 1984).

The present paper analyses the results of a multi-disciplinary investigation aimed at clarifying two little known aspects of isidia: the first stages of their ontogeny and the influence they exert on the assimilative capacity of the thallus. The locally common fruticose lichen, *Pseudevernia furfuracea* (L.) Zopf, was selected for the study because: (a) it was readily available, (b) young lobes devoid of isidia coexist on the same thallus with old lobes completely covered with isidia, and the two types of lobe can easily be separated; (c) this species is often used in ecophysiological (Türk 1983; Manrique *et al.* 1993; Scheidegger *et al.* 1995*b*; Vidergar-Gorjup *et al.* 2001; Kranner *et al.* 2003) and biomonitoring (Niewiadomska *et al.* 1998; Bari *et al.* 2001; Vingiani *et al.* 2004) studies.

Material and Methods

Lichen sampling

Thalli of *Pseudevernia furfuracea* were collected in January and December 2003 from the lower branches of isolated Larch (*Larix decidua* Mill.) trees, near Sauris di Sotto (Carnic Alps, Italy), at 1500 m altitude for studying isidia development (Figs 1–4) and gas exchange measurements (Figs 7–10). Thalli were air-dried in the forest, then transported immediately in plastic bags to the laboratory, where they were cleaned of bark fragments and debris under a dissecting microscope. The negative reaction to C (sodium hypochlorite, 10%), which is diagnostic for var. *furfuracea* (Culberson *et al.* 1977), was verified in 100 randomly selected samples; less than 3% of the samples tested belonged to var. *ceratea* (Ach.) D. Hawksw.

Further samples were collected in June 2004 for quantifying the increase in external surface area due to the presence of isidia (Fig. 5), and for studying water loss processes (Fig. 6). Thirty randomly selected thalli were collected with a short piece of the twig on which they were growing, put in closed plastic boxes, *c.* 4 × 4 × 5·5 cm, without touching them, and transported to the laboratory for further processing.

Isidium ontogeny

Observations were carried out by SEM, bright field and fluorescence light microscopy on small apical portions of thalline lobe tips, *c.* 0·5–1 cm long. For SEM dried thallus fragments were directly coated with a 200–220Å thick layer of gold in a sputter coater and then examined in a Philips 515 SEM at 20 kV.

For bright field microscopy thalline fragments were fixed in buffered formalin (Pearse 1985) for 24 h, dehydrated in an ethanol series (30, 50, 70, 80, and 95%) and embedded in JB4 resin (Polysciences, Inc. Warrington, UK). Sections 7–10 µm thick were cut with a glass knife on a Reichert OM2 ultramicrotome. The following histochemical tests were performed on the JB4-embedded sections: (1) periodic acid-Schiff (PAS) for general water-insoluble polysaccharides localization (Pearse 1985); (2) Toluidine Blue O (TBO) 0·05% in acetate buffer pH 4·4 for 1 min as metachromatic stain (for mechanism of TBO metachromatic reaction, see Giordani *et al.* 2003). Another series of unfixed and cryostatic sections was used to show acid phosphatase activity according to the azo dye method of Burstone (1958).

For fluorescence light microscopy, thalline fragments were embedded in Technovit 7100 (Hereaeus Kulzer, Wehrheim). Sections 7 µm thick were mounted serially and observed unstained to show autofluorescence or treated with 0·001% DAPI (4′,6-diamidino-2-phenylindole, Sigma-Aldrich, St Louis, USA) for nuclear staining (Regan & Moffatt 1990). Observations were made using an optical microscope Leitz Dialux 22EB equipped with an epi-illuminating condenser mounted with types A (UV=340–380 nm) and H2 (blue=390–490 nm) fluorescence filter blocks. Photomicrographs were taken using Kodak Ektachrome 160T colour reversal film.

Control reactions for all histochemical methods were carried out according to the methods suggested by the respective authors.

Estimation of isidial density and increase in external lobe surface area

Thirty-six isidiate lobes were selected randomly from 12 thalli stored in plastic boxes, and observed under a stereomicroscope Wild M420 with a Leica DC300 camera connected to a computer. Entire and broken isidia were counted on the computer monitor covered with a plastic film, marking them with a felt-tip pen, continuously adjusting the focus of the image. The dry weight of each sample was measured after 24 h at 120°C, and its surface area was measured with a LI3000A Leaf Area Meter (Licor Inc., Lincoln, Ne, USA), using enlarged, calibrated photocopies. The isidia were then removed. A randomly selected subsample of 100 isidia was mounted in glycerine, observed

with a transmitted light microscope at × 100 magnification and their length and basal diameter were recorded. The isidium lateral surface area was approximated to that of a cylinder with the same basal diameter. The surface area of each lobe was then estimated as the sum of the two faces, plus the sum of the lateral surface areas of the isidium population, taking into account also fallen isidia.

Thallus water loss

Water loss was measured gravimetrically after soaking silica dried thalli of similar weight (*c.* 0·22 g each) for 5 min in distilled water, blotting or shaking dry, and the rate of weight change was followed by weighing at increasing time intervals (every minute for the first 10 min, then every two minutes for half an hour, and finally every 30 min until air dry). Between weighings, samples were simultaneously placed on a latticework placed on a laboratory bench in dim light. The measurements were carried out at 27°C, 40%RH; temperature and air humidity were monitored with an Assmann aspired psychrometer. Two sets of thalli, strongly isidiate, and weakly isidiate, were used; each set consisted of two samples. At the end of experiments, the thalli were dried to constant weight at 120°C.

CO_2 gas exchange

Treatment of samples

Three sets of samples were prepared: isidiate lobes (IL), non-isidiate lobes (NIL), and isidia (I). The latter were obtained by gently shaking plastic bags containing air-dried thalli. The samples of all materials, *c.* 500 mg each, were dried over silica gel for 24 h, sealed in Petri dishes and kept at −32°C until use. Concomitant chlorophyll fluorescence measurements demonstrated the particularly high stability of light reactions of *P. furfuracea* after 6 months of low temperature storage, in good accordance with the results of Feige & Jensen (1987).

Before the experiments IL and NIL samples, consisting of 12 to 16 lobes taken from different thalli, were re-hydrated for 18 h in the dark in a closed chamber filled with wet paper, then immersed in distilled water for 30–40 sec, and then gently hand centrifuged. The lobes were then placed in a Petri dish, covered with a thin mesh to avoid the curling of wetted lobes, and used in the CO_2 gas exchange measurements. If necessary, in order to maintain hydration, the material was gently sprayed with distilled water after each series of measurements.

A different protocol was adopted for the samples of isidia (I). These were also kept for 18 h in a humid chamber, but then positioned with a soft brush on a disc of commercial horticultural fleece (“non-woven fabric”), stretched on a plastic ring of *c.* 10 cm diam. The disc was laid for 120 min on a layer of Whatman no. 1 filter paper soaked in distilled water, in a Petri dish, which was placed in a yet larger petri dish lined with wet paper. Half an hour before starting the CO_2 gas exchange measurements, samples were exposed to a photosynthetic photon fluence rate (PPFR) measured over the waveband 400–700 nm of 100 μmol photons m^{-2} s^{-1}, weighed after removing excess water on the fleece disc with absorbing paper, and then used in the experiments. Thallus relative water content (RWC) was expressed as a percentage of dry weight (i.e. RWC% = [(wet weight − dry weight)/dry weight] × 100). Following this protocol, hydration of isidia was constantly around 150%, but only two hydration cycles per day could be carried out, with a loss of *c.* 3–4% of the isidia per cycle.

Water content, CO_2, and light response curves

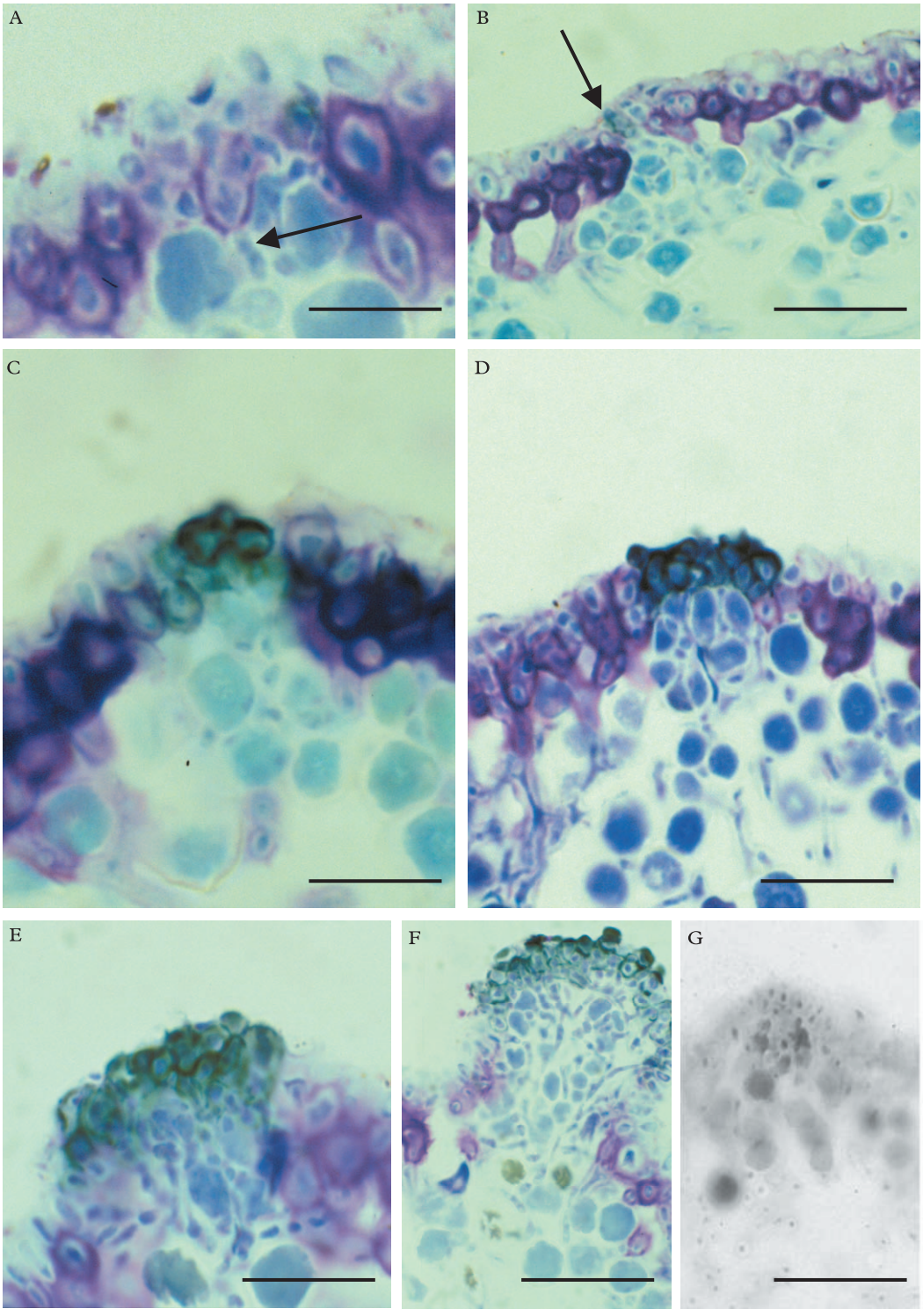
The CO_2 exchange rates of the three sets of samples (IL, NIL, I) were characterized by building up response curves to water content, CO_2, and light, at a thallus temperature of 17 ± 1°C. Maximum net photosynthesis (Ph_n) and dark respiration (R_d) at different CO_2 concentrations and light conditions were calculated as the average of three to six values measured at optimal thallus water content. Gross photosynthesis (Ph_g) was calculated as the sum of Ph_n and R_d. At the end of the CO_2 gas exchange experiments, samples were dried to constant weight over silica gel, and divided into two parts, one for the estimation of their photosynthetic pigments (see below), and the other for measuring their dry weight at 120°C for 24 h, and then put over silica gel for 2 h.

The CO_2 exchange rates were measured in a closed system with a LICOR-6250 infrared gas analyzer (IRGA) (Licor Inc., Lincoln, Ne, USA; estimated precision ± 0·2–0·3 ppm CO_2; cuvette volume 4000 cm^3; internal volume 130 cm^3; maximum flow rate 1000–1100 μmol s^{-1}), connected to a data-logger. During the experiments, a natural light spectrum generated by a halogen incandescent lamp was provided by a Fl-400 fiber illuminator (Walz, Effeltrich, Germany) and the PPFR was measured using a Licor Quantum meter model LI 185A inserted within the cuvette at the same position as the lichen thalli.

Chlorophylls and carotenoids

Samples of IL, NIL (n=10), and I (n=16), each *c.* 50 g, were dried over silica gel for 24 h, then repeatedly washed with 5 ml aliquots of pure spectrophotometric grade acetone, until the solution did not turn yellow after the addition of a drop of conc. KOH, to remove the lichen substances (Brown & Hooker 1977). Approximately, 8–10 washings were necessary for IL and NIL, and 20–22 washings for I. Further samples, consisting of seven single isidiate lobes, were cut in half along their axis into two portions, *c.* 13–22 mg in weight, and from one half isidia carefully removed under a dissecting microscope; both groups of samples, with and without isidia, were washed as above.

The samples were homogenized with a small aliquot of polyvinyl polypyrrolidone in 15 ml DMSO for 50 mg of material under dim, green light, the tube left overnight at room temperature in the darkness, and then centrifuged at 5000 rpm. The absorbance of supernatant was measured with a spectrophotometer Perkin-Elmer mod. 554. Estimation of chlorophylls and total carotenoids was carried out using the equations of



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Pfeifhofer *et al.* (2002), and the ratio OD_{435}/OD_{415} was calculated according to Ronen & Galun (1984).

Statistics

Statistics were carried out with the program Excel97 (Microsoft) and Statistica 6.0 (StatSoft). Polynomial curves were obtained with the program Curvefit.

Results

Isidium ontogeny

Three zones can be recognised in a growing lobe of *Pseudevernia furfuracea*: the pseudomeristematic apical tip, covered by an amorphous epicortex (Rikkinen 1997) darkened by melanin pigments; the differentiation zone (or elongation zone *sensu* Honegger 1993), where the epicortex progressively deteriorates; and the fully differentiated zone, where the epicortex is completely absent. In the latter zone the upper cortex consists of two morphologically and histochemically different layers: the outer layer, formed by anticlinally oriented hyphae with free, swollen, PAS positive, TBO negative tips, and a cementing, PAS positive and TBO metachromatic matrix at their base, and the inner layer, consisting in a scleroplectenchyma of hyphae conglutinated by a PAS positive, TBO metachromatic matrix (Figs 1B, 2B, 3A & B).

The earliest developmental stages of an isidium, according to our observations, can be observed only in the fully differentiated zone. The first event consists in the increased aplanosporogenesis of a small clump of algae, and is immediately followed by the proliferation of the neighbouring medullary hyphae, that become oriented upwards (Figs 1A & B, 2A). At this stage the photobiont cells are small in size, the smallest occurring towards the upper cortex, and have a reduced plastidial pigmentation due to a lower chlorophyll content (Fig. 2D). The hyphae surrounding these algae have thin walls, intensely stained cytoplasm and large nuclei, perfectly visible in DAPI stained sections (Fig. 2B & C). The intense metabolism of both myco- and photobiont is shown by the pronounced activity of acid phosphatase enzymes (Fig. 1G), associated with dephosphorilative processes, which are presumably involved in active intercellular movements of glucids (Gahan 1984). Further algal proliferation forms a sort of wedge penetrating into the upper cortex (Figs 1B–D, 2A & B). Only at this stage the conglutinated cortical cells in the immediate proximity of the primordium show an increased, anti- and periclinally oriented division, becoming easily recognisable for their large lumen and strongly stained nucleus (Figs 1C & D, 2B). Also the composition of their wall is progressively modified, because both metachromatic reaction to TBO and DAPI blue fluorescence are weakened.

The growth of cortical cells, further algal divisions and the constant intrusion of medullary hyphae carrying the algal cells upwards to produce a small protuberance above the thallus surface (Fig. 1C–E). At this stage the protruding tips of the cortical cells bordering the young isidium appear thicker and almost brown-black due to deposition of dark brown pigments. These pigments turn dark green when stained by the ferrous ions technique, have a positive green reaction with TBO, are bleached only by strong oxidising agents and therefore can be identified as melanins (Pearse 1985). The

FIG. 1. Light micrographs of transverse sections showing developmental stages of isidium formation in *Pseudevernia furfuracea*. A, first stage, algal cells and medullary hyphae (arrow) penetrate the upper cortex, TBO test; B, aplanosporogenesis of algae and oriented tip growth of neighbouring medullary hyphae in an initial stage of development, the arrow points to a wall darkening due to early melanin deposition in the cortical cell walls, TBO test; C–E, formation of an isidial primordium, subsequent anti- and periclinally oriented growth of cortical cells, intrusion of medullary hyphae and algal division elevate a protuberance above the thallus surface, cortical cells bordering an isidial primordium show darkening and thickening at their protruding tips, forming a brown-black cap above the protuberance, TBO test; F, cortex, algal layer and medulla differentiated in a young isidium, TBO test; G, Burstone test showing the strong reactivity for acid phosphatases of algal and cortical cells next to protuberance of a young isidium. Scales: A=10 μm; B & F=30 μm; C=20 μm; D & E=25 μm.

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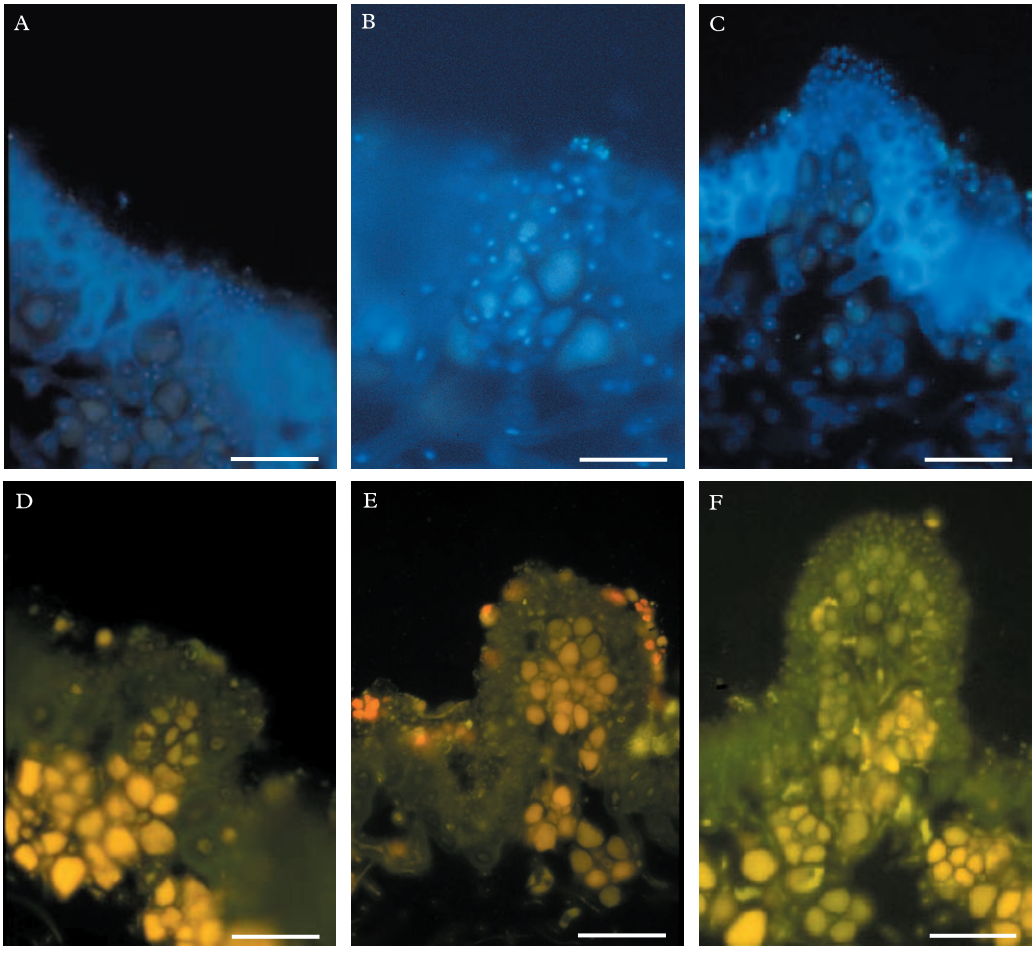

FIG. 2. Fluorescence light micrographs of transverse sections showing early developmental stages of isidium formation in *Pseudevernia furfuracea*. A–C, DAPI staining, UV-A excitation light; D–F, autofluorescence, blue excitation light. Scales: A–D = 10 μm; E & F = 20 μm.

surface of the emerging structure is similar to that of the pseudomeristematic zone at the apex of growing lobes, being formed by the spherical to ovoid protruding tips of perpendicularly arranged hyphae (Figs 1E & F, 2F, 3C). Internally, the young isidium is completely filled by algae (Fig. 2E). When it attains a height of *c*. 200–250 μm, however, a well-developed medulla is already differentiated, and is connected to the medulla of the lichen thallus (Figs 1F, 3D).

Mature isidia have a simple or branched cylindrical body and are often constricted at their bases (Fig. 3A). Scars left by detached isidia may be frequently seen in untouched or carefully handled thalli. Recent scars can be easily recognized because they expose photobiont cells and medulla. Older scars are covered by a newly formed cortical layer, and therefore are recognized as shallow depressions on the thallus surface (Fig. 3B).

Further cortical structures of *P. furfuracea*, which are present on both faces of the dorsiventral thallus (Fig. 4A–C, F), are superficially similar to young, developing isidia. Almost spherical in shape (30–50 μm diam.) (Fig. 4A–F), they can be recognized by their intense dark brown, verging upon

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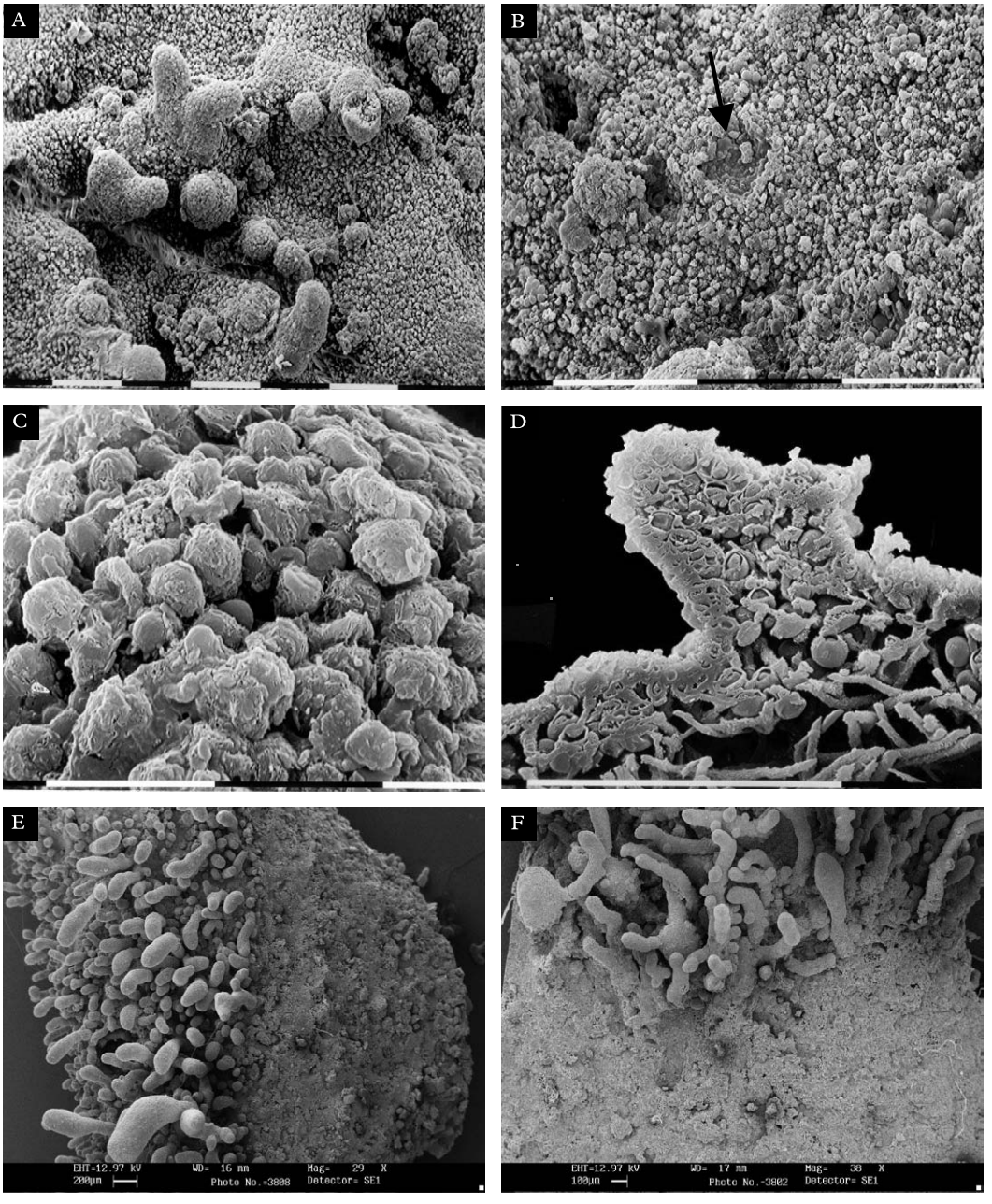

FIG. 3. SEM micrographs of surface features in *Pseudevernia furfuracea*. A, simple and branched mature isidia occurring in a non-epicorticate thalline area, note the roughness of mature cortical surface due to minute crevices separating protruding hyphal tips; B, scars left by detachment of isidia, the arrow points to recent scars exposing photobiont cells, older scars are visible as depressions in the thallus surface covered by a newly formed cortical layer; C, top of an isidial primordium, the surface shows an irregular structure of spherical to ovoid protruding tips of anticlinally oriented cortical hyphae; D, transverse section through a mature isidium showing the continuum of medullary spaces between isidium and lobe. E–F: surface of a lobe after the mechanical removal of isidia. Scales: A, B, D & F=100 µm; C=10 µm; E=200 µm.

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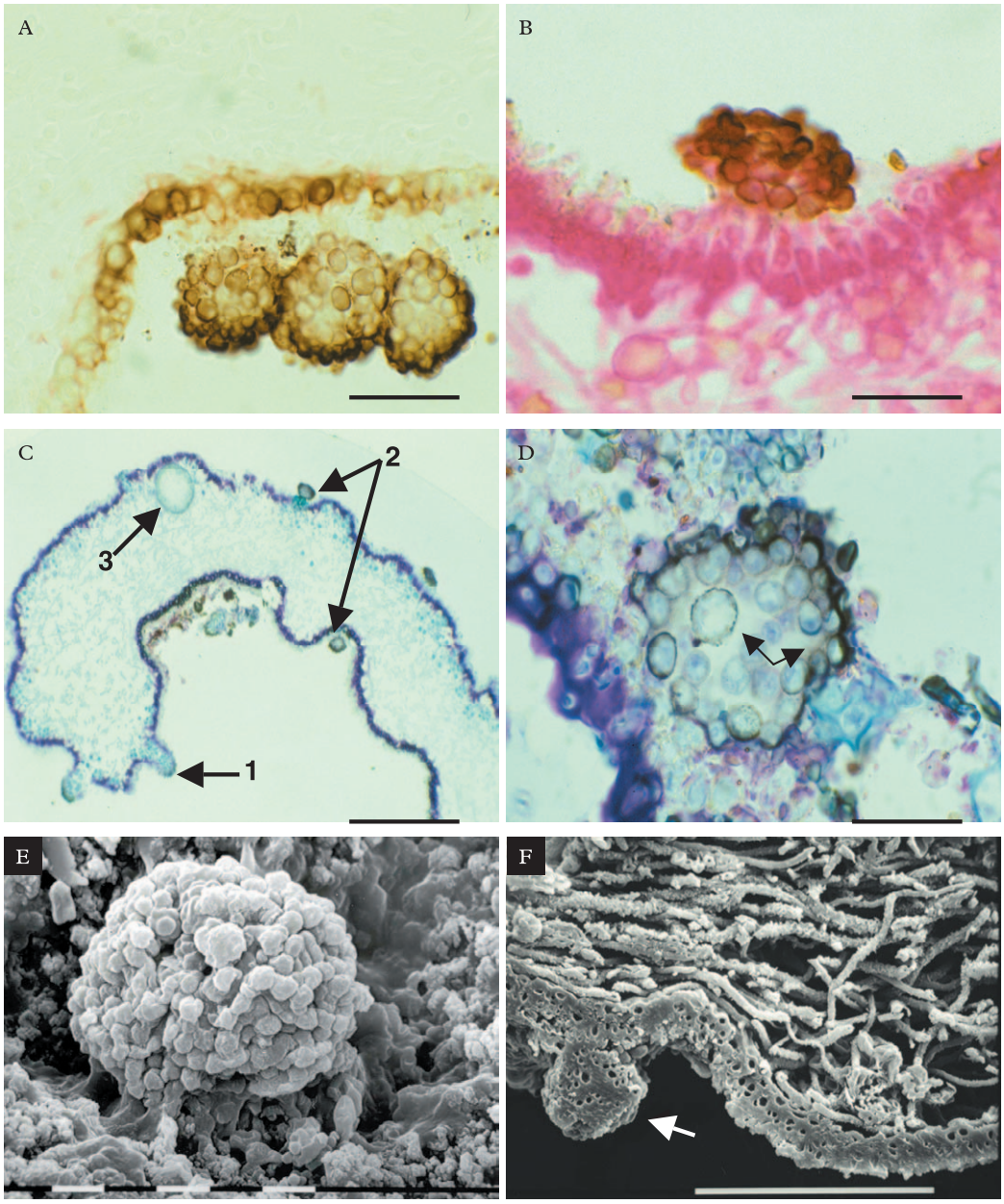

FIG. 4. Light and SEM micrographs of thalloconidia in *Pseudevernia furfuracea*. A, unstained section of the lower cortex with three thalloconidia, the cutting plane omitted their base; B, thalloconidium on the upper cortex, the base forming a continuum with the cortical cells, PAS test; C, cross-section of lobe with young isidium (arrow marked '1'), epi- and hypothalline thalloconidia (arrows marked '2'), and pycnidium (arrow marked '3'), TBO test; D, cross section of thalloconidium, TBO test; the arrows point to deposition of melanins; E & F, SEM micrographs of thalloconidia: external surface (E), and cross section (F). Scales: A=50 μm; B & D=25 μm; C=250 μm; E=10 μm; F=100 μm.

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black pigmentation. These structures are completely devoid of algae and are derived from the proliferation of the cortical external layer, to which they remain connected by a very short strand of hyphae (Fig. 4B & C, E & F), but forming a clear basal constriction. The outgrowth, however, is not accompanied by the intrusion of medullary hyphae as during isidium ontogeny. Their external surface (Fig. 4) consists of strongly adpressed, dematiaceous, more or less isodiametric cells, *c.* 3–4 µm diam., whereas in the internal portion wide intercellular spaces divide somewhat larger cells (*c.* 6–8 µm diam.), characterized by scarcely pigmented walls, in which deposits of polyphenolic pigments (precursors of melanins), turning green in TBO, are present (Fig. 4D). The cytoplasm of the inner cells is less dense than in the hyphae of a growing isidium (compare Figs 1A–G, 2A–F with Fig. 4A–F) and they have larger vacuoles and histochemical tests for lipophilic substances show the presence of numerous lipidic bodies.

Estimation of isidium density and increase in external lobe surface

Isidium density ranged between 24 and 111 isidia mm^{-2}, with a mean value of 55 ± 18 isidia mm^{-2}. Broken isidia were observed on all lobes, although their frequency was rather variable, ranging from 1 to 13%. The density of isidia and frequency of broken isidia were not statistically correlated. Isidia were morphologically rather heterogeneous, and therefore estimates of their individual external surface ranged between 0·017 and 0·146 mm^2, with a mean value of $0{\cdot}063 \pm 0{\cdot}027$ mm^2, and a basal thickness of 82 ± 16 µm ($n=100$). The frequency distribution of homogenous classes of 0·011 mm^2 each is shown in Figure 5.

The increase in external lobe surface caused by the development of isidia was estimated to range between 107 and 211%. This noteworthy figure, obtained with a minimum increase in weight, *c.* 14–63%, is possibly still higher in deformed, over-isidiate lobes, which were purposely excluded from the present investigation.

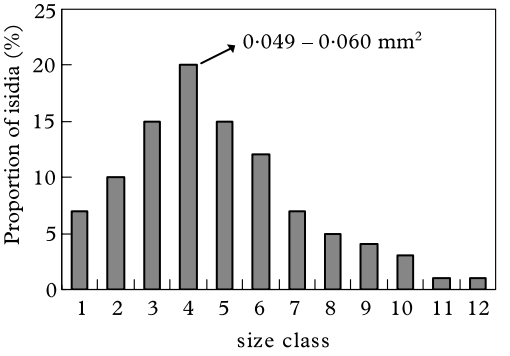

FIG. 5. Size distribution (by surface area) in 100 randomly selected isidia of *Pseudevernia furfuracea* (size classes increment by 0·011 mm^2).

Thallus water loss

Isidiate and non-isidiate thalli clearly differed in water storage capacity and speed of water loss (Fig. 6). Isidiate thalli reached a maximum RWC of 240–245%, against 150–160% for non-isidiate thalli, and retained water more efficaciously. This can probably be explained by the increased external capillary system of isidiate thalli, consisting of the open spaces among isidia (Rikkinen 1997), and the greater quantity of polysaccharides and other hygroscopic materials covering, as shown before, the external surface of isidia.

CO_2 gas exchanges

The response curves of CO_2 gas exchanges of I, NIL, and IL to thallus water content, CO_2 concentration, and light intensity are presented in Figures 7–9, respectively. Compared to NIL and IL, data for I were exceptionally homogeneous, only small differences being observed among duplicates of the same material. In contrast, striking differences were observed between the three different materials, particularly in Ph_n and its dependence on thallus water content (Fig. 7). Depression of Ph_n regularly occurred at high thallus water contents (>150%) in NIL but was only slight in IL and absent in I, with an optimum at *c.* 100–120% RWC (see also Scheidegger *et al.* 1995*b*). In contrast, no significant difference was detected in R_d, which remained relatively constant (from

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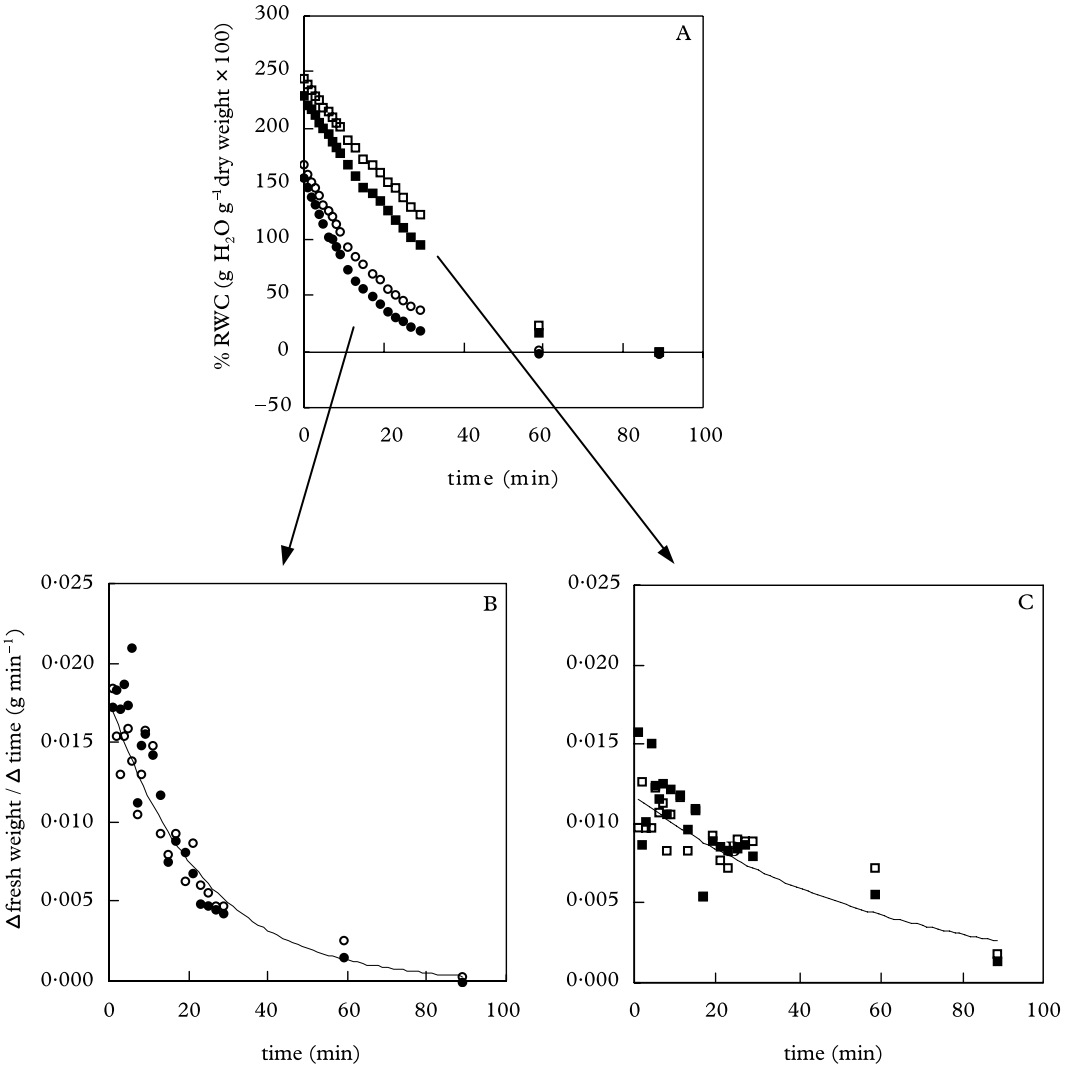

FIG. 6. Variation over time of thallus relative water content (A), and rate of water loss in non-isidiate (B) and isidiate (C) thalli of *Pseudevernia furfuracea* under the same environmental conditions. Duplicate data for isidiate (□, ■) and non isidiate (○, ●) thalli.

0·7 to 1·0 mg CO_2 g^{-1} h^{-1}) within a broad range of RWC values. It must be emphasised that the I desiccation curves started from a maximum RWC of 150%, and therefore ignore what happens at higher values, although a considerable depression of CO_2 gas exchange similar to that observed in NIL seems to be unlikely.

The differences in Ph_n (and Ph_g) noted in Figure 7, with I>NIL>IL, are confirmed by data in Figure 8 where further differences include the Ph_g saturation value to external CO_2 concentration, that was >600 ppm in I, 350 ppm in NIL, and only *c.* 270 ppm in IL. The estimated CO_2 compensation point was lowest in I (9 ppm CO_2), and highest in NIL (40 ppm CO_2) (Table 1).

The three materials also showed a different reaction to increasing light (Fig. 9), although the difference between IL and NIL

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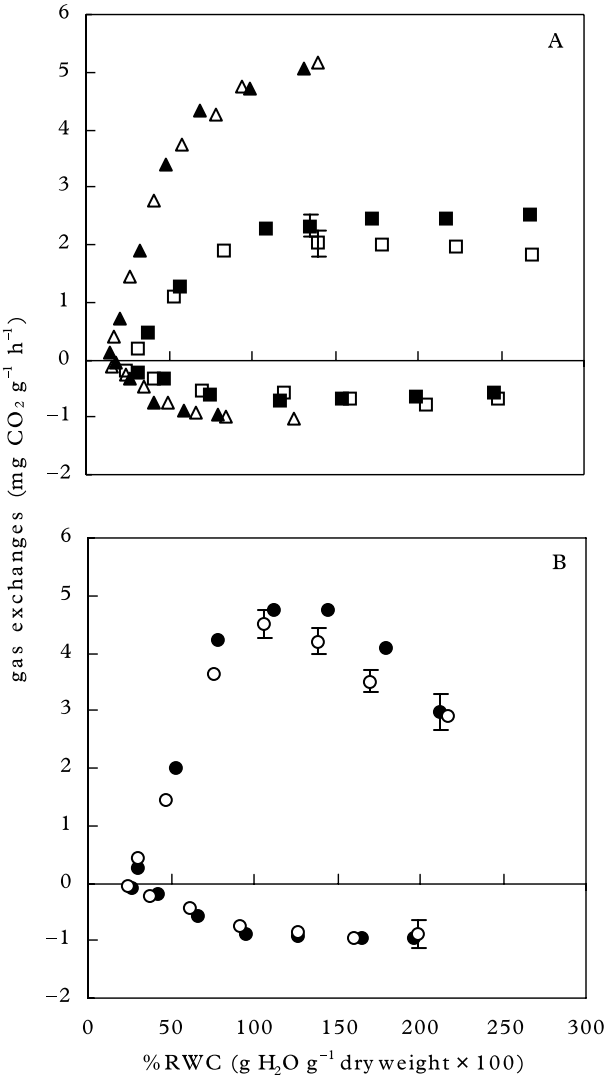

FIG. 7. Dark respiration (negative values) and net photosynthesis (positive values) of *Pseudevernia furfuracea* at 17 ± 1 °C, 270 µmol m^{-2} s^{-1} PPFR, and 360 ppm CO_2. A, isidia (△, ▲) and isidiate lobes (□, ■); B, non-isidiate lobes (○, ●). Two samples (open vs closed symbols) were used for each type of material. Means (n=3) are plotted ± SD except where it is exceeded by the symbol.

was less evident. The highest rate (*c.* 9·6 mg CO_2 g^{-1} h^{-1} at saturation) was again recorded in I, being approximately double that of IL. However, when expressed on a chlorophyll *a* basis (data from Table 2), this difference was considerably reduced, although Ph_n still remained slightly higher in I than in IL and NIL. Isidia also had higher quantities of total carotenoids (Table 2), in accordance with the fact that in this material we observed a particularly high content of lichen substances. It is noteworthy that NIL and I had practically identical Chl_a/Chl_b (3·57 ± 0·07 against 3·57 ± 0·11), and OD_{435}/OD_{415} ratios (1·28 ± 0·04 against 1·30 ± 0·01), which were statistically higher than in IL (3·20 ± 0·11, and 1·19 ± 0·05, respectively).

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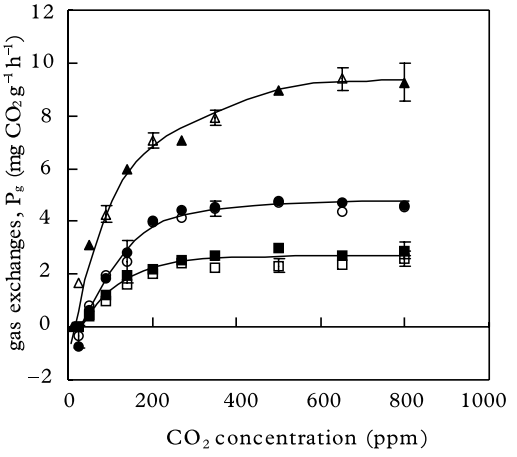

FIG. 8. CO_2 dependence curves of gross photosynthesis (mg CO_2 g^{-1} h^{-1}) in isidia (△, ▲), and isidiate (□, ■) and non-isidiate (○, ●) lobes of *Pseudevernia furfuracea*, at optimal water content, 17 ± 1 °C, and 350 µmol m^{-2} s^{-1} PPFR. Two samples (open vs closed symbols) were used for each type of material. Means (n=6) are plotted ± SD except where it is exceeded by the symbol.

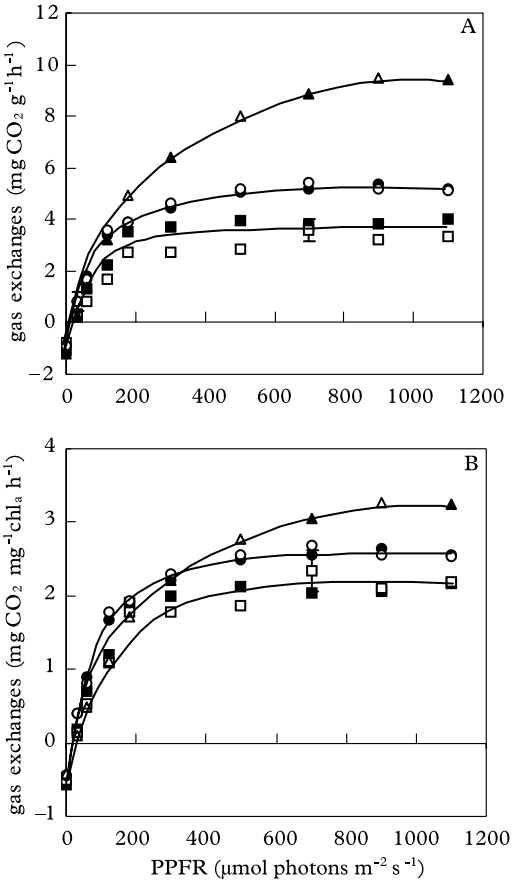

FIG. 9. Light dependence curves of net photosynthesis in isidia (△, ▲), isidiate (□, ■), and non-isidiate (○, ●) lobes of *Pseudevernia furfuracea*, at optimal water content, 17 ± 1 °C, and 360 ppm CO_2. A, expressed on the basis of dry weight (mg CO_2 g^{-1} h^{-1}); B, expressed on basis of chlorophyll *a* content (mg CO_2 mg^{-1} chl_a h^{-1}). Two replicates (open vs closed symbols) were used for each material. Means (n=3) are plotted ± SD except where exceeded by the symbol.

In all experiments the lowest rates of both Ph_n and Ph_g were always measured in IL, an apparent contradiction with the observation that I had impressively high rates. The importance of isidium development for increasing the CO_2 gain of mature lobes is evident from Fig. 10, in which two desiccation curves of the same IL sample, before and after the mechanical removal of isidia, are compared. Whereas R_d did not change significantly, Ph_n drastically decreased to 54%, with a concomitant reduction of *c*. 28% in both chlorophyll *a* and total carotenoids (Table 2). It must be noted that the mechanical removal of isidia, carried out with tweezers used under a stereomicroscope at high magnification, was less traumatic than expected, because SEM observations revealed that the cortical surfaces were still relatively intact, with very few medullary hyphae emerging from the broken cortex (Fig. 3E & F).

Discussion

Surprisingly, morphogenesis of isidia has attracted little attention, probably because these structures have long been interpreted as simple cortical outgrowths ('hernies', see Ozenda 1963). Except for a very few anatomical studies (Rosendahl 1907; Moreau & Moreau 1919; Dughi 1933; Honegger 1987; Ott *et al.* 1993), most work deals with the external morphology of these structures from a systematic point of view (Du Rietz 1922, Beltman 1978). Little is known about the first stages of isidium development, and the factors that trigger their phenotypic

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TABLE 1. *Main significant physiological parameters inferred from Figs 7–9 relative to isidiate lobes (IL), non-isidiate lobes (NIL), and isidia (I) of* Pseudevernia furfuracea

	Isidiate lobes	Non-isidiate lobes	Isidia
Optimal RWC (%)	110–250	100–150	>150
CO_2 compensation point (ppm)	22	40	9
CO_2 saturation point (ppm)	270	350	>600
Light compensation point (μmol photons m^{-2} s^{-1})	26	15	27
Light saturation point (μmol photons m^{-2} s^{-1})	300	500	900

TABLE 2. *Photosynthetic pigments content (μg mg^{-1} dry weight; $C_{(x+c)}$=total carotenoids; Chl_a, Chl_b=chlorophyll* a, b*) in non-isidiate and isidiate lobes, and isidia of* Pseudevernia furfuracea, *and per cent variation recorded in isidiate lobes after the mechanical removal of isidia*

n	Non isidiate lobes 10	Isidiate lobes 10	Isidia 16	After isidia removal 7	Wilcoxon matched pairs test *P*-level
$C_{(x+c)}$	0·59 ± 0·07†	0·49 ± 0·06	0·85 ± 0·05	− 27% ± 11	0·018*
Chl_a	2·03 ± 0·10	1·70 ± 0·26	2·90 ± 0·17	− 29% ± 14	0·018*
Chl_a/Chl_b	3·57 ± 0·07	3·20 ± 0·11	3·57 ± 0·11	− 5% ± 7	0·091
OD_{435}/OD_{415}	1·28 ± 0·04	1·19 ± 0·05	1·30 ± 0·01	− 5% ± 5	0·028

*Statistically significant differences (last column) (P<0.02).
†Mean values ± 1 SD.

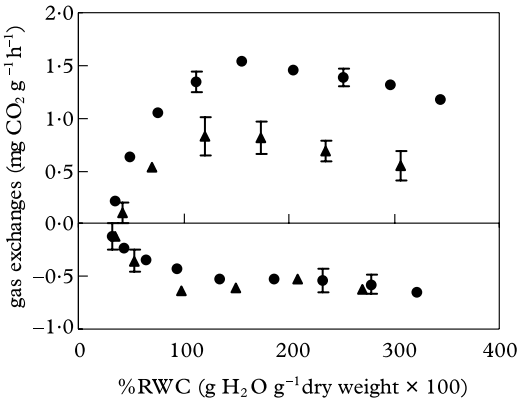

FIG. 10. Dark respiration (negative values) and net photosynthesis (positive values) in isidiate lobes of *Pseudevernia furfuracea* at 17 ± 1 °C, 270 μmol m^{-2} s^{-1} PPFR, and 360 ppm CO_2, before (●) and after (▲) mechanical removal of isidia (see Fig. 3E–F); n=3.

expression are still unknown. It is worth noting that in *Pseudevernia furfuracea* isidia start to develop only after the disappearance of the melanin-darkened epicortex. The presence of a true epicortex in this species had been excluded by Hale (1973), but it was later correctly recognized by Rikkinen (1997), who also showed that the development from a smooth continuous epicortex to the rough surface topography typical of old *P. furfuracea* lobes seems to involve transitional stages in which the epicortex first achieves a pored structure and then later deteriorates. Our observations fully confirm these results. Rikkinen (1995: 239) also suggested that in *P. furfuracea* an initial increase in algal proliferation could be triggered by a local increase in irradiance within a light-limited algal layer. These observations are potentially important for explaining the first stages of isidium development. The progressive disappearance of the brown melanin-pigmented epicortex could both cause an increase in the intensity and quality of light reaching the photobiont layer, with an enrichment of the blue component, since

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melanins, having a broad monotonic absorption curve that decreases from the UV region (Turkovskii & Yurlova 2002), would absorb more in the blue than in the red region. These factors suggest that phytochrome or more probably cryptochrome-mediated processes are triggered in the algae, with a cascade series of events leading to the well-coordinated morphogenetic process described earlier. In the lichen symbiosis phytochrome-mediated processes had already been hypothesized (but not demonstrated) by several authors (Avalos & Vicente 1985, 1986; Giles 1970), for example to explain the formation of parasoredia in the foliose lichen *Pseudoparmelia caperata* (L.) Hale (Raineri & Modenesi 1986). On the other hand, the presence of cryptochrome-mediated processes has been neglected so far, although the role of this molecule as a morphogenetic regulator is well known in green algae (Suetsugu & Wada 2003), and also in fungi (Suzuki *et al.* 1977; Ross 1985; Galland & Senger 2001).

The importance of events in the algal layer for the process of isidium formation was recognized by Nilson-Kajanus (1903, 1911), who however attributed the proliferation of algae to an excess in humidity. More recently, Ott *et al.* (1993) recognized the proliferation of photobiont cells as a key event, but only in *Parmotrema crinitum* (Ach.) M. Choisy, and not in *P. furfuracea*. In the latter species they described an increased division rate of the cortical layer as a primary event, which only later was followed by an increase in algal division beneath the growing cortex. The medulla only participated after isidia had already emerged from the cortex in the final differentiation stage, when hyphae of the medulla then grew towards the base of the protuberance (Ott *et al.* 1993: 67). In our opinion, this series of events is not confirmed by the limited iconography reported. In contrast, the importance played by medullary hyphae in isidium formation can be inferred from Jahns (1984), who holds that in *Parmelia saxatilis* (L.) Ach. isidia appear not in corticated areas, but in the crevices of the pseudocyphellae, i.e. where there is no cortex, and medullary hyphae are directly exposed (see, on this point, also Beltman 1978).

In *P. furfuracea* there are also cortical outgrowths derived from proliferation of cortical hyphae, and which are devoid of algae (Fig. 4). Their morphology is quite different from that of very young isidia, and therefore they cannot be confused with them. It might be argued that they are formed by a parasite, but the reactivity to histochemical tests of their cellular components is identical to that of neighbouring tissues, and they form an evident continuum with the upper layer of the cortex. Alternatively they might be galls induced by the infection of a fungus, whose hyphae have not been demonstrated by the dozens of staining techniques used in this and other concurrent studies (Giordani *et al.* 2004) or they might be thalloconidia. We strongly support the latter hypothesis, because they are practically identical to the thalloconidia described by Hestmark (1990) in *Umbilicaria esculenta* and *U. mammulata* (*loc. cit.* figs 9, 13, respectively). As in our species, the vegetative mycobiont propagules of the two *Umbilicaria* species are derived by direct proliferation of cortical scleroplectenchymatous tissue, the phenomenon being limited to the lower cortex, although in *Umbilicaria* species their frequency is considerably higher (Hestmark 1992). Interestingly, thalloconidia have also been reported from some crustose members of the *Parmeliaceae* (e.g. *Protoparmelia*) (Poelt & Obermayer 1990), but they were not known to occur in foliose or fruticose members of the family. If confirmed by further experimental work, the presence of thalloconidia in *P. furfuracea* would represent an important novelty in our knowledge of the reproduction strategy of this species, which relies strongly on isidium dispersal (see Nienburg 1919).

Our experimental data demonstrate that the growth of isidia strongly modifies the relationships of the thallus with its environment, particularly CO_2 assimilation economy, as already suggested by Smith (1921). The high surface/volume ratio typical of isidia, the proximity of their algal cells to the cortex, and the high content of

photosynthetic pigments (Table 2) are probably the basis of the high carboxylation efficiency and very low CO_2 compensation point (9 ppm CO_2) (Fig. 8 and Table 1). Being young, rich in actively growing pseudomeristematic tissue and algal cells, isidia have relatively high R_d and, above all, spectacularly high Ph_n, although at 17°C (the temperature chosen for our experiments) it is only 50% of the maximum, which occurs at *c.* 5°C (Türk 1983). The high rates of CO_2 assimilation by isidia on the one hand, and the low rates by isidiate lobes on the other, are only superficially a contradiction: the quantity of isidia produced on the surface of old lobes is not enough to equal the Ph_n rates typical of young, non-isidiate lobes. However, the Ph_n of isidiate lobes would be considerably lower if isidia were absent (see Fig. 10). From this point of view, isidium production might be considered a process by which old lobes are rejuvenated. This is exactly the opposite to what happens when soredia develop, because in the parmelioid lichens investigated so far, sorediate lobes have lower R_d and Ph_g, than esorediate lobes due to a lower chlorophyll content (Tretiach & Carpanelli 1992).

The formation of isidia has two further effects that positively influence CO_2 gain: the reduction of Ph_n inhibition at high thallus water contents (Fig. 7), and a retarded dehydration (Fig. 6). Both are caused by the fact that the formation of a thick layer of isidia increases the water holding capacity of the thallus by storing large quantities of water in the capillary spaces between the bases of isidia. The tops of isidia tend to remain free of liquid water, because they are often raised above the level of the water film eventually present (Scheidegger *et al.* 1995*b*), and have positive water potentials (Rikkinen 1997). Consequently, the CO_2 assimilation of the most active parts of the thallus, the isidia, is not limited by the increased CO_2 diffusion resistance caused by the presence of liquid water (Green *et al.* 1994; Lange *et al.* 1998, 1999); on the contrary their hydration is guaranteed by the water present at their base. On the other hand, the smooth surfaces of non-isidiate lobes are covered by a thin water film at high RWC, and this strongly affects the CO_2 gas exchange (Scheidegger *et al.* 1995*b*). Thus, whereas non-isidiate lobes suffer the typical Ph_n inhibition at high RWC, isidiate lobes and isidia maintain relatively constant rates in a wide range of RWCs (Table 1).

The capillary forces and the high content of mucopolysaccharides probably also explain the lower rate of thallus dehydration of isidiate thalli (Fig. 6C). These observations agree with those of Valladares *et al.* (1993), who demonstrated that isidiate *Lasallia pustulata* has a higher water storage capacity than other anatomically similar species of the same genus devoid of isidia. From this point of view, it might be of some interest to study the behaviour of isidiate and non-isidiate morphotypes exposed to humid air currents, because the development of the small, three-dimensional irregular structures might cause a strong increase of condensation phenomena, as observed by Rundel (1974) in *Ramalina menziesii* Taylor, and by Lange *et al.* (1990) in *Teloschistes capensis* (L.f.) Vain. Also in our case any difference could be explained by morphological features only, because preliminary measurements indicate that the two morphotypes have similar water potentials (data not shown). Of course, the increase in water condensation capacity might be off-set by a higher evaporative loss due to the thermal increase caused by melanin-darkening of the epicortex, a factor not influencing the results of the water loss experiments of Fig. 6, which were carried out in dim light.

It is noteworthy that although isidia are very efficient in increasing CO_2 gain in older lobes, isidiate lichens are less frequent than sorediate ones. The lichen flora of Italy, for instance, has only 85 isidiate species (3·7%), whereas sorediate species are four times more frequent (15·1%) (data derived from Nimis 2003). Similar results were given by Bowler & Rundel (1975) in their comprehensive study of the reproductive strategies in lichens. These observations can be explained by two non-mutually exclusive hypotheses. Either isidia are less effective in

securing the establishment of new thalli, probably because they are less efficient in dispersal and in the first stages of fixation to the substratum (Jahns 1984; Kärnefelt 1990; Scheidegger *et al.* 1995*a*; Zoller *et al.* 2000) and/or the morphogenetic events leading to the formation of isidia are more complex, being under the control of a larger number of genes. The latter hypothesis seems to be the less supportable: isidia are known from many unrelated taxa, and isidiate species often have close relatives without isidia (Bowler & Rundel 1975). The capacity to produce isidia can apparently be acquired and lost quite easily, probably because it requires only minor changes in the chain of events that controls thallus growth.

We thank Lucia Muggia (Trieste), and Laura Carletti (Siena) for assistance in the field, Antonio Corallo (Genova) and Tito Ubaldini (Trieste) for help with SEM photographs, and Laurence Baruffo (Trieste) for assistance in the laboratory. This study was carried our in the framework of the COFIN 2002 project "Sviluppo di metodologie per il monitoraggio biologico dell'inquinamento atmosferico da metalli in traccia nelle aree urbane ed industriali italiane", co-ordinated by Roberto Bargagli (Siena).

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Accepted for publication 9 *May* 2005