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Induction of protonemal gemmae and gametophyte of *Cratoneuron decipien* (Brid.) G. Roth using IAA and kinetin

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Abstract

The protonemal gemmae and gametophyte inducing effect of the IAA and kinetin was examined in moss *Cratoneuron decipiens* growing using solid media. Cultures were raised from gametophyte tips on a solid basal medium containing Knop's major salts and Nitsch and Nitsch's trace elements. Aseptic chopped gametophytes were inoculated onto four types of concentrations (10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M) of IAA and kinetin. High frequency of bud and gemmae were selected then subcultured to same supplemented of IAA and kinetin. Protonema produced gemmae, callus and gametophytic bud. Production of protonemal gemmae varies according to the concentration of IAA and kinetin, whereas low concentrations of growth regulators promoted gemmae formation and bud induction. Gametophytes were regenerated from protonema and calli after subculture. IAA regulated gametophytes induction and growth. Kinetin influenced gemmae formation and gametophyte regeneration also. All aspects of development of this moss species are governed by the external growth regulators.

Keywords: Gemmae, gametophyte, growth regulators, protonema.

Abbreviations: IAA, indole-3-acetic acid; M, mole; NaOCl, sodium hypochlorite; ANOVA, analysis of variance.

Introduction

Mosses are clonal organisms. All mosses are able to spread vegetatively through fragments and propagules. Mosses vegetative reproduction includes fragments, gemmae and vegetative diasporas. Magill (1990) likewise used the concept of Goebel to define gemmae as vegetative diaspores with no apical cell and that always must begin growth with a protonemal phase. These units then include caducous leaves and endogenous gemmae, as well as those specialized, oval, round, or irregularly shaped structures we have always called gemmae in the strictest sense. *In vitro*, *C. decipiens* produces gemmae as well as gametophytic buds on protonema.

Mosses hormones operate very much as they do in tracheophytes (Maravolo, 1980). In bryophytes, auxins are transported directionally, permitting apical dominance to occur, and their activity is concentration dependent. The highest concentrations of auxin occur at the tip and base of the upright gametophore, with distribution throughout the stem, as demonstrated in *Physcomitrella patens* (Bierfreund et al., 2003).

Cytokinins can increase gametophyte production in several mosses (Bopp, 1963). Hahn and bopp (1968) have employed budding as a specific test for exogenous cytokinins. Developments of mosses are influenced not only by the internal environment, but also by the external environment. These responses are typically mediated by the hormones. Known bryophytes hormones regulate growth and gametangial production, protonemal bud formation, branching gemmae formation and senescence. The mode of control of these growth regulators are poorly understood in bryophytes, although in most cases they seem to act similarly to their mode of action in tracheo- phytes. The effect of IAA and kinetin on the gemmae formation and gametophyte induction is reported here.

Materials and methods

Plant materials and culture establishment

Cratoneuron decipien (Brid.) G. Roth was collected from Cheongju, S. Korea. Mature gametophytes were maintained in 50% shady places under greenhouse condition. Mature gametophytes tip were surface sterilized with 1% NaOCl water for 1 minute and washed repeatedly with sterile water. Gametophyte tips were cultured on a solid basal medium containing Knop's (1865) major salts and Nitsch and Nitsch's (1956) trace elements solution with 1% sucrose, 0.8% agar and pH was adjusted to 5.7. The cultures were maintained under an illumination of 2000±200 lx for 14h/d, at 25±1 ºC.

Table 1. Effect of IAA and kinetin on differentiation of *Cratoneuron decipiens* protonema.

	IAA				Kinetin			
	10^{-8}M	**10^{-7}M**	**10^{-6}M**	**10^{-5}M**	**10^{-8}M**	**10^{-7}M**	**10^{-6}M**	**10^{-5}M**
Number of bud/culture vessel (mean±SE)	31.4±1.2	27.4±1.5	17.1±0.5	5.3±0.9	46.7±2.3	33.5±1.8	25.2±1.1	17.3±1.5
Size of bud[z]	+++	++	+	+	+++	+++	++	+
Estimated caulonema	20-30%	20-30%	10-20%	5-10%	30-40%	20-30%	20-30%	10-20%

[z] -, no bud development; +, small buds, most of them few cells; ++, buds with more than 10 cells, +++, large buds, some leaves developed

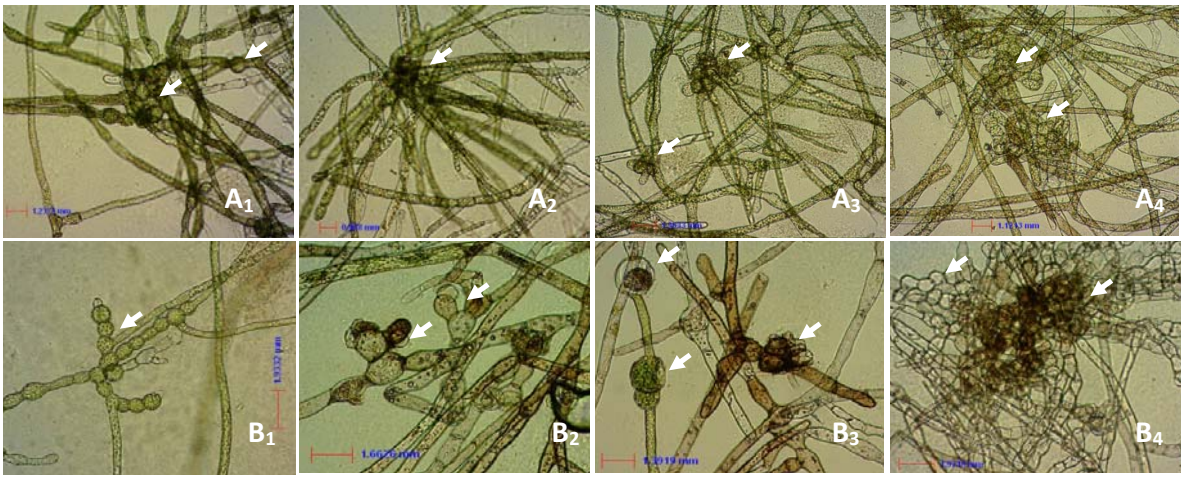

Fig 1. Effect of IAA (A_{1-4}) and Kinetin (B_{1-4}) on gemmae induction from secondary protonema. A_1= 10^{-8} M of IAA, protonemal branches showing round shaped gemmae; A_2= 10^{-7} M of IAA, developing composite gemmae; A_3= 10^{-6} M of IAA, composite gemmae; A_4= 10^{-5} M of IAA, mature, composite gemmae. B_1= 10^{-8} M kinetin and protonema branches with early stage of gemmae; B_2= 10^{-7} M kinetin and formation of composite gemmae; B_3= 10^{-6} M of kinetin and composite gemmae ready for germination; B_4= 10^{-5} M kinetin, composite gemmae germinated to protonema. Photographs were taken from 4 weeks of subcultures.

Gemmae induction

Aseptic chopped gametophyte (1g inoculums per 300ml culture vessel) were inoculated onto four types of concentrations (10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M) of IAA and kinetin under same cultivation conditions. After two weeks, caulonemata were observed using stereomicroscope. Then three weeks of incubation, 1g of protonemata was taken from the surface of solid medium and gemmae and buds were observed. A high frequency of bud and gemmae was chosen for next subculture.

Gametophyte induction

After 30 days of culture, High frequency of bud and gemmae were selected and subcultured to four types of supplemented of IAA and kinetin (10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M) under same cultivation conditions.

Experimental setup and statistical analysis

All experiments were repeated 3 times. Parameters was analyzed by one-way ANOVA with DMRT (Duncan multiple range test) post hoc test. All analysis was carried out using PASW Statistics 17. Photographs of gemmae cells were taken using AM423X-Dino-Eye USB digital eyepiece camera.

Results

Culture establishment

Protonemata were developed from inoculated gametophyte tips. One month of inoculation gametophyte tips produced many erect gametophytes. About 5-10 gametophytes formed around each gametophyte tip. Secondary protonema also developed from inoculated gametophyte tips.

Table 2. Effect of IAA for *in vitro* gametophyte production of *Cratoneuron decipiens* moss.

Con. of IAA (M)	Gametophyte height (cm)	No. of gametophyte/flax	Fresh weight (mg)/flax	Dry weight (mg)/flax
10^{-8}	1.36±0.10 a	388.20±11.12 a	874.56±2.77 a	179.26±0.81 a
10^{-7}	1.00±0.05 b	322.20±6.38 b	770.46±5.35 b	156.38±1.42 b
10^{-6}	0.56±0.05 c	272.80±6.10 c	646.62±10.29 c	126.21±1.68 c
10^{-5}	0.32±0.37 d	194.45±5.87 d	366.22±9.90 d	88.83±2.58 d

Data from 60 days old culture and represents mean with standard errors and data followed by the same letter are not significantly different at 5% level (DMRT test).

Table 3. Effect of Kinetin for *in vitro* gametophyte production of *Cratoneuron decipiens* moss.

Con. of Kinetin (M)	Gametophyte height (cm)	No. of gametophyte/flax	Fresh weight (mg)/flax	Dry weight (mg)/flax
10^{-8}	2.90±0.11 a	225.40±6.81 a	2738.20±31.48 a	358.10±4.19 a
10^{-7}	2.54±0.14 a	125.20±7.59 b	1602.00±59.29 b	219.18±8.33 b
10^{-6}	2.02±0.05 b	99.80±3.18 c	1163.80±6.76 c	215.38±1.14 b
10^{-5}	0.86±0.15 c	77.60±2.89 d	1134.80±7.63 c	176.34±1.60 c

Data from 60 days old culture and represents mean with standard errors and data followed by the same letter are not significantly different at 5% level (DMRT test).

Proliferation of *C. decipiens* was successfully give rise enough, purified and sterile plant materials.

Gemmae induction

Fine chopped gametophytes were tested to produce secondary protonema and then induced callus, gemmae and gametophytic bud. Chopped gametophytes on solid medium were allowed to grow numerous gametophytes through secondary protonema and fragmentation. Table 1 summarizes the effects of exogenous IAA and kinetin on differentiation of protonema. The media containing 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}M IAA induced 31.4±1.2, 27.4±1.5, 17.1±0.5 and 5.3±0.9 buds per culture vessel respectively. Kinetin induced higher number of bud than IAA and it was 46.7±2.3, 33.5±1.8, 25.2±1.1 and 17.3±1.5 buds per culture vessel with the media containing 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}M kinetin respectively. Buds in 10^{-8} and 10^{-7}M were larger than in 10^{-6} and 10^{-5}M for both growth regulators. The percentage of caulonema was reduced with increase of concentration of both hormones.

Protonema differentiated to caulonema and gemmae. After 3 weeks of growth development, the caulonemal branches started with a number of divisions and produced gemmae. These gemmae were intercalary and multicellular (Figure 1). The medium containing 10^{-8}M of IAA and kinetin was produced green, oval, most times intercalary gemmae. The gemmae color was turned brown with increase of strength of the both hormones (Figure 1). Gemmae were mainly restricted to the prostrate filaments at the centre of protonemal patch. Most of the time gemmae still attached to protonema and developed into leafy shoots via germination. Attached gemmae germinating on the same medium (i.e. medium on which they were produced) developed two or three filaments of restricted growth (Figure $1B_3$). This in turn formed gemmae at their tips, thus resulting in composite gemmae (Figure 1 A_3, A_4, B_3 B_4). After subculture of gemmae to higher concentration of growth regulators, produced normal protonema (Figure 1 B_4). The colors of gemmae were light to brown with the increase of growth regulators.

Gametophyte induction

The medium with 10^{-8}M of kinetin was produced highest frequency of buds and gemmae and it was selected for source of gametophyte induction. Biomass (calli, protonema, gemmae and buds) from selected lines were subcultured onto 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}M of IAA and kinetin. Protonema of this species was still green and cullus induction was failed in 10^{-8}M of IAA and kinetin. After subculture callus and gemmae production was still occurred in 10^{-6}M and 10^{-8}M of both growth regulators. Redifferentiation into protonema or many erect gametophytes through protonema was found. The lengths of gametophytes were significantly different among all strength of IAA and kinetin media (Table 2 & 3). Longest gametophyte was detected on 10^{-8}M of IAA and kinetin. The number of gametophyte was significantly different in all types of hormone concentrations. The maximum numbers of gametophyte were produced in 10^{-8}M of both growth regulators. Higher concentration of IAA (10^{-5}M) induced less number of bud and size of gametophyte were minified (Figure 2 A & B). After subculture regeneration of gametophyte were stimulated at low concentration of kinetin and IAA. Fresh and dry

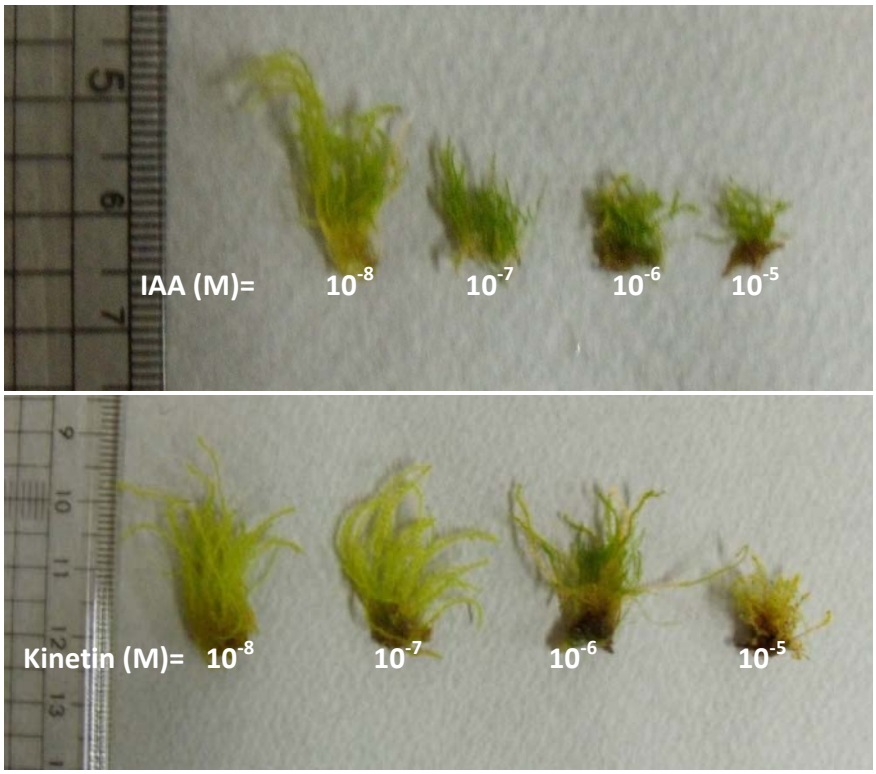

Fig 2. Subcultured calli and protonemal gemmae inducing gametophyte using different concentration of IAA (A) and kinetin (B). Size of gametophyte reduced with the increase of concentration of growth regulators.

weights of biomass were significantly highest in 10^{-8}M of both hormones (Table 2&3).

Discussion

Vegetative reproduction includes fragments, gemmae and vegetative diasporas. Fragmentation was especially common propagation system for *C. decipiens* moss. Propagula differed from gemmae in having an apical cell that can grow directly into a leafy shoot without a protonema. At the early research stage on bryophytes gemmae and gametophyte induction, most of them were induced from spores on culture media containing several organic additions. Plants excreted hormones and affected by external hormones (Beutelmann and Bauer, 1977). Bryophytes were no exception to these external regulators. Both cytokinins and IAA appear to be important in controlling bryophyte growth and propagation (Chopra and Gupta, 1992).

Our results suggested that lower concentrations of both IAA and kinetin stimulated bud induction even size of buds. Cytokinin in the presence of auxin promoted buds (Gorton and Eakin, 1957). Szweykowska (1963) found she could get *Ceratodon purpureus* to initiate buds in the dark by adding kinetin (a cytokinin), but could get no buds even in light without it. The percentage of caulonema was reduced with increase of concentration of IAA and kinetin. At high concentrations inhibit caulonemata (Cove et al., 1978, 1979).

Hormone seemed to play an important role in gemma formation. Stange (1983) suggested that gemmae required auxin transport from the parent plant, based on disruption of gemma differentiation in *Riella helicon-phylla* when treated with an auxin antagonist. However, when ethylene and IAA were applied together, the combination had positive, additive effects on cell elongation (Stange and Osborne, 1988). On the other hand, gemmae generally failed to germinate while still on the parent thallus. After subculture of gemmae to higher concentration of growth regulators, produced normal protonema. In *Hyophila crenulata*, gemmae occurred on the protonema (Olarinmoye, 1981) and *Tetraphis pellucida*, gemmae had a broad range of germination conditions (Forman, 1964).

Gametophyte developments were influenced by lower concentration of IAA and kinetin. IAA regulated growth of gametophyte. IAA seems to be essential for normal stem elongation (Bidwell, 1979). Auxins promoted stem elongation at low concentrations and inhibit it at high ones, presumably due to induction of ethylene (Goodwin and Mercer, 1983), and concentrations that promoted growth in one part of a plant may inhibit it in another. In reviewing the mode of action of auxins in both non-tracheophytes and tracheophytes, Cooke and coworkers (2002) were surprised to find bryophytes exhibited most of the same physiological mechanisms for regulating IAA and for IAA-mediated responses as did the tracheophytes. Kinetin in this species remained low concentrations for gametophyte induction and develop-

ment. Cytokinins in bryophytes remained elusive until very recently because of their low concentrations. Nevertheless, a possible cytokinin known as Factor H, an adenine derivative (Bhatla and Dhingra-Babbar, 1990), has been known for much longer as a stimulant for increasing the number of gametophore buds (Klein, 1967).

In conclusion, using IAA and kinetin, protonemal gemmae and gametophyte production was successful. Also, *in vitro* moss regeneration was established. *C. decipiens* moss used in present study merit further research due to their economically importance. Moreover, *in vitro* moss regeneration system could provide ample, purify and sterile experiment materials for suspension culture and other studies. Furthermore, this study might be useful for tissue culture of other mosses.

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