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The moss *Dicranella varia* (Hedw.) Schimp, from spore to gametophore under *in vitro* conditions

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Abstract
In this study, spore germination features, protonema development and gametophyte differentiation of *Dicranella varia* were studied under *in vitro* culture conditions for the first time. The results show that germination pattern of this species is exosporic and sporelling type is Bryum type. Spore swelling and germination was observed in the Distilled Water media supplemented with or without 1.5 % (w/v) sucrose and half strenght Murashige and Skoog medium without sucrose (germination percentage 50%, 48% and 56% respectively). In our experiments, protonemal form is not affected by different nutrient contents. Filamentous protonema formation has been observed in all three media. Protonemal development were faster in DW medium with sucrose than the medium without sucrose. Caulonema and gametophyte differentiation were observed in Distilled Water media supplemented with or without sucrose (approximately 90 gametophyte per petri). Caulonema and gametophyte formation were not observed in half strenght Murashige and Skoog media without auxin. Auxine type and concentration effected gametophyte formation. Gametophore differentiation was observed in half strenght Murashige and Skoog medium containing 1 mg/L Indole-3- acetic acid.

Keywords: *Dicranella varia*, *in vitro,* spore germination, distilled water, protonema, gametophore.

1. Introduction

Bryophytes have peculiar importance in the studies of life cycle because of their different life strategies as a response to a wide spectrum of ecological factors. A life cycle of a bryophyte starts with a spore or gemmae germination and continues till to next spore formation and its release to adequate microhabitat. The events between these two points include all biological processes of a species but it is not easy (or in some cases not possible) to observe this processes in nature. *In vitro* techniques provide a good practical solution to observe the stages of life cycle of bryophyte species. Hohe and Reski (2005) [12] well summarized bryophyte axenic culture studies in the last century and draw its horizons in plant biotechnology. Vujicic *et al*. (2012) [31] and Sabovljević *et al*. (2012a) [27] conducted studies aiming to use axenic cultures in species conservation. Decker and Reski (2007, 2008) [7, 8] have established moss bioreactors to improve biopharmaceuticals. A number of studies on bryophyte axenic cultures has been published on different species in the recent years (Basile and Basile, 1988 [2]; Kowalczyk *et al*. 1997; Sabovljević *et al*. 2003, 2006 [22, 24], 2009, 2010, 2012b [25, 26, 28]; Duckett *et al*. 2004 [9]; Rowntree, 2006 [20]; Erdağ *et al*. 2015) [10]. However, many data are still in controversial state and numerous species react in different ways or express different pattern of development under the same conditions (Bijelović *et al*. 2004 [4]; Sabovljević *et al*. 2005) [23].

This manuscript represents the first study on the early developmental stages of *Dicranella varia* (Hedw.) Schimp. under *in vitro* conditions. We investigated the spore germination characteristics, stages of protonema and gametophore development, and the effect of different plant growth regulators on the number of gametophores in *D. varia* under *in vitro* conditions.

2. Materials and Methods

Mature plant specimens were collected from Balıkköy (province Aydın) at the end of March 2014. The vaucher specimens are kept in the herbarium of Adnan Menderes University (AYDN 3404).

The cultures were initiated using mature spores from closed capsules. The capsules were surface sterilized by dipping in 1 % NaOCl for 6 minutes and rinsed three times in sterile distilled water. Sterilized intact capsules were opened by using sterile forceps and dissection needle and then spores were released in eppendorf tubes containing 1 ml distilled water to gain 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.

Distilled water (DW), half and full strengths of Murashige and Skoog (1/2 MS and MS) Murashige and Skoog, 1962) media were used in the experiments. These media were supplemented with 1.5 % (w/v) sucrose or were sucrose free.

Protonemal fragments were collected from the cultures and then they were transferred to half strengths MS media supplemented with different concentrations and type of cytokinins and auxins two months after the first incubation.

Cultures were kept at 24±2 °C with 16:8 photoperiod. Illumination provided by cool white fluorescent lamps at 20 µmol m^{-2} s^{-1}. The same experiments were also conducted in dark conditions. 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.

All trials were performed with 2 petri dishes for each type of media and in 3 repeats. Spore germination characteristics and spore germination percentages in three medium types were determined at the end of week 8. In these experiments, randomly selected approximately 100 spores were observed under light microscope and germ tube formation was the main criteria to evaluate spore(s) as "germinated". Gametophore numbers per petri were determined at the end of month 3.

Microscopic photographs were taken using an Olympus E 330 digital photograph machine attached to an Olympus CX 31

optical microscope and Leica EC3 digital photograph machine attached to Leica S8APO stereomicroscope.

3. Results and Discussion

Spores of *D. varia* are isosporic and spherical. 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. Among mosses, the requirement of light for spore germination is species- dependent event (Meyer, 1948) and this phenomenon is considered as a kind of life strategical response (Silva *et al*., 2010) [29].

Spore size of *D. varia* varies between 25-28 µm (Figure 1a). Swollen spores (7 th week of cultivation) reached 36-40 µm diameter (Figure 1b). Germination of the spores was exosporic. 10 days after spore swelling, germ tube formation was occured as spore wall ruptures. 1-2 celled young protonema was developed from this germinated spores. Germination polarity was predominantly monopolar, with seldom bipolarly germinated spores (Figure 1c).

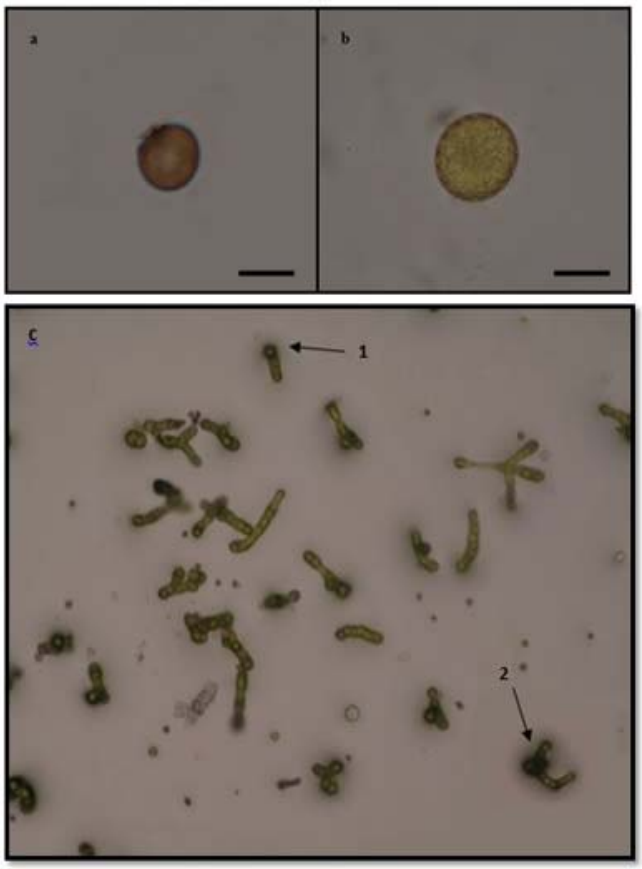

Fig 1: a. Mature spore
b. Swollen spore
c. Monopolar germination (1), bipolar germination(2)

Spore swelling and germination was observed in the DW media supplemented with 1.5 % (w/v) sucrose, DW media sucrose free and half strenght MS medium sucrose free (germination percentage 50%, 48% and 56% respectively). Addition of sucrose to DW medium slightly increased germination percentage and again slightly decreased germination time. Similar result on earlier germination (2-4 days) of spores in media enriched by sucrose has been reported by Sabovljević *et al*. (2012) [31]. The highest germination percentage was held in half strenght MS medium without sucrose, and inhibition of spore germination was observed in half strenght MS medium enriched with sucrose. As a possible explanation for this observation, half strenght Murashige and Skoog medium contains relatively high amount of salts and this may cause excessively negative osmotic potential when half strenght MS medium is supplemented with sucrose. Finally, this contraversial situation may inhibite water uptake by spores.

Early protonemal development was more or less similar in three media. Subglobose and globose cells of earliest germination step was followed by elongated cylindrical cells. Apical cells of branches were obtuse or rounded. Crowded appearance of branches was a result of quick and rather intensive cell division in the middle of these branches. In this case, wedge shaped cells were inserted among the square-like cells. The length of elongated cylindrical cells vary between 2 to 15 times of the length of square shaped cells. Chloroplasts in these cells were scattered and few. Contrary, chloroplasts seem to be more intensive and crowded due to decreasing cell size in crowded celled parts of these branches.

Sporelling type in *D. varia* is “Bryum type” due to formation of subglobose and globose cells were followed by elongated cylindrical cells and general appearance of spore germination. This type is characterized by filamentous form protonema having long cylindric cells and contains only chloronema or chloronema and caulonema together (Nehira, 1983) [18]. In our experiments, caulonemal filament were also observed with normal formation of chloronemal threads. Globose, subglobose cells and then elongated cells were present in all media. In early stages of development, numerous primary chloronemal branches had formed on the main chloronemal filament; these primary branches were then followed by secondary branches in all three media (Figure 2a, b and c). Protonemal development was also similar in these media. Information on mineral nutrition of bryophytes is not presented sufficiently in literature. It has been suggested that bryophytes needs macronutrients and micronutrients closely similar to those of higher plants (Bates, 2000 [3]; Zechmeister *et al*., 2002) [32]. Although bryophytes exhibit normal development on a spectrum of media, there are some reports that this may be changed by varying the concentrations of single element or relative concentrations (Bopp, 1983) [5]. By contrast, deveopment is not materially altered by 1/10 or even 1/100 dilution of the entire nutrient solution (Duckett *et al*. 2004). In these trials, it is interesting to observe healthy protonemal development in DW media solidified only by agar with no nutrients. The may be explained by:

1. Some excreted substances by germinating spores may support the development,
2. The development may be supported by cations from agar.

This may represents an adventage for the species when their spores reached a vey poor sites having limited amount of nutrients.

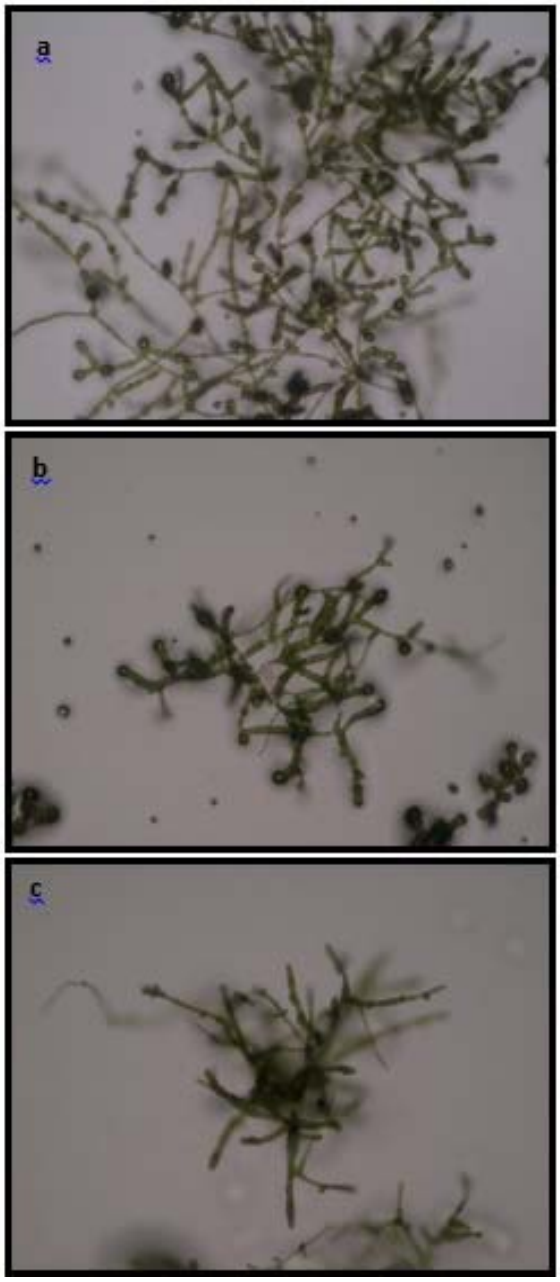

Fig 2: a. Protonemal development in DW medium containing sucrose
b. Protonemal development in DW medium without sucrose
c. Protonemal development in ½ MS medium without sucrose

Protonemal development were faster in DW medium with sucrose than the medium without sucrose. Addition of sucrose to bryophyte cultures is not strictly necessary (Rowntree *et al*. 2011) [21]. Bryophytes usually grow photoautotrophically (sugar free) in *in vitro* culture conditions (Takami *et al*. 1988 [30]; Hohe *et al*. 2002 [11]; Rowntree 2006) [20]. Thus it may be suggested that plant physiology and development *in vitro* systems is probably quite similar when compared to the plant in its natural environment. However, sucrose can have a beneficial effect on development processes in some species (Sabovljević *et al*. 2005) [23].

In our experiments, in the early stages of caulonema it contains chloroplast as remnants of previous chloronemal stage in DW media with or without sucrose. Gametophore initiation points were scattered on caulonemal filaments (Figure 3a and b). Gametophore initiation points on these caulonema seems to be a callus like structure formed by intensive globose cell accumulation rather than a leafy bud in DW medium without sucrose (Figure 4 a). On the other hand, it becomes like a compact mass of cells in DW medium with sucrose (Figure 4 b). In further steps of gametophore formation, the gametophore becomes leafy by appearance (Figure 5a and b) as widely known in many species. Numbers of gametophore were 90 in average per petri dishes in both media but gametophores were developed faster in media with sucrose.

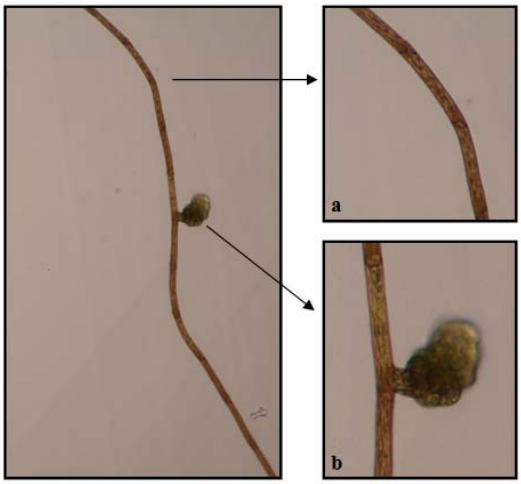

Fig 3: a. Caulonemal filament
b. Callus-like gametophore bud.

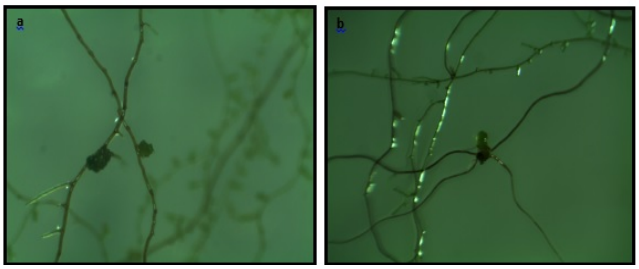

Fig 4: a. Callus-like gametophore buds in DW medium without sucrose
b. Gametophore bud in DW medium containing sucrose

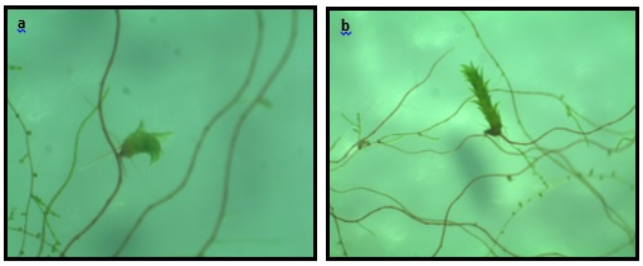

Fig 5: a. Leafy gametophyte
b. Developed leafy gametophyte in DW medium without sucrose

Caulonemal development and gametophore formation were not observed half strenght MS medium. Some of medium components although not considered as plant growth regulators, have been shown to exhibit a distinct effect on plant development. For example, when the moss *Physcomitrelle patens* is cultured, the addition of ammonium tartrate to the medium promotes the development of chloronema and surpresses differentiation of caulonema cells (Ashton and Cove,

1977 [1]; Jenkins and Cove 1983) [13]. Similary, in our experiments, chloronemal development continued but gametophyte differentiation was cease in half strenght MS medium containing high amount of salts.

In the experiments on the effect of plant growth regulators for gametophyte formation, protonemal fragments were incubated in half strenght MS media containing different amounts of cytokinins and auxins (Table 1). In our experiments, responses to cytokinins was observed as continuing vitality in medium with 2 mg/L KIN and callus formation besides vitality in medium with 1 mg/L KIN. In medium with 1 mg/L KIN one month later of callus formation, secondary chlroronemal branches were produced and then dwarf calli were formed on this filaments (Figure 6). Any concentration of BA gave gametophore formation and protonemal filaments started to die (becoming brownish and loss of chloroplasts). While auxins promote caulonema formation in *Physcomitrella patens*, cytokinins promote bud formation depending on their concentrations (Decker *et al*. 2006) [6]. Lower concentrations support only chloronemal cells and higher concentrations support caulonemal cells (Reski and Abel, 1985). In our experiments, caulonemal filaments were not seen in ½ MS medium. Probably, cytokinin concentrations did not promote cell diferentiation to caulonema but caused calli formation. It was reported that exogenic high concentration of cytokinins promote calli like bud formation but these buds never differentiated to leafy gametophores (Reski, 1998).

Table 1: Effects of auxins and cytokinins on protonemal fragments on ½ MS media

	Protonemal vitality	Gametophyte differentiation	Callusing
0.1 mg/L IAA	-	-	-
1 mg/L IAA	+	+	-
2 mg/L IAA	+	-	-
0.1 mg/L IBA	-	-	-
1 mg/L IBA	-	-	-
2 mg/L IBA	+	-	-
0.5 mg/L KIN	-	-	-
1 mg/L KIN	+	-	+
2 mg/L KIN	+	-	-
0.5 mg/L BA	-	-	-
1 mg/L BA	-	-	-
2 mg/L BA	-	-	-

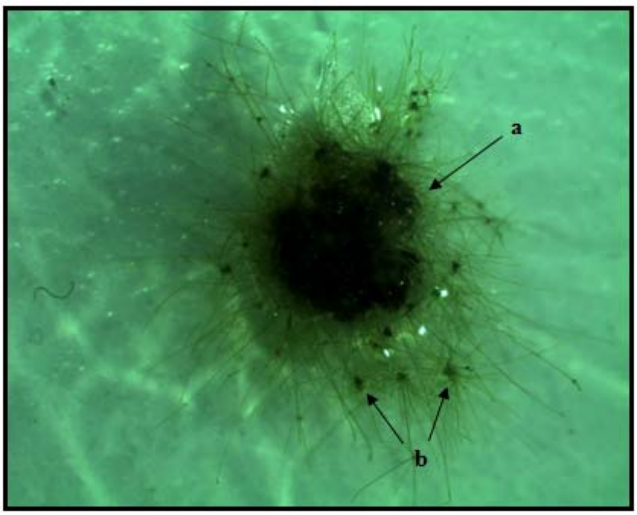

Fig 6: Calli on ½ MS medium containing 1mg/L KIN a. Mother callus b. Miniature calli on secondary chloronemal filaments

Results of our experiments show that gametophore differentiation is highly affected by type and concentrations of exogenically applied auxines in half strenght MS media. Beside that protonemal vitality was conserved in the media with 1 and 2 mgL IAA and 2 mgL IBA. Only gametophyte diferentiation was observed in medium containing 1mgL IAA (130 gametophyte per petri) (Figure 7). Bijelović *et al*. (2004) [4] had previously reported that a corelation between protonemal growth and the gametophore formation, and this corelation is affected by the concentration of auxines.

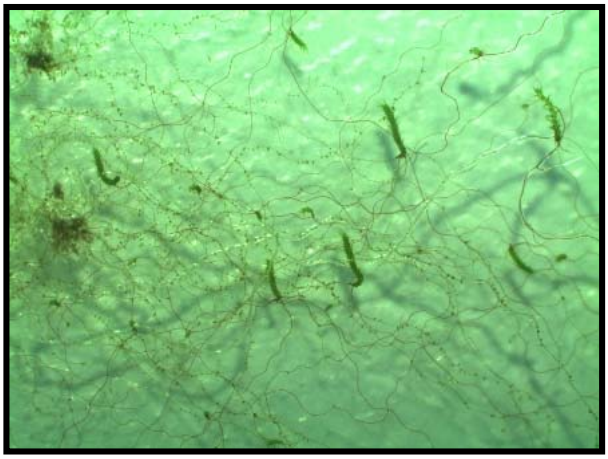

Fig 7: Developed gametophytes in ½ MS medium containing 1 mg/L IAA.

In the present study, the spore germination characteristics and the stages of protonema and gametophore formation of *D. varia* have been investigated under *in vitro* conditions. We hope these data will contibute to our knowledge on moss biology.

4. Acknowledgements

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