V. Spadaro, F. M. Raimondo & P. Colombo

Endophytic evidences in *Helleborus* (*Ranunculaceae*)

Abstract

Spadaro, V., Raimondo, F. M. & Colombo, P.: Endophytic evidences in *Helleborus* (*Ranunculaceae*). — Fl. Medit. 17: 277-286. 2007. — ISSN 1120-4052.

The results of a morphoanatomical study carried out on various organs of *Helleborous bocconei* subsp. *intermedius*, an endemic to South Italy and Sicily, which roots are traditionally used to treat pulmonary diseases of cattle. S.E.M. observations have shown diffuse fungal structures in the internal organs, perhaps spreading out on the exterior of the plant. The endophyte, which will be studied in detail in future, has been temporarly referred to *Botrytis* sp. Such study, extended for verification also to *H. lividus* subsp. *corsicus*, endemic to Corsica, has shown analogous fungin structures, confirming thus in another species of *Helleborus* the same endophytic manifestations observed in *H. bocconei* subsp. *intermedius*. Such evidence, contextually verified on two isolated taxa of *Helleborus* and even on other findings of congeneric species belonging to Palermo's Botanical Garden, lead the authors into extending this endophytic relation to the whole genus, which unpublished in *Helleborus*, was already known for *Ranunculaceae*, since already observed in *Ranunculus bulbosus*.

Key words: *Botrytis*, Endophytism, Morphoanatomy, ethnopharmacobotany.

Introduction

During a biological and ethnopharmacobotanical study on *Helleborus bocconei* subsp. *intermedius* (Guss.) Greuter & Burdet, endemic to Sicily and Southern Italy, well known in Sicilian folk tradition for its efficacy against pneumonia in livestock (Raimondo & Lentini 1990), some elements of phytochemical and biological importance have been reported (Spadaro 2006). These are unpublished aspects that may contribute to explain the pharmacological effect on which the empirical employment in veterinarian treatment founds its bases when treating pulmonary diseases with dried roots of such plant, locally known as "radicchia". Besides the phytochemical study – which has however produced isolation and characterization of new biologically active organic molecules, object of a specific contribution (Rosselli & al. 2006) – the strictly biological one has brought to light a particular form of symbiosis between a fungin endophyte and the host plant. Such peculiar case of endophytism, still unknown in *Helleborus* L, is here reported for its biological interest and implications in the veterinary therapeutical treatment – after being verified on other taxa of the same genus – both as remarkable applicative result of the study carried

out and as further discriminant characteristics of the *Helleborus* genus within the family of *Ranunculaceae*.

Materials and methods

The study has initially concerned *Helleborus bocconei* subsp. *intermedius* endemic taxon to mid-south Italy and Sicily. For comparison, *H. lividus* subsp. *corsicus* (Willd.) Tutin, endemic to Sardinian and Corsican, has been studied too.

H. bocconei subsp. *intermedius* is a rhizomatous geophyte, occurring in open and submediterranean woods. Basal leaves are glabrous, persistent, with 5-11 segments, all or mostly divided to the middle and margins are irregularly and deeply dentate (Pignatti 1982).

Helleborus lividus subsp. *corsicus* is a hemicryptophyte scapose, occirring in stone grounds, has long petiolate leaves with 3 coriaceous segments. The central segment is elliptical while the side ones have equal dimensions and are sub-lobate at their bases. Lamina is 8-20 cm wide, dark green on the adaxial surface and greyish-blue on the abaxial one; margins are characterized by quite hard thorn-like ends.

As for *Helleborus bocconei* subsp. *intermedius*, the study material was collected in Mt. Pizzuta (Palermo), Portella del Garrone (Piana degli Albanesi, Palermo), Piano Ferla (Santa Cristina Gela, Palermo), Serra di Prato (San Mauro Castelverde, Palermo).

The material concerning *H. lividus* subsp. *corsicus*, was collected in the Palermo Botanical Garden, coming from a mountain stand in Corsica situated exactly just above the town of Corte.

The following operations have been carried out:

- stomatic replications of adaxial and abaxial surfaces of wintering mature leaves and young leaves of the same year, as well as replicas of cauline and bracteal leaves and petals, in order to acquire information about their micro-morphology;
- sections and fresh colouring by means of vital dye in order to assess dislocation of various types of tissues in main organs (leaves, stalks, roots);
- dehydration by means of increasingly-concentrated ethlyl alcohol of main organs in order to perform final paraffin permeation so to obtain permanent semifine sections in order to verify microscopic anatomy;
- clarification by means of Fuchs' method to determine xylematic leaf pattern;
- S. E. M. observations to study fine tridimensional structures of adaxial and abaxial epidermic cuticular complexes as well as the "stomata complex" of leaf surface.

Results and discussion

Basal leaves of *Helleborus bocconei* subsp. *intermedius* are glabrous, dorsiventral, hypostimatic; the stomatic complex is anomocytic; stomatic density on the adassial surface is 160 stomata × mm^2; stomata are variable in size with rime lalways parallel to the leaf main axis. Epidermic cells - 343 × mm^2 on average - are large (132 × 66 mm) with irregularly sinuous margins and deep cuticular striae variously oriented.

The abaxial surface is characterized by less numerous (288 × mm^2) and smaller (40 × 13 μm) deep and thick walled epidermic cells. Both adaxial and abaxial surfaces are crossed by rectangular, long cells, multiple lined, with parallel cuticular striae. Rows are variable in number, correspond to innervations throughout the leaf mesophyll and thus are expression of the neuration order. Through S. E. M. epidermic cells on adaxial surface, at 300 ×, appear convex, big and crossed by variously displayed cuticular striae, in contrast with the thick and parallel striae of the rectangular cells that subtend nervation traces (Fig.1).

The abaxial surface shows superficially more convex cells, with a repand wall and with still more irregular striae. Stomata appear neatly elliptical, with highly convex and smooth guard cells and with rima subtended by particular thick cuticle on the margins. Among intercostals panels of the adaxial surface there are visible vesicolous, clavate hair with foot inserted among closer epidermic cells radially displayed. *H. bocconei* subsp. *intermedius* leaves of different provenance are all fypostomatic, dorsoventral with similar micromorphology, but with **P. E. F.** (**F**oliar **E**pidermic **P**arameters) peculiar as for number per mm^2, in length, width and thickness, as well as in cuticular striae morphology.

S.E.M. observations put in evidence, firstly along traces of nervations and secondly among intercostal panels cut out from traces of nervatures, peculiar structures that only at the end of the whole anatomic study were identified as vesicolous conidiogenous, mostly located in intercostal panels, clearly distinguishable by their morphology from hairs (Fig. 2).

In cross section, the leaf is dorsiventral, with both adaxial and abaxial single-layered epidermis; single-layered palisade with wide intercellular spaces; spongy tissue with wide spaces and lacunae containing collateral vascular bundles of variable diameter since the cross section meets nervations of various order. They all have a parenchymatous sheath; particularly evident is the midrib which is under a deep invagination of the adaxial surface and that protrudes from the abaxial one by means of a very prominent parenchymatous keel. Equally structured ribs, only smaller, are also present under major side nervations (Table 1).

Leaf edges are linear and slightly folded towards abaxial surface.

In *H. lividus* subsp. *corsicus* – of which only leaves have been studied – the lamina is glabrous, with dark green adaxial surface while glaucous, dorsiventral, hypostomatic on the abaxial surface. The adaxial surface is characterized by isodiametric cells, with cellular walls strongly sinuous, 114 × 89 μm and with a number of 251 × mm^2; mingled with epidermic cells there are long and narrow rectangular cells, deeply sculptured with parallel cuticular plicae, multiple-lined, that branch out to define epidermic panels of various dimensions; these are the leaf traces that underline nervations spreading out in the mesophyll (Fig. 3).

On the abaxial surface cells are even more sinuous, smaller (72 μm) but with numerous stomata (91 × mm^2); striae are more marked, prominent and randomly displayed in most cells. On the adaxial surface of the leaf of *H. lividus* subsp. *corsicus* the same conidiogenous cells as in *H. bocconei* subsp. *intermedius* have been observed, though here surrounded by radially displayed and with parallel striae.

In cross section the leaf is dorsoventral with both adaxial and abaxial epidermis single-layered; its chlorenchymatic palisade is also single-layered with short collecting one-layered cells connecting the palisade with a wide spongy tissue, with wide intercellular spaces and a chlorenchyma with small and round cells. The mesophyll completely includes the

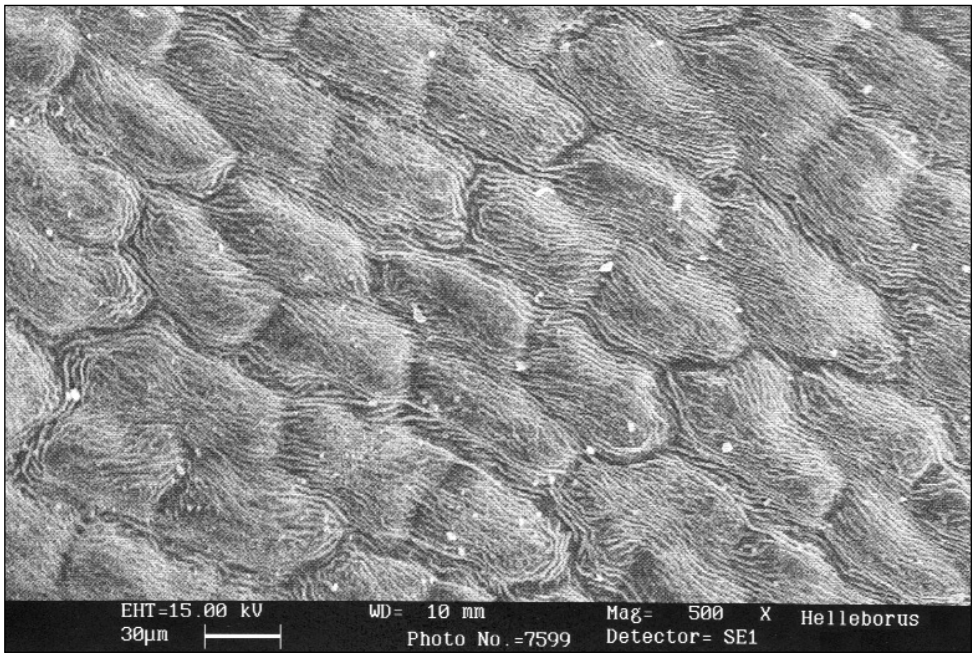

Fig. 1. Adaxial surface of persisten basal leaf of *Helleborus bocconei* subsp. *intermedius,* at S.E.M.

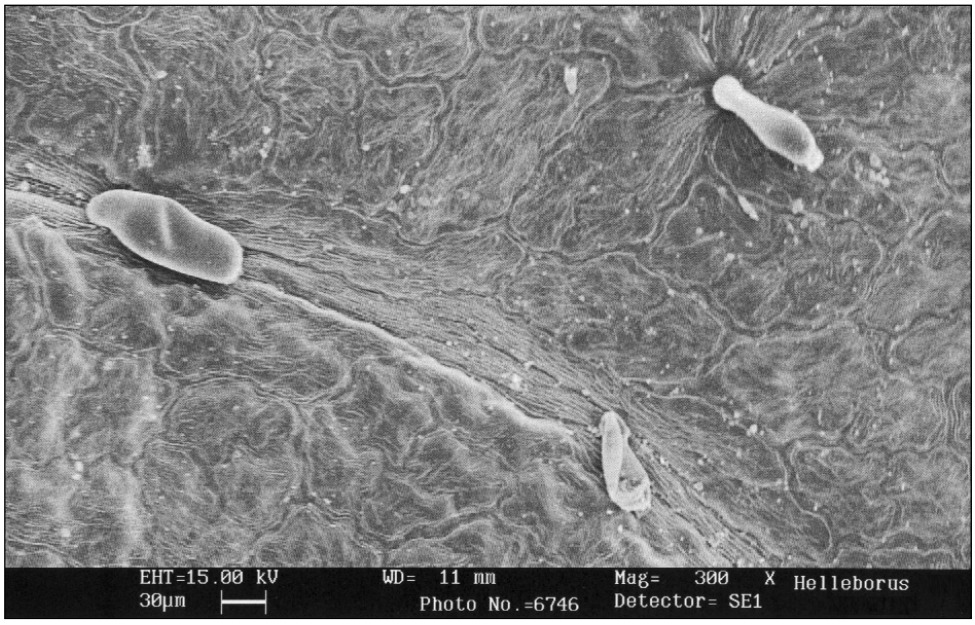

Fig. 2. Adaxial surface of persistent basal leaf of *Helleborus bocconei* subsp. *intermedius* at S.E.M., highlighting conidiogenous cells.

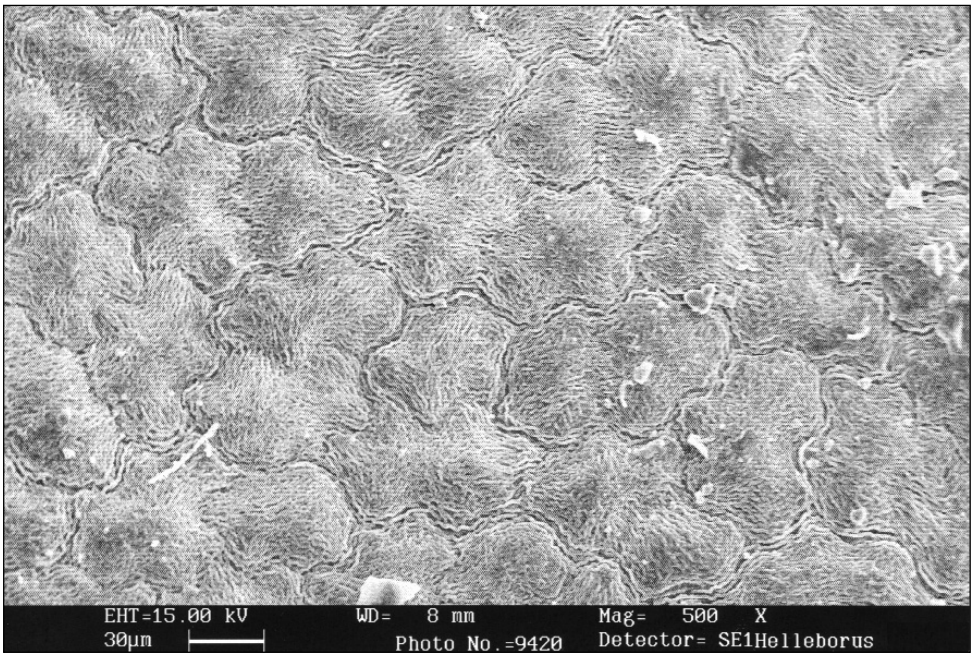

Fig.3. Adaxial surface of the leaf of *Helleborus lividus* subsp. *corsicus,* at S.E.M.

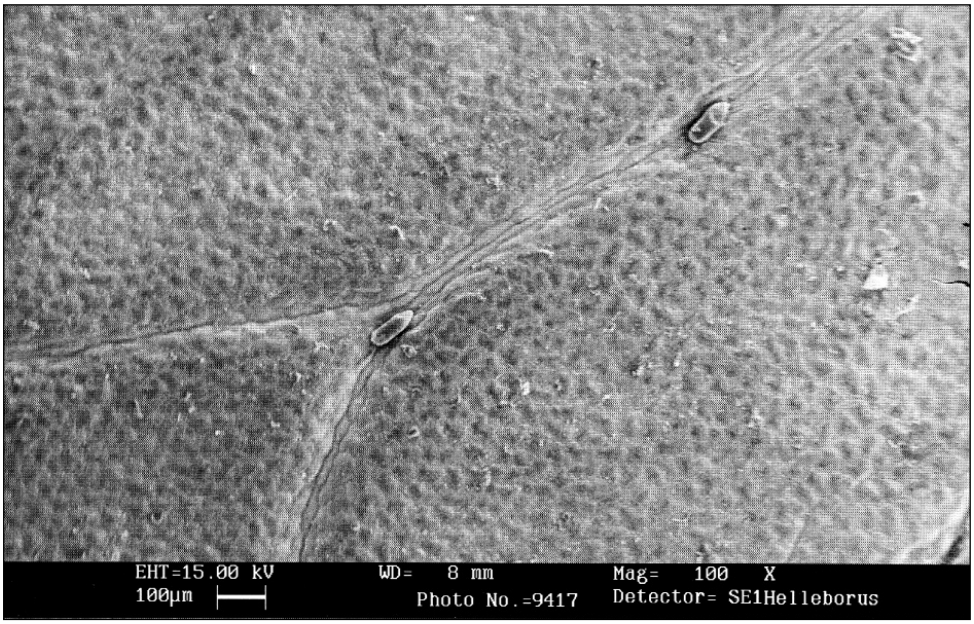

Fig. 4. Adaxial surface of the leaf of *Helleborus lividus* subsp. *corsicus,* with emergent conidiogenous cells, at S.E.M.

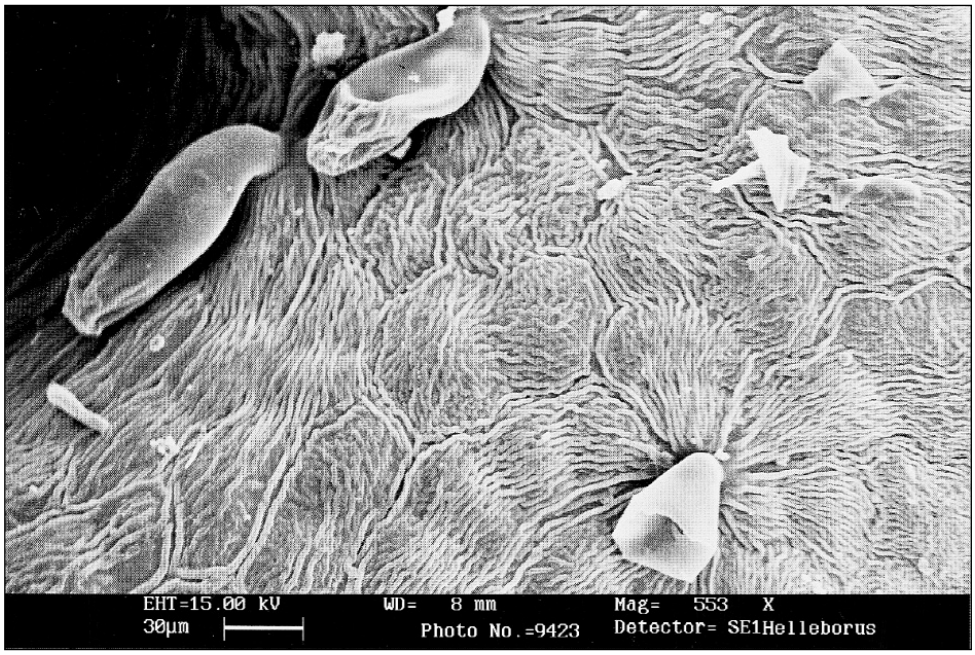

Fig. 5. *Helleborus lividus* subsp. *corsicus*: clearly visible conidiogenous ampulla-shaped cells of the endophyte mycete, at S.E.M.

smaller vascular bundles, which are corresponding to nervations from the third order to up; the second order bundles are more visible because they are characterized by a depression on the adaxial surface and by a slight protrusion on the abaxial one. The midrib (first order) remarkably protrudes from the adaxial surface, but with a strong invagination on the adaxial one, in correspondence with which the palisade chlorenchyma breaks off. Here thickness is 310 µm; the other morphological parameters are displayed on table 1.

On the adaxial surface of the leaf of *H. lividus* subsp. *corsicus* the same conidiogenous cells as in *H. bocconei* subsp. *intermedius* have been observed, here surrounded by radiating cells which present parallel striae.

Floral stalk

The floral peduncle in *Helleborus bocconei* subsp. *intermedius* presents monostratified epidermis under which, in centripetal direction, there is a double layer of lamellar collenchyma followed by back up cortical parenchyma containing a ring of collateral bundles, each displaying a cap with thin sclerenchymatic cells separated by multiserial medullary rays. Thepith is made up of big cells similar to those found in the cortex, having a reserve function. The peduncle's peculiarity is the presence, in all cells of cortex and medulla, of spiralate fungin hyphae in the inside. Among spirals, one or more bodies are found. They, at first, might seem cellular nuclei but, by at a greater magnification, are actually numerous and are identified as the symbiont fungus' hyphae plentiful present also on the organ's

Table 1. Leaf morphological parameters (thickness in μm) in studied *Helleborus*.

	Whole lamina	Level midrib	Epidermis upper and cuticle	Lower epidermis and cuticle	Palisade	Lacunose
H. bocconei subsp. *intermedius*	250	400	25	23	58	152
H. lividus subsp. *corsicus*	175	310	17.5	17.5	30.5	74

surface. This also explains the particular morphology of the exterior xylem which might appear more lignified than its due. Actually, the massive presence of hyphae obstructing the vases together with counterstain (safranine hydroalcoholic) give the impression of an abnormal lignification, which accumulates around the xylema due to excessive concentration of structures (Fig. 6).

Most likely hyphae reach the floral bud through the thalamus coming to the ovarian follicles; thus, after the anthesis, during their formation, seeds result already colonized by the fungus whose spores would contextually germinate.

This is an unpublished yet relevant case of endophytism that has never reported for *Helleborus* species and which is object of investigation such as isolation of the mycete and cultivation for taxonomic purposes (Fig. 7).

STEM

In cross section the stem of *Helleborus bocconei* subsp. *intermedius* is rhizomatous bearing, in fact its subspecies is a rhizomatous geophyte. The cross section shows various external layers of suberized cells forming a multi-layered exoderm protecting a cortex made up of numerous layers of storage parenchymatous cells of different kinds: both amylaceous and tiny crystals already spotted in the root and other organs. Moreover, there are numerous leaf traces at different level of the cortex (Fig. 8) that, in succeeding sections, come into the vascular bundles located in centripetal direction, in one and only row, without continuous change but limited to the different-sized bundles (greater bundles alternate with smaller ones), separated by multiserial pith rays. Within each bundle there is a small cap of sclerenchyma protecting the liber, riche of cribose elements and parenchyma. Xylem consists of numerous proto and metaxylematic elements, not lignified, with a narrow diameter, with paratracheal parenchyma and thin tissues, simple, and with numerous perforations on radial walls. In the center there is a wide medulla, rich with storage parenchymatous cells in which crystals and other formations are abundant.

Particularly interesting are the cells of the cortical parenchyma since the inside shows some thickly spirally wrapped fungin hyphae have been observed; through porocanals they connect to surrounding cells, reaching the central cylinder thus penetrating the inside of xylematic vases.

ROOT

Roots are tetrarchic with suberose exoderm just hinted only at an early stage; it involves only the external layer of epidermic cells, while it appears more conspicuous, multi-layered and worn-out for soil friction in older roots. Proceeding in centripetal direction we see

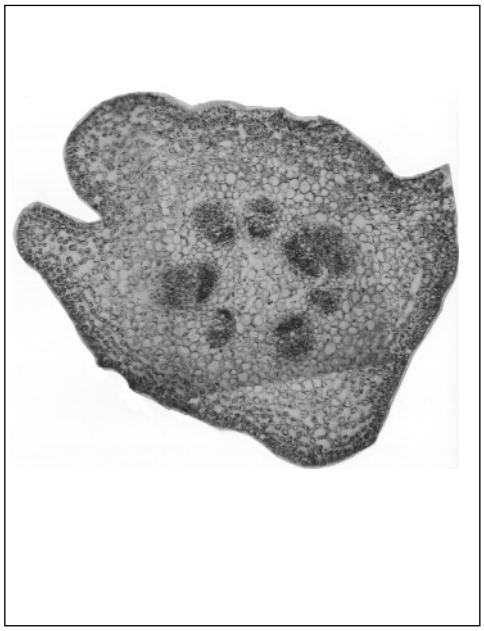

Fig. 6. Cross section of floral stalk of *H. bocconei* subsp. *intermedius* (× 100).

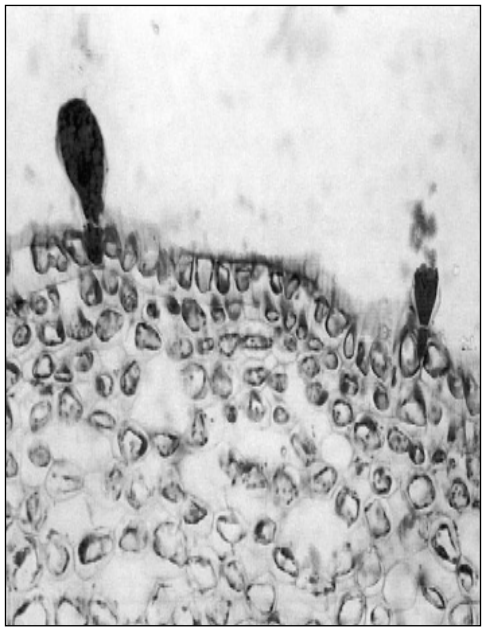

Fig. 7. Detail of cross section of floral stalk of *Helleborus bocconei* subsp. *intermedius* (× 200) highlighting conidiogenous cells of the endophyte, at edges.

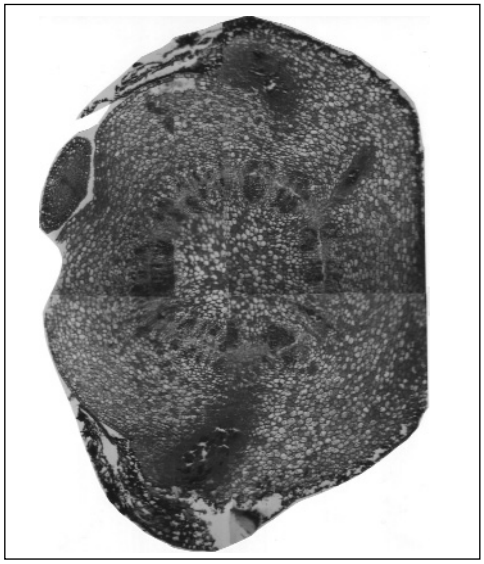

Fig. 8. Cross section of stalk of *Helleborus bocconei* subsp. *intermedius* (× 100).

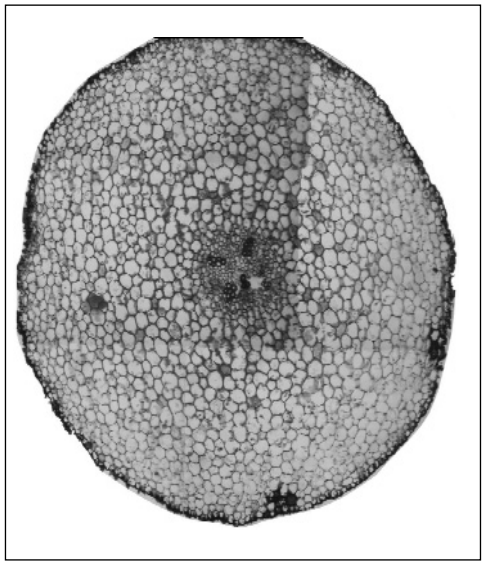

Fig. 9. Cross section of root of *Helleborus bocconei* subsp. *intermedius* (× 100).

a very wide cortex is, as usual in this organ, with thin wall parenchymatous cells, many of which, randomly displayed, bearing numerous crystals observed in other organs, too. Still proceeding in a centripetal direction we meet a thin endodermis and, at last, a tetrarchic actinostele and a parenchymatous pith.

In most cortical cells some fungin structures have been spotted as well, as in epigeal organs.

Therefore, in the organs studied of both species, fungin structures that assume a symbiotic relation between fungus and plant, original of hellebore species studied, have been observed.

On leaves, conidiogenous ampulla-shaped cells are mainly located along main nervations and in correspondence with the leaf edge, in the lobes forming among thorn-shaped protrusions (Bell 1993). The organs' anatomy of the investigated taxa, exspecially in *H. bocconei* subsp. *intermedius* as in *H. lividus* subsp. *corsicus,* of which only the leaf has been analyzed, has allowed to follow the route of the hyphae that go inside roots directly from the soil through lesions of the exoderm or through scars caused by the fall of radical hairs, both of main roots and those of superior order; through the cortical parenchyma the endodermis and the pericycle reach the xylema invading in *H. bocconei* subsp. *intermedius* rhizomatous stalk. Through the cauline knots, they reach the leaf petiole invading the lamina. Some other hyphae reach the flower buds and colonize the floral peduncle, the thalamus, segments of the perianth and reproductive structures. We believe it a steady and permanent fungin colonization, since it has been spotted in both investigated species, and as for *H. bocconei* subsp. *intermedius* even in plants of other stands coming from studied material. Such a symbiosis, besides the two studied taxa, has been studied in findings of *H. virdis* L. and *H. orientalis* Lam. In the collection of Palermo Botanical Garden (Spadaro & al. 2007) – introduced by seed respectively from the Milan Botanical Garden (Brera) in 1962 and from Austria, by means of a correspondent, in 1957 – as evidence of a congenital and generalized symbiosis.

Conclusions

Given the presence of the fungal symbiosis, focused not only in *H. bocconei* subsp. *intermedius* and *H. lividus* subsp. *corsicus* but also in other taxa of the same genus, the authors assume that such symbiosis is a constant characteristic of the genus. In this regard, it has been observed that cases of endophytism among *Ranunculaceae* were seen only in *Ranunculus bulbosus* L. (Holm 1913).

What needs to be defined now is the specific entity of the studied species. As regards *H. bocconei* subsp. *intermedius*, the tillage isolation of the responsible mycete has allowed to refer (momentaneously) the endophyte to *Botrytis bissoidea* Walker (Spadaro & al. 2007). It is not excluded that in *H. lividus* subsp. *corsicus*, *H. virdis* and *H. orientalis* the endophyte is made up of the same, or other, fungin species and so it may result in a fungus/plant specificity. All this makes up a stimulating hypothesis of study for whose confirmation authors are still working.

Acknowledgment

Special thanks Dr. A. M. Terranova for English translation. Studies have been carried out within the PhD "Risorse vegetali" of Palermo University, XVIII cycle and supported by Università degli Studi di Palermo.

References

Bell, A. 1993: La forma delle piante – Bologna.

Esau, K. 1965: Plant anatomy. – New York.

Fuchs, C. 1963: Fuchsin staining with NaOH clearing for lignified elements of whole plants or plants organs. – Stain technology **38:** 141-144.

Hickey, L. J. 1979: A revised classification of the architecture of dicotyledonous leaves. – Pp. 25-39 in Metcalfe, C. R. & Chalk L., Anatomy of the dicotyledons, 2° ed., **1.** – Oxford.

Holm, T. 1913: *Ranunculus bulbosus* L. – Merck's Rep. **22:** 2002-2004

Lentini, F., Raimondo F. M. 1990: Indagini etnobotaniche in Sicilia. IV. L'uso tradizionale delle piante nel territorio di Mistretta (Messina). – Quad. Bot. Amb. Appl. **1(1990):**103-117.

Metcalfe, C. R., Chalk, L., 1957: *Anatomy of the dicotyledons,* **1.** – Oxford.

Pignatti, S. 1982: Flora d'Italia, **1.** – Bologna.

Raimondo, F. M., Lentini F. 1990: Indagini etnobotaniche in Sicilia. I. Le piante della flora locale nella tradizione popolare delle Madonie (Palermo). – Naturalista Sicil., s. 4, **14(3-4):** 77-99.

Rosselli, S., Maggio, A., Senatore, F., Spadaro, V., Bruno M. 2006: Indagine fitochimica sulle radici di *Helleborus bocconei* Ten. subsp. *intermedius*. Atti XV Congresso Italo-Latinoamericano di Etnomedicina. Perugia-Roma 23-28.9.2006.

Spadaro, V. 2006: Studio biologico ed etno-farmacobotanico di *Helleborus bocconei* subsp. *intermedius* (*Ranunculaceae*) – Phd Thesys, Università di Palermo.

—, Colombo, P., Raimondo F. M. 2007: Contributo allo studio dell'endofitismo in *Helleborus* L. (*Ranunculaceae*). – P. 134 in Atti 102° Congresso S. B. I., Palermo 26-28 settembre 2007. – Palermo.

—, Tempesta, S., Colombo, P., Raimondo, F. M., Rambelli A. 2007: An interesting endophyte isolated from *Helleborus bocconei* subsp. *intermedius* (*Ranunculaceae*) in Sicily. – P. 195 in Venturella, G. (ed), Results XII OPTIMA Meeting. Pisa 10-15 settembre 2007. – Pisa.

Address of the authors:
Vivienne Spadaro, Francesco M. Raimondo & Paolo Colombo, Dipartimento di Scienze botaniche, via Archirafi, 20. I-90123 Palermo. E-mails: vspadaro@unipa.it, raimondo@unipa.it, pcolombo@unipa.it.