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Brood cells in the rare, epiphytic moss *Tayloria rudolphiana* (Garov.) Bruch *et* Schimp. (Splachnaceae)

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Abstract – *Tayloria rudolphiana* (Garov.) Bruch *et* Schimp. was grown in axenic culture, from spores and gametophyte fragments to study its development patterns for the first time. It was grown for 16 months on Parker's growth media under a 14h light/16°C-10h dark/14°C cycle with daylight fluorescent lighting. The expansion of protonemata filaments and branch formation in *T. rudolphiana* followed the typical tip growth pattern seen in mosses. All types of protonemata cells were observed (chloronemata, caulonemata and rhizoids) in specific developmental sequences, depending on their origin. Protonemata (caulonema) derived brood cells were observed for the first time in *T. rudolphiana*. Brood cells formed at the ends of the caulonemal filaments as chains of short, relatively thick-walled, spherical cells, containing abundant chloroplasts and some lipid droplets. Brood cells developed after 4 months in culture on colonies initiated from spores.

***Tayloria rudolphiana* / brood cells / *in vitro* culture / Splachnaceae / protonemata development / moss**

Résumé – *Tayloria rudolphiana* (Garov.) Bruch *et* Schimp. a été mise en culture sous conditions axéniques, à partir de spores et de gamétophytes, afin d'étudier pour la première fois son développement en culture *in vitro*. L'espèce a été cultivée dans un milieu Parker pendant 16 mois sous un régime de lumière et de température 14 h jour/16 °C – 10 h nuit/14 °C avec un éclairage fluorescent reproduisant la lumière du jour. L'expansion des filaments de protonéma et la formation de branches dans *T. rudolphiana*, ont suivi le schéma typique observé chez les mousses. Tous les types de cellules du protonéma ont été observés (chloronéma, caulonéma et rhizoïdes) dans des séquences de développement spécifiques selon leur origine. Des cellules nichées derivées du protonéma (caulonéma) ont été mises en évidence pour la première fois chez *T. rudolphiana*. Les 'brood cells' se sont formées aux extrémités des filaments de caulonéma en formant des chaînes de cellules courtes, vertes et sphériques, avec des parois relativement épaisses. Ces cellules contenaient des chloroplastes en abondance et quelques gouttelettes lipidiques. Elles ont été observées après 4 mois de culture dans les colonies engendrées à partir de spores.

***Tayloria rudolphiana* / cellules de propagation / culture *in vitro* / Splachnaceae / développement du protonema / mousse**

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INTRODUCTION

The Splachnaceae

The Splachnaceae, or dung mosses, are unique amongst the mosses in their preference for organic substrates (frequently animal dung), known as coprophily, and their adaptations in favour of entomophily. In general, coprophilous species are assumed to be entomophilous, whereas epiphytic or humicolous species are assumed to be anemophilous (Goffinet & Shaw, 2002; Koponen, 1982a). Some of the main characteristics of the Splachnaceae are linked to entomophily, namely the large, brightly coloured apophysis and the emission of volatile compounds (Koponen *et al.*, 1990; Pyysalo *et al.*, 1978, 1983); capsule walls that shrink and the presence of a pseudocolumella which ensures that spores are pushed out of the capsule; "sticky" spores which clump together and attach to the bodies of insects (Koponen & Koponen, 1978; Koponen, 1982a); and reflexed or erect peristome teeth after dehiscence so that spores come into contact with the insects' bodies (Koponen, 1977, 1982a, b, 1990; Koponen & Koponen, 1978; Vitt, 1981). The relationships between members of the Splachnaceae and their organic substrates have been investigated in some detail (Cameron & Wyatt, 1989; Gonzalez *et al.*, 2006; Koponen, 1990; Marino, 1988a, b; Marino *et al.*, 2009). In contrast, gametogenesis and vegetative propagation in members of this family have not been widely documented (Duckett *et al.*, 2004; Mallón *et al.*, 2006).

Brood bodies

Correns (1899: 146) illustrated gemmae from *Tayloria serrata* (Hedw.) Bruch et Schimp. and briefly described them, also citing an earlier description of gemmae on the tomentum in the upper stem leaves of *T. serrata* by Limpricht (1893: 147) "*Wurzelfilz trüb purpurm, mit gebräunten, schmal elliptischen, fünfgliederigen Brutkörpern.*" Nishida & Iwatsuki (1980) illustrated spore germination in *Tayloria hornschuchii* (Grev. *et* Arn.) Broth., showing uni-polar germination and protonemata development into densely branched filaments with long, cylindrical cells of the *Bryum*-type. Duckett *et al.* (2004), based on Correns' illustration of "clavate multicellular protonemal gemmae with what appear to be clearly defined tmema cells," grew all eight British species of the Splachnaceae to investigate protonemal morphogenesis in the family. None produced vegetative structures in culture and no gemmae were found during an investigation of herbarium material of various *Tayloria* species, leading to the conclusion that the material illustrated in Correns (1899) was taxonomically erroneous and likely to belong to the genus *Zygodon* Hook. *et* Taylor. More recently however, Mallón *et al.* (2006) reported brood cells and choloronemata bulbils from *in vitro* cultures of *Splachnum ampullaceum* Hedw. This was the first report of vegetative reproduction for this species and supported the hypothesis that brood cells are produced in the Splachnaceae.

Tayloria rudolphiana (Garov.) Bruch *et* Schimp.

Tayloria rudolphiana has been reported from 26 historical or extant localities in four countries from Europe and Asia: Austria, Germany, Switzerland, China (Gao & He, 2003; Grims & Köckinger, 1999; Hofmann, 2009; Meinunger & Schröder, 2007). Koponen (1992) synonymised the Chinese *T. delavayi* (Besch.)

Besch. with *T. rudolphiana* based on their identical sporophytes and similarities in their gametophytes. This synonymy has not been universally recognized, thus *T. rudolphiana* is considered as a European endemic by some authors (ECCB, 1995; Hofmann *et al.*, 2006). The largest extant populations of this species are known from Switzerland where 9 of the 11 original localities checked have living populations which are being monitored (Hofmann, 2009). Within Switzerland, *T. rudolphiana* is listed as vulnerable (VU) on the Bryophyte Red List (Schnyder *et al.*, 2004) and it appears in the *Ordonnance sur la protection de la nature et du paysage* on the list of protected Swiss plants, Annex 2. Factors contributing to the rarity of *T. rudolphiana* in the wild are not yet fully understood, but the main threat to the long-term survival of this species, at least in Europe, appears to be its low population density combined with the loss of its specific habitat. It is usually found on the prominent, horizontal branches of mature *Acer pseudoplatanus* L., *A. campestre* L. or *Fagus sylvatica* L. situated in open pastures or open forests on north-facing slopes or in gorges where ambient humidity levels are high (Hofmann *et al.*, 2006). It is found from 1000-1800 m in the Alps (Koponen, 1992; ECCB, 1995) and from 3800-4400 m in China (Gao & He, 2003). It is frequently found growing adjacent to or within populations of other bryophytes, such as *Leucodon sciuroides* (Hedw.) Schwägr. Literature sources indicate that this species is anemophilous, prefers nitrogen rich substrates and that it is found on bird faeces (ECCB, 1995; Hofmann *et al.*, 2006; Koponen, 1992), although recent field observations have not confirmed this.

***In vitro* culture in literature**

In vitro culture has been recognised as an important tool in bryophyte *ex situ* conservation and reintroduction trials (Pence, 2004; Rowntree & Ramsay, 2005; Rowntree 2006; Sarasan *et al.*, 2006), as well as being a key technique facilitating the study of the germination and development of mosses (see reviews in Duckett *et al.*, 2004 and Hohne & Reski, 2005; Nehira, 1983; Goode *et al.*, 1992; Duckett *et al.*, 1998). Such observations can contribute novel information on species (Duckett *et al.*, 2004), such as the presence of vegetative reproduction, which is not always seen in the wild, and on subsequent development patterns of vegetative propagules (Duckett & Ligrone, 1992; Duckett *et al.*, 2001), specific nutrient requirements (Sabovljevic *et al.*, 2003), or responses to desiccation (Mishler & Newton, 1988; Rowntree *et al.*, 2007), which can be of use in interpreting certain aspects of their biology.

In the light of this, *Tayloria rudolphiana* was grown for the first time under axenic conditions with the two-fold aim of studying its developmental patterns for the first time and assessing whether this species can be propagated under artificial conditions for future reintroduction trials in Switzerland (Martinez, 2009).

MATERIALS AND METHODS

Four herbarium samples of *Tayloria rudolphiana* from the canton of Bern were used for culture initiation (localities Schwarzwaldalp – G00048238, Tschingel – G00048239, Spieggegrund – G00048240, Mäscherchopf – G00048241; the first collected in 2006 and the rest in 2007). Herbarium specimens, donated by

H. Hofmann through the NISM project, are housed in G. One to four gametophyte fragments and spores from 2 operculate or deoperculate capsules per population were used. All plant fragments from the same plant were placed into a single Petri dish and 1 dish was used per capsule. The selected stems were rinsed under running water for 15 minutes, surface sterilised for 3 minutes in 0.25% Sodium dichloroisocyanurate (NaDCC) solution and then rinsed twice in sterile deionised water (see Rowntree, 2006). Operculate sporophytes were rinsed in sterile deionised water for 1 minute before being immersed in 1% NaDCC solution for 5 minutes and then rinsed twice with sterile deionised water. Deoperculate capsules were not sterilised. The gametophyte tissue and spores were placed into Petri dishes (5.5 cm dia × 1.5 cm or 9 cm dia × 1.5 cm) containing either Parker's growth media (Klekowski, 1969) or MS media (Murashige & Skoog, 1962), both solidified with 1% Phytogel. Cultures were kept in a RUMED 1301 growth chamber on a cycle of 14h light/16°C and 10h dark/14°C with daylight fluorescent lighting. Contaminated material was re-cultured from visibly clean parts of the plates until contaminant free material was obtained. Specimens were observed daily after culture initiation, weekly once protonemata had developed and monthly after they were one month in culture. Cultures were transferred to new media plates every few months during the 16-month period. Observations on growth and development were made using a Leitz Dialux 20 optical microscope or a Leica $MZ7_5$ dissection microscope, both of which could be attached to a Leica DFC290 digital camera. The specimens were either photographed on the culture medium or were removed from it and mounted in water on microscope slides with coverslips. A total of 17 petri dishes were initiated into culture resulting in 103 axenic Petri dishes at the end of the study.

RESULTS

Gametogenesis

Tayloria rudolphiana was grown successfully on Parker's medium for 16 months and on MS medium for 8 months. All types of protonemata cells were observed (chloronemata, caulonemata and rhizoids) in specific sequences (see below) depending on their origin. *Tayloria rudolphiana* has *Bryum*-type spore development (see Nehira, 1983) and mostly unipolar germination, although occasionally two choloronema filaments were produced per spore. Spores of *T. rudolphiana* swelled after a few days in culture, produced the first protonemal cell within the first 7 days and subsequently branched into choloronema and caulonema filaments before producing rhizoids and buds on mature cultures after 28 days. In cultures that were initiated from gametophyte fragments, protonemata (caulonemata) were produced, which quickly differentiated into a protonemal network including caulonemal branches, rhizoids and buds. Chloronema were not seen in gametophyte initiated cultures. The expansion of protonemata and branch formation in *T. rudolphiana* followed the typical tip growth pattern already described in mosses (Goode *et al.*, 1992; Menand *et al.*, 2007). Chloronemata were identified by their perpendicular cell walls and short cells with large green chloroplasts. Caulonemal cells were identified by their oblique cell walls which became slightly brownish with age, a reduced number of chloroplasts (transparent appearance) and cells that were longer than those of chloronemal filaments. Rhizoids were identified by their oblique cell walls, brownish-reddish coloration and lack of chloroplasts (see Goode *et al.*, 1992). We found that *T. rudolphiana* grew well on Parker's and MS media for over 16 months and was able to rapidly produce new protonemal networks and buds when re-cultured. These observations indicate that the species is unlikely to be an obligate nitrophile.

Brood cells

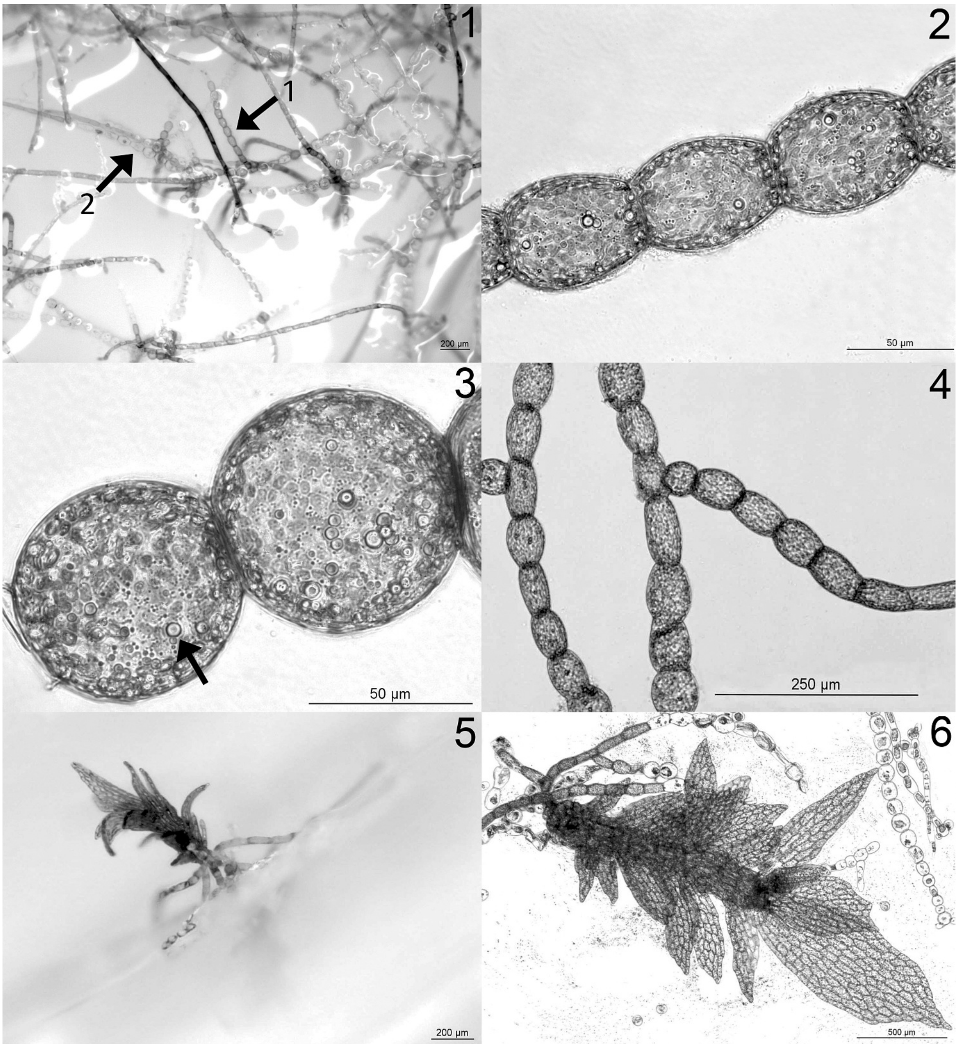
Brood cells were observed in several cultures of *Tayloria rudolphiana* initiated from spores after four months of growth on the same medium. The brood cells in *T. rudolphiana* were short, relatively thick-walled, spherical cells with abundant chloroplasts and some lipid droplets (Figs 1-3). They formed in groups at the ends of protonemal (chloronemal) branches (Fig. 1) as previously reported in Mallón *et al.* (2006) for *Splachnum ampullaceum*. They randomly developed on choloronema filaments (as previously reported for other mosses: Duckett & Ligrone, 1992; Duckett *et al.*, 2004), at the extremities of the colonies. The brood cells dropped off the parent filament in branches from four-five cells long, via separation of cells by the middle lamellae. Brood cells could be seen scattered on the medium where some subsequently germinated, by differentiating into chloronemal and caulonemal filaments (Fig. 4) and following the developmental scheme of spore-initiated cultures (chloronema, caulonema then buds and rhizoids) formed shoots (Figs 5-6). Culture dishes where brood cells were seen differed from the cell cultures that did not develop brood cells, as new buds and shoots were scattered all over the culture plates due to the migration of the brood cells across the surface. Brood cells were not observed in the herbarium samples used in this study.

Field samples, herbarium specimens and cultures

Observations made of two *T. rudolphiana* populations in Switzerland (Scwarzwaldalp, Bern) revealed that spores were "sticky" and clumped together at the capsule mouth above the reflexed peristome. They were not easily dislodged and did not disperse when blown upon. Instead the spore clumps readily stuck to objects that brushed against them, indicating that this species is not likely to be anemophilous. No bird faeces have been seen in association with Swiss populations of *Tayloria rudolphiana* observed in the field (Hofmann, *pers. comm.*). Herbarium specimens of Swiss populations indicate that this species appears to be present in the same localities (presumably on the same trees) for many years. At the same Swiss locality as above 20 potential host trees were identified and only two had populations of *T. rudolphiana* present. Recent transplant experiments revealed that re-located populations of *T. rudolphiana* were able to establish on new host trees (Hofmann, 2009). These observations indicate that *T. rudolphiana* may be able to maintain itself *in situ* over time (is a good establisher/competitor) but is a weak disperser.

DISCUSSION

The growth patterns of *Tayloria rudolphiana* in culture, from spore and gametophyte initiates, are widespread in mosses, in that nutrient-rich media promoted the proliferation of filamentous protonemata (Duckett *et al.,* 2001) and



Figs 1-6. Brood cell formation in *Tayloria rudolphiana* (Garov.) Bruch *et* Schimp., in axenic cultures initiated from spores on Parker's medium grown for 4 months without re-culturing. **1.** Brood cell formation (1) in the protonemal network with dead brood cells present (2) on some of the filaments; protonemal network is within Parker's medium on a Petri dish. **2.** Brood cell filament illustrating typical short globular cell shape. **3.** Brood cells with developed chloronemal cells and lipid droplets (arrow). **4.** Two brood cell filaments with a side branch initial (on left) and a side branch (right) which has differentiated into a caulonemal filament. **5.** Young shoot which developed from brood cells stuck on the wall of a Petri dish. **6.** Young shoot derived from brood cells, which are here dead.

that protonemata elongation and proliferation from spores followed a certain developmental sequence (Nehira, 1983). Similar patterns of protonemal development were described for *T. hornschuchii* (Nishida & Iwatsuki, 1980). All the types of protonemata filaments described in literature were observed in the spore derived cultures of *T. rudolphiana*: chloronemata, caulonemata and rhizoids. Their order of appearance was also representative of past observations, in that the first filaments to germinate were made up entirely of chloronemal cells which then differentiated into caulonemal filaments which in turn formed rhizoids (see Goode *et al.*, 1992). A typical pattern of cell differentiation was also observed: chloronemal cells differentiated into caulonemal cells or brood cells, and caulonemal cells differentiated into rhizoidal cells as documented in other studies (Pressel *et al.*, 2008). When cultures were initiated from gametophyte fragments it was observed that protonemal networks and buds developed faster than those for spore initiates. In contrast, chloronema was not seen in gametophyte initiated cultures. Ward (1960) observed that in *Polytrichium commune* Hedw. regular reculturing of protonemata caused them to re-differentiate into buds at an accelerated rate compared with non-manipulated colonies, and that thinner protonemal networks grew more slowly than denser ones. His study demonstrated that estimates of growth rates in cultured specimens are likely an artefact of manipulation, but his research also raises interesting questions about protonemal development and substrate colonisation in nature.

As *T. rudolphiana* is rare in the wild and assumed to be a nitrophile, it was expected that it would be difficult to culture in the laboratory. However, it grew rapidly in culture and produced brood cells, an event reported for the first time in the present study of this species. It is estimated that brood bodies occur in approximately 25% of all mosses (Duckett *et al.*, 1998), although recent studies have revealed that they may be much more common in the mosses than previously thought (Rowntree *et al.*, 2007). Brood bodies, formed mainly on chloronemal filaments, rhizoids, leaves or on specialized gemmae-bearing structures, comprise any structures that function as vegetative propagules, including gemmae, tubers, reduced branches, bulbils or brood cells (Duckett & Ligrone, 1992).

Brood cells are thought to be produced in response to either the drying of the medium, the accumulation of secondary compounds or the depletion of nutrients in the medium, as they were seen in older cultures from four months onwards and in colonies that remained on their original medium (see Goode *et al.*, 1993). Experimental manipulation of cultures of *Splachnum ampullaceum* using abscisic acid (ABA) revealed a direct relationship between ABA concentration (high) and the formation of brood cells on the chloronemata, indicating that brood cell production may be in response to desiccation events (Mallón *et al.*, 2006), something already observed in other taxa (Duckett *et al.*, 1993; Goode *et al.*, 1993).

Brood cells in *T. rudolphaina* were composed of chains of spherical cells that contained numerous chloroplasts and lipid droplets. It was noted that the brood cells detached easily from the parent structure through the separation of the middle lamellae, and dispersed easily across the culture medium when plates were removed from the culture chamber for observation. Tmema cells, as illustrated in Correns (1899: 146), were not present in *T. rudolphiana*. The brood cells observed in this species differed from those illustrated by Correns in *T. serrata* in both their structure and position (on the chloronema versus from the tomentum).

The main role of brood bodies is as perennating organs: produced as survival mechanisms when conditions worsen (Rowntree *et al.*, 2007), although some species such as *Dicranoweisia cirrata* produce them in response to elevated nutrient availability (Duckett *et al.*, 2001). In *T. rudolphiana* brood cells may be produced in response to seasonal desiccation and may play a role in the *in situ* population maintenance of this species on its host trees over time.

Although its rather specific habitat preferences may restrict this species geographically, other factors contributing to its rarity locally have yet to be studied in detail. More field observations of *T. rudolphiana* are necessary to confirm its status as insect or bird-dispersed. Its ability to spread to suitable trees within the same locality appears to be low, indicating that either dispersal events are very rare, or that establishment is difficult on the already well-populated branches of other trees. *Tayloria rudolphiana* is an autoicous, freely fruiting species and each population usually has abundant capsules with clumped spores. The role of the spores in the re-population of the host trees should also be studied as clumped spores may fall from the capsule and germinate *in situ*, providing an alternative means to out-compete or grow on top of potential competitors. *In situ* recruitment, through vegetative reproduction (brood cells) or mass spore germination, may have an important role to play in the maintenance of populations of *T. rudolphiana*. The relationship between brood cell production in wild populations of *T. rudolphiana* and environmental stress is unknown. Based on our observations of the cultured of *T. rudolphiana*, brood cells may be produced in response to desiccation.

Final Statements

Based on our observations of *T. rudolphiana* in the laboratory and in the field, the following hypotheses are proposed: 1) its rarity in the wild is linked to its dispersal capacities rather than to its poor potential for growth or nutrient requirements, e.g. nitrophily; 2) it is insect or bird dispersed based on its “sticky” spores that clump together at the capsule mouth, or it has lost its disperser; 3) brood bodies and spores have a role in its long-term population maintenance *in situ*. This study also shows that populations of *T. rudolphiana* can be successfully bulked up through *in vitro* culturing. The technique could therefore be employed for *T. rudolphiana* as both a method of *ex situ* conservation and, following reintroduction trials, to potentially replenish wild populations.

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