European Journal of Biotechnology and Bioscience
ISSN: 2321-9122
Impact Factor: RJIF 5.44
www.biosciencejournals.com
Volume 5; Issue 5; September 2017; Page No. 53-58

Spore germination and protonemal features of some mosses under *in vitro* conditions

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Abstract
In this study, spore germination features and protonema development of *Grimmia dissimulata*, *Syntrichia ruralis*, *Syntrichia laevipila* and *Syntrichia princeps* were investigated under *in vitro* culture conditions for the first time. All of these species are positively photoblastic with exosporous germination. Spore swelling and germination was observed in the Distilled Water medium supplemented with 1.5 % (w/v) sucrose (*Grimmia dissimulata, Syntrichia ruralis, Syntrichia laevipila and Syntrichia princeps* with germination percentages 94%, 34%, 54% and 43% respectively) and half strenght Murashige and Skoog medium without sucrose (for only *Grimmia dissimulata*; germination percentage was 51%). The sporeling types were identified as "Bryum type" for *G. dissimulata* and *S. laevipila* and "Encalypta type" for *S. ruralis* and *S. princeps*.

Keywords: *in vitro*, spore germination, sporeling type, *Grimmia dissimulata, Syntrichia princeps, Syntrichia ruralis, Syntrichia laevipila*, bryophyte biology

Introduction
Spore germination and protonemal development are two important areas to understand moss biology (Liu *et al.* 2016) [6] but observing these process *in situ* is not an easy attempt. *In vitro* culture techniques can be used as an alternative to *in situ* observations that can be very difficult and long time requiring studies. Many researchers have conducted *in vitro* culture techniques on different species of mosses under controlled conditions (Basile and Basile, 1988 [2]; Kowalczyk *et al*. 1997 [5]; Sabovljević *et al*. 2003 [9]; Duckett *et al*. 2004 [3]; Rowntree, 2006 [12]; Sabovljević *et al*. 2009 [10], Bagdatlı and Erdağ, 2015 [4], Erdağ *et al*. 2015 [4], Liu *et al*. 2016) [6]. However, no studies have been performed to understand spore germination characteristics and protonema development of a relatively higher number of moss species.
This manuscript represents the first study on the early developmental stages of *Grimmia dissimulata* E. Maier, *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr, *Syntrichia laevipila* Brid., and *Syntrichia princeps* (De Not.) Mitt. under *in vitro* conditions. We investigated the spore germination characteristics and stages of protonema development in these species under *in vitro* conditions.

Materials and methods
Plants with mature sporophytes were collected from two different areas of Turkey between March and April of the years 2013 and 2014. *Grimmia dissimulata* E. Maier (AYDN 3405) and *Syntrichia princeps* (De Not.) (AYDN 3406) were collected from Balıkköy province Aydın. *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr (AYDN 3407) and *S. laevipila* Brid. (AYDN 3408) were collected from Canyon of Geyik province Ula, Muğla. The vaucher specimens were deposited in the herbarium of Adnan Menderes Üniversity (AYDN).
Spore suspensions were prepared by following a method reported in Bagdatlı and Erdağ (2015) [4]. Briefly, undehiscent capsules were surface sterilized by dipping in 1 % NaOCl for 6 minutes and rinsed three times in sterile distilled water. Sterilized capsules were opened by using sterile forceps and dissection needle and then spores were released in eppendorf tubes containing 1 ml distilled water for spore suspensions. Approximately 10 µL of the suspension were taken by a micropipette and transferred to sterile petri dishes (90 mm diameter) containing 25 ml nutrient medium. All treatments were conducted in laminar air flow cabinet.
Three media were evaluated for spore germination experiments: Distilled water (DW), half and full strengths of Murashige and Skoog (1/2 MS and MS) (Murashige and Skoog, 1962) [7] media. All the media were supplemented with 1.5 % (w/v) sucrose or were sucrose free.
Cultures were kept at 24±2 °C with 16:8 photoperiod and dark. Illumination provided by cool white fluorescent lamps at 20 µmol m^{-2} s^{-1}. In all experiments, the pH of medium were adjusted to 5.8 and 0.8 % agar-agar (w/v) (Sigma) was added before autoclaving at 105 kPa for 15 min at 121 °C.
Six replicates of petri dishes were used for each type of media treatment. Spore germination characteristics and rates in three media types were determined at the end of weeks 5 or 6. Approximately 100 spores from each petri dish were randomly selected under light microscope.
Microscopic photographs were taken using an Olympus E 330 digital photograph camera attached on Olympus CX 31 optical microscope and Leica EC3 digital photograph camera attached on Leica S8APO stereomicroscope.

Results and Discussion
No contamination was recorded from inoculated spore suspensions. Spores of all species are isosporic and spherical shapes. The resulting data showed that no spore germination

was observed in the dark culture. In the media under constant dark conditions, the spores were swollen, but had failed to continue to the further stages of germination. Spore germination was only observed under photoperiod conditions. All of them were positively photoblastic with exosporous germination.

***Grimmia dissimulata* E. Maier**

Spore size of *G. dissimulata* varies between 8-13 mikrometers diameter (Figure 1a). Swollen spore size in 4 th week of cultivation was 20 µm (Figure 1b). 10 days after of the first day, spore walls were ruptured, and then germ-tube formation and also 1-2 celled protonema was observed. Germination polarity was predominantly monopolar but seldom bipolar germination was also observed (Figure 1c). Germination success was observed only in the media of distilled water with 1.5 % (w/v) sucrose and ½ MS medium without sucrose (respectivelly 94 % and 51 %). In MS medium and distilled water without sucrose medium there was no germination. As known well, MS medium has high amount of minerals and high amounts of nutritive minerals may inhibite germination by causing negative osmotic potential. Succesful germination in distilled water with 1.5 % (w/v) sucrose may exhibits requirement of a carbon source during germination process. A similar statement on external carbon source requirement for germination had been reported by Vujivic *et al.* (2012) [13]. Yet germination succes in ½ MS without sucrose was 51% whereas no germination in ½ MS with 1.5 % sucrose sounds a paradox but this contrary situation is probably due to negative interaction between sucrose and nutritive minerals. During germination in both media, no difference has been observed in lateral branches were formed on main axis and followed by the secondary branche formation on the new lateral branches (Figure 1d, e, f). The first cell of chloronema was elongated and continued to development to be more elongated and narrower. This elongating cells were around 20 µm long.

Sporeling type of *G. dissimulata* is "Bryum type" because of thread like protonema with long cylindrical cells. This type may cover both chloronema and caulonemal formation (Nehira, 1983) [8] but caulonema formation was not observed in our experiments for the species.

After three months of incubation, gametophore differentiation was not observed in protonemal colonies in both media.

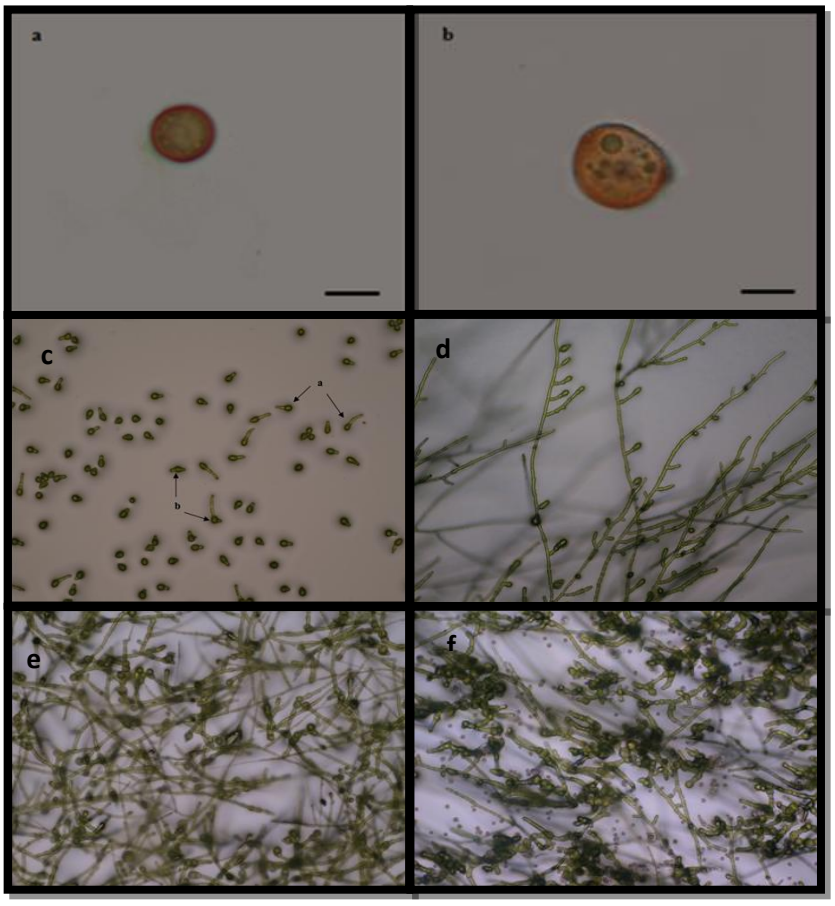

Fig 1: *Grimmia dissimulata* E. Maier
a. Mature spore (Bar: 10 µm)
b. Swollen spore (Bar: 10 µm)
c. Monopolar germination (a), bipolar germination (b)
d. Primary and secondary branches
e. Protonemal development in DW medium containing sucrose
f. Protonemal development in ½ MS medium without sucrose

***Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr**

The spores of *S. ruralis* are 10-12 µm wide. Six weeks after of cultivation, they became 40-50 µm wide and spore coats were ruptured and separative cell wall was formed to create sister cells in these swollen spores. By reason of successive divisions a massive protonema with 1-2 globose cell(s) was developed. In this primary protonemata, massive globose cells further developed to filamentous protonema of chloronemal filaments. By these properties, sporeling type in *S. ruralis* is "Encalypta type" (Nehira, 1983) [8]. Germination polarity is mainly monopolar but rarely bipolar cases were encountered (Figure 2a). Swelling and germination of spores were observed only in distilled water containing 1.5% sucrose in our experiments (34%). In other media, no germination was observed. Observing germination in only media containing sucrose sounds possible effect of sugars on germination of spores. This effect was also reported by Sabovljevic *et al.* (2012) [11, 13] and Vujicic *et al.* (2012).

In our experiments, a colour change (blackish) was observed when spores cultivated in a crowded mass but chloronema filaments were developed depending by time among this blackish mass. These chloronemal filaments were not transformed to caulonema (Figure 2b, c, d).

The chloronema filaments with long cylindric cells (spore dispersed more efficiently in culture media) became knotty and then took a kind of nostocoid appearance. Apical part of these nostocoid chains bear a along cylindric cell. Increasing branching on the chains create more and more chloronema filaments in culture media (Figure 2e). Transformation to caulonema was rapid and colour of these filaments changed from green to brown (Figure 2f). Vujicic *et al.* (2012) reported that presence of sugar in media promotes chloronema-caulonema transformation. In a normal cycle, it is expected to see gametophore initiations following caulonema phase but there was no gametophore development after caulonema formation in our experiments. Sabovljevic *et al.* (2012) [11, 13] has reported that sugars promoted germination but inhibited caulonema and gametophore formation.

In order to obtain gametophores, exogenically plant growth regulators were added to culture but no gametophore development was observed. Six months after from first culture experiments, the culture was stil in caulonema phase.

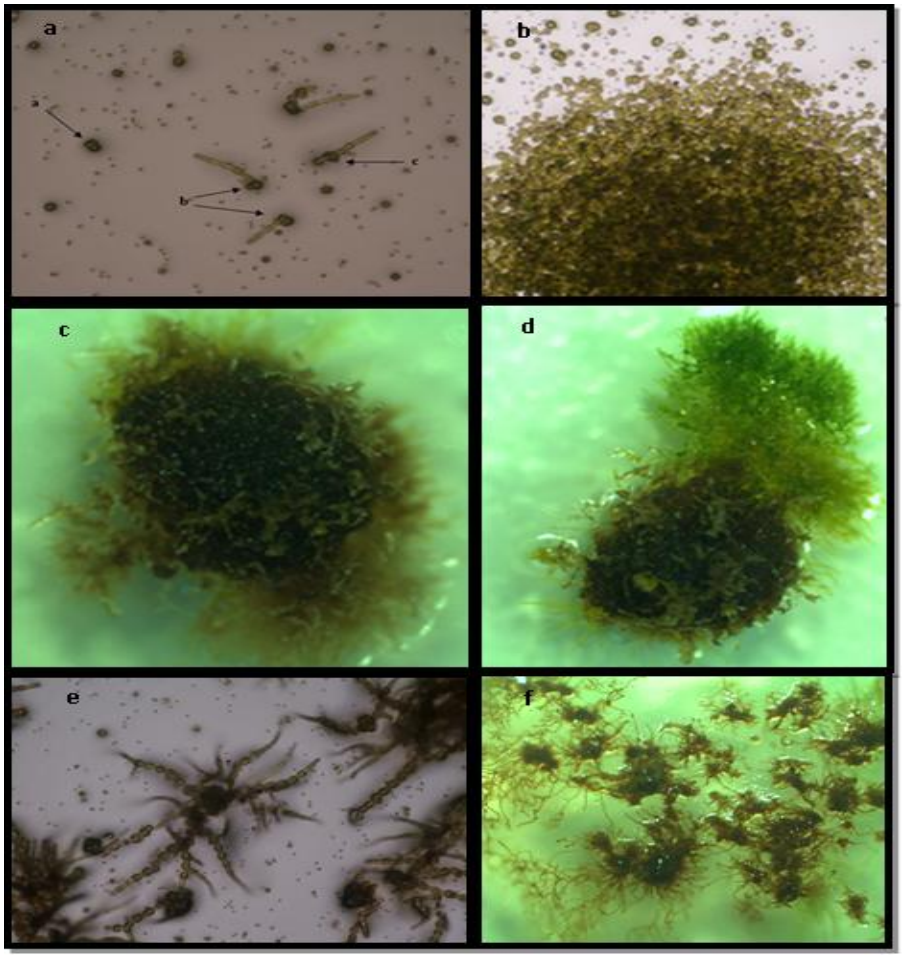

Figure 2: *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr
a. Massive protonema with globose cell(a) Monopolar germination(b) Bipolar germination (c)
b. Crowded spore mass
c. Blackish spore mass
d. New developing chloronemal filaments
e. Protonemal development in DW medium containing 1.5% sucrose
f. Caulonemal development in DW medium containing 1.5% sucose

***Syntrichia laevipila* Brid.**

Spores of *S. laevipila* are 16-20 µm wide (Figure 3a). At fourth weeks of the first inoculation, the spores became 35-40 µm wide (Figure 3b) and spore coats were ruptured, a germ tube was developed and the first protonema filaments with few cells was formed at sixth weeks. Germination was generally monopolar but a few bipolarly germinated spores were observed (Figure 3c). Protonema beared long cylindrical cells at the beginning. Sporeling type is "Bryum type".

Germination of spores were observed only in DW medium containing 1.5% sucrose (germination percentage 54%). In the other media, spore swelling or germination were not observed as in *S. ruralis.*

The first branching node was very close to spore coat and it was generally around the third cell of filament. In some filaments which are ca. 300 µm long, no branching was developed (Figure 3d). In secondary or tertiary branches of a branched filament the long cylindric cells became ovoid and served as a new branching point (= nodium). Nearly all chloronemal filaments was strongly tend to transform caulonema (Figure 3e). During transformation to caulonema, swollen cell groups became fragmented into few celled subgroups. These subgroups probably continued to develope filaments but because of crowded condition due to developing many filaments was an obstacle to observe them further. We preferred to comment them (swollen cells) as Tmema cells (Figure 3f). The tmema cells are the cells leaving protonema for vegetative development, behaving like gemmae. This event can be observed in certain species having short lived protonemas. The chloronemal filaments were transformed to caulonema as expected but gametophores were not developed even in the case of exogenically addition of plant growth regulators. At the six months of the first inoculation, the culture was stil in caulonema phase.

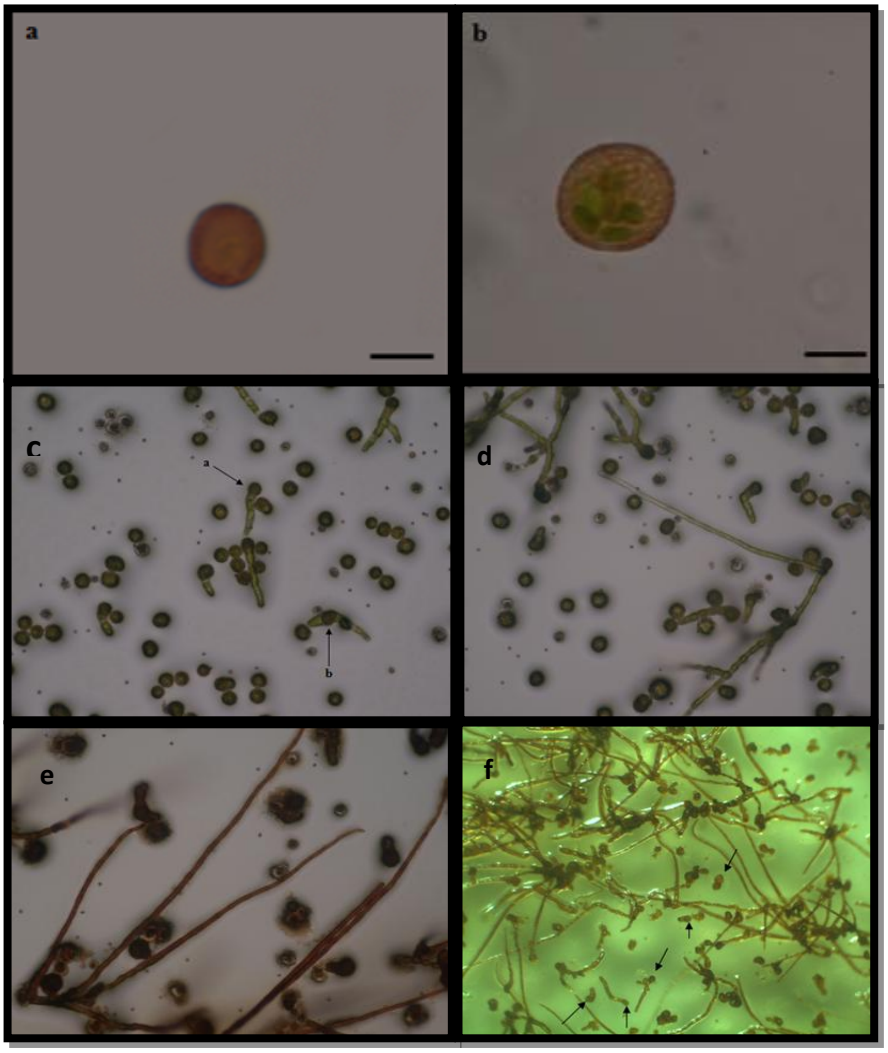

Fig 3: *Syntrichia laevipila* Brid.
a. Mature spore (Bar: 20 µm)
b. Swollen spore (Bar: 20 µm)
c. Monopolar germination (a), bipolar germination (b)
d. Chloronemal branching in DW medium containing sucrose
e. Caulonemal filaments in DW medium containing sucrose
f. Tmema cells (arrows)

***Syntrichia princeps* (De Not.)**

Spores of *S. princeps* are 10-12 µm wide (Figure 4a). Swollen spores after 5 weeks of culture were 45-55 µm wide (Figure 4b). Spores formed a massive protonema (as determined by Nehira, 1983) [8] containing isodiametric cells by 1-3 mitotic divisions. Germination was mainly monopolar but bipolarly germinated spores can be seen in cultures (Figure 4c).

Germination of spores were observed only in DW medium containing 1.5% sucrose (germination percentage 43%). In the other media, spore swelling or germination were not observed as in *S. ruralis and S. laevipila.*

In early stages of germination, appearance of culture was similar to a cushion containing numerous protonemal filaments which were derived multicelled groups. These groups contained 2 to 8 cells (Figure 4d). In the further steps, increasing cell number in the culture made impossible to count cells of filaments.

Developing chloronema filaments became a nostocoid chain by means of swollen cylindric cells at their middle part (Figure 4e). During this formation, cell walls of these swollen cells became brownish (caulonema formation). In addition, the cell walls are oblique at the end of this process. Aerial caulonema filaments were also formed, in curly forms (Figure 4f).

Exosporic germination and filamentous protonema of chloronema and caulonema from massive globose cell groups confirm "Encalypta type" sporeling in *S. princeps* (Nehira, 1983) [8].

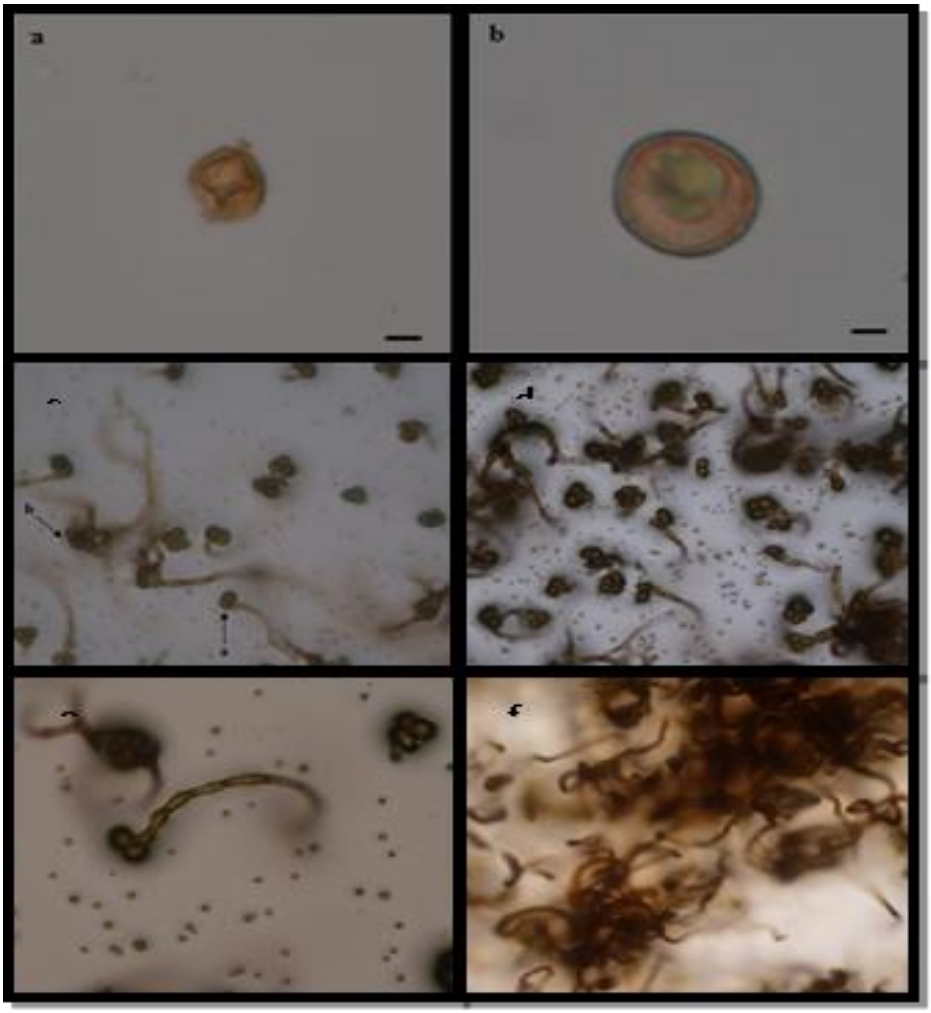

Figure 4: *Syntrichia princeps*
a. Mature spore (Bar: 5 µm)
b. Swollen spore (Bar: 5 µm)
c. Monopolar germination (a), bipolar germination (b)
d. Massive globose cell groups with protonemal filaments
e. Chloronemal filament in DW medium containing 1.5% sucrose
f. Aerial caulonemal filaments in DW medium containing 1.5% sucrose

Conclusion

Information on germination and early development of species which were used as materials in our experiments are shown in Table 1. Obtained results are contributive to enlight biology of these species and will be possibly helpful for creating a base for future studies on morphology, physiology, genetics and biochemistry of these species.

Table 1: Information on spore germination and early development of species

	G. dissimulata	***S. ruralis***	***S. laevipila***	***S. princeps***
Spore size	8-13 µ	10-12 µ	16-20 µ	10-12 µ
Swollen spore size	20 µ	40-50 µ	35-40 µ	45-55 µ
Time of germ tube formation	5 weeks	6 weeks	5 weeks	6 weeks
Germination type	Exosporic	Exosporic	Exosporic	Exosporic
Sporeling type	Bryum	Encalypta	Bryum	Encalypta
Germination polarity	Monopolar, Bipolar	Monopolar, Bipolar	Monopolar, Bipolar	Monopolar, Bipolar
Massive protonema	-	+	-	+
Chloronema	+	+	+	+
Caulonema	-	+	+	+
Protonema apical cell	Obtuse rarely acute	Long- cylindric	Long- cylindric	Long- cylindric
Tmema cell	-	-	+	-
Swolling in protonemal filament	-	Nostocoid appearance	+	+
Aerial caulonema filament	_	_	_	+

Acknowledgements

This study supported by the Scientific Research Project Found of Adnan Menderes University (Project number: FEF-13024). We would like to give our special thanks to Prof. Dr. Adnan Erdağ for his helpful comments and contributions.

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