Studies on Versatile Methods to Control the Development of Shoot and Root Apical Meristems of Bamboo and Some Model Grass Species through Plant Cell Tissue and Organ Culture Techniques

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List of Abbreviations

The following abbreviations have been used throughout the text:

PCTOC	Plant cell tissue and organ culture
SLCE	Small scale liquid culture environment
SD	Standard deviation
BA	6-benzyl adenine.
KIN	6-furfuryl amino purine/ Kinetin/ Furfuryladenine
TDZ	Thidiazuron
NAA	Napthaleneacetic acid
2,4-D	2, 4 dichlorophenoxy acetic acid
PG	Phloroglucinol
COU	Coumarin
MS	Murashige and Skoog (1962) medium
MS0	Growth regulator free MS medium
½ MS	Half strength of MS medium
PGR	Plant growth regulator
UV	Ultraviolet
RGB	Red green blue
HSB	Hue saturation brightness
VB	Vascular bundle
ROI	Region of interest
LED	Light-emitting diode
FN	First node
MN	Middle node

TM	Top meristem
DAC	Days after culture
SAM	Shoot apical meristem
RAM	Root apical meristem

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General Introduction

The term ‘Plant culture’ usually refers to planting in pots, greenhouses, farms and fields. After the concept of totipotentiality of plant cells (Haberlandt 1902) has been arise, Hanning (1904) developed a new method of plant culture called embryo culture, from which he obtained a full growing plant. Since 1920, many types of cultures were developed such as callus culture, organ culture, hypocotyl culture and cell suspension culture etc. After 1945, all these types of culture were grouped under a collective term ‘plant tissue culture’ or ‘*in vitro* culture’ as it was initiated in glass vessels under sterile environment. Since the1950s, *in vitro* culture methods have contributed to the study of plant cell totipotency and enabled the propagation of plant materials for research or commercial purposes (Rosspopoff *et al.* 2017). Plant cell tissue and organ culture (PCTOC) refers the *in vitro* growth on an artificial media under aseptic and controlled environment (Hussain *et al.* 2012). At present, PCTOC protocols such as micro-propagation via organ culture, callus culture, organogenesis, and somatic embryogenesis are used in all aspects of plant biotechnology (Ogita 2015).

Although, there are complicated factors affecting the success of PCTOC protocols, the most accurate way to establish an efficient PCTOC protocol is thought

that 1, to select the best explant, and 2, to set up a suitable environmental condition. In case of micropropagation, for example, terminal bud which has the great capacity to produce the whole plant organs such as node, internode, leaf, root, and lateral bud portions, is an ideal explant. In other word, if we can establish a new method to control the developmental processes of shoot apical meristem (SAM) and root apical meristem (RAM) in *in vitro*, it provides a versatile method of PCTOC techniques.

The present study focused on PCTOC of bamboo and some practical model grass species, such as rice, barley and *Brachypodium*, since these plants are well-known for their great economic, social, cultural, and scientific values (Buckingham *et al.* 2014, Ye *et al.* 2017, Brkljacic *et al.* 2011). These plants belong to the family Poaceae, and with 11,500 species, the grass family Poaceae is economically, ecologically, and evolutionarily one of the most successful species-rich groups (Hodkinson 2018). For example, bamboo used as food, construction materials, handicrafts, paper pulping, and as efficient agents for conservation of water and soil (Ye *et al.* 2017). Throughout the East and Southeast Asia, especially in China, Japan, Korea, bamboos are one of the material for making chopsticks. It considered as a prime renewable resource of biomass production and this quality showed their importance in bio refinery production (Truong

and Le 2014). Rice and Barley also considered as one of the staple foods in the world and also considered as important energy crops, thus have importance in biorefinery production (Sharma *et al*. 2018, Truong and Le 2014). 60% staple foods in the world under Poacease family (Hodkinson 2018) and being our staple food family, researchers are trying to develop and modify their quality to ensure the sustainability and food security to the world. Rice, barley and *Brachypodium* are one of the important practical model grass species, because of that, these species has been used in genetic breeding and biotechnology research for genetic improvement of food and energy crops (Xie and Peng 2011, Holubova *et al.* 2018, Harwood 2012, Li *et al*. 2014).

Grass species have jointed stem called 'culm' and each culm segment begins and ends with a solid joint called 'node'. As node portion contains 3 unique meristems such as intercalary meristem, SAM, and RAM, it is recognized as a meristem abundant area. This morphological uniqueness will be a great advantage for my research objectives. Here, I listed main objectives of the thesis research which will be presented as chapters.

i. To check effects of solid and liquid media on the growth of bamboo node cultures.

ii. To establish a small-scale liquid culture environment (SLCE) for controlling the development of SAM and RAM.

iii. To select the best node portion from a shoot by using anatomy, fluorescent microscopy, histochemical observation, digital imaging analysis and also checking *in vitro* growth performances.

iv. To apply phenolic compounds for further promotion of SAM and RAM development in SLCE system.

Sood *et al.* (2002) tested the effects of agar-solidified medium and liquid medium on shoot multiplication, whereby root formation of *Dendrocalamus hamiltonii* was investigated; it was found that a liquid culture condition is more suitable for the tissue culture of this bamboo species. The basic protocol for setting a suitable culture environment, I used *in vitro* node culture stocks of two major bamboo species, both *Phyllostachys meyeri* McClure (Pm) and *Bambusa multiplex* Raeush (Bm) according to the liquid culture method as previously reported (Ogita *et al.* 2008). In Chapter I, using these node culture systems, I checked the effects of solid and liquid media on growth of bamboo shoots. Based on the result of Chapter I, I have selected liquid MS (Murashige

and Skoog 1962) medium, which is the base medium condition for my further experiments. In Chapter II, I focused on using a 6-well microplate that contains 2 mL per well of a liquid medium, which provides a SLCE for optimizing the culture protocol of the bamboo within a short period. Three types of node portions—the first node (the base node near a rhizome tissue), middle nodes (upper nodes near the 1st node), and the top meristem—were independently cultured in the SLCE to select the explant which showed the best growth performance. By culturing the first node in the SLCE system, I performed a quick survey for detection of *in vitro* SAM and RAM development. Another main topic of Chapter II is the autofluorescence measurement technique. Autofluorescence (primary fluorescence) is the fluorescence of naturally occurring substances, such as chlorophyll, collagen, and fluorite. Most plant and animal tissues show some autofluorescence when excited with ultraviolet light (e.g., light with a wavelength of approximately 365 nm) (Rost 1999). In the previous studies, we focused on evaluating the autofluorescence intensity of target plant cells, which reflects the histochemical features of the plant cell wall (Ogita *et al.* 2011), and accumulation of specific secondary metabolites (Ogita *et al.* 2015). The technique was also used digital imaging analysis to measure the growth features in a protoplast co-culture assay by

Ogita and Sasamoto (2017). Currently, in the field of life sciences, the concept and application of autofluorescence measurement have developed with the requirement of non-destructive detection of a target cell and tissue (Croce and Bottiroli 2014, Zheng *et al.* 2016, Wena *et al.* 2019). I specified the color variation of explants as the relative fluorescent intensity by assessing the autofluorescence properties with digital imaging analysis using ImageJ software. Some staining techniques such as Wiesner reaction, safranin staining, DAPI staining were also applied to identify histochemical characteristics of bamboo nodes. In Chapter III, I checked the effects of two phenolic compounds, both phloroglucinol (PG) and coumarin (COU), on controlling the SAM and RAM development in Bm and Pm bamboo plants and other model grass species, i.e. rice, barley and *Brachypodium*. Several utilities and possibilities of the SLCE system were discussed in the present research.

Chapter I

Effects of solid and liquid media on growth of shoots in bamboo node culture system

1.1. Introduction

Solid medium has been generally recommended for PCTOC. On the other hand, several advantages of liquid medium condition have also been pointed out (Saxena 1990, Kaladhar 2017, Roy *et al.* 2014). Actually, liquid medium condition is sometimes preferred for tissue culture (Ogita *et al.* 2008, Shirin and Rana 2007, Das and Pal 2005, Sood *et al.* 2002). A tissue culture protocol of bamboo (*Phyllostachys meyeri* McClure, Pm) was successfully achieved by Ogita *et al.* (2008) specifically regarding *in vitro* germination of the caryopses and plant regeneration from nodal segments of both the germinated seedlings and tissue-cultured clone plants. *In vitro* node culture stocks of (*Bambusa multiplex* Raeush, Bm) have also been established using the method for Pm, as previously reported. Hence, several clonal small plantlets of two major bamboo species that have a high ability to form multiple shoots were obtained in a regular period (ca. every 1–2 months). Throughout regular maintenance cultures of bamboo nodes, I

found a wide range of variation on shoot growth in solid and liquid MS medium. In the present research, using our node culture system, I checked the effects of solid and liquid media on the growth of bamboo node cultures of Pm and Bm.

1.2. Materials and methods

1.2.1. Plant material

Two major bamboo species both Mohaichiku (*Phyllostachys meyeri* McClure) which is monopodial and temperate (10˚C - 30˚C) type having chromosome number 2n = 48 and Horaichiku (*Bambusa multiplex* Raeush) which is sympodial and tropical (20˚C - 40˚C) type having chromosome number 2n = 72, were used in this experiment. *In vitro* culture stocks of these bamboos were maintained at Plan Cell Manipulation Laboratory, Prefectural University of Hiroshima, Shobara, Japan (**Figure 1.1, A- B**). Briefly, half strength MS (Murashige and Skoog) (Murashige and Skoog 1962) liquid medium containing 30 g/L sucrose was prepared as a standard medium unless otherwise specified. The pH of the medium was adjusted to 5.7 before autoclaving. All the cultures were maintained at 25°C, with a 16-h photoperiod under a fluorescent illumination (65 μmol m^{-2} s^{-1}).

1.2.2. Culture environment setting

In this experiment, growth performance of node tissues on solid and liquid medium conditions were compared. As shown in **Figure 1.1**, a square plastic culture box containing 50 mL of half strength MS medium was prepared. As a solidifying agent, 3 g/L gellan gum (Wako Pure Chemicals Industries Ltds) was added when it needed. 1.5 -2.0 cm of nodes were excised from *in vitro* shoots of *P. meyeri* and *B. multiplex* and transferred in the culture boxes and incubated at 25°C, with a 16 h photoperiod under a fluorescent illumination (65 μmol m^{-2} s^{-1}).

1.2.3. Collection of data and analysis

Growth performance of all cultures were observed according to the following indexes, i.e. plant height (cm), number of shoots/explant, number of roots/explant, root length (cm) and weight increasement. Student's t-test has been performed to identify the significant difference between solid and liquid condition.

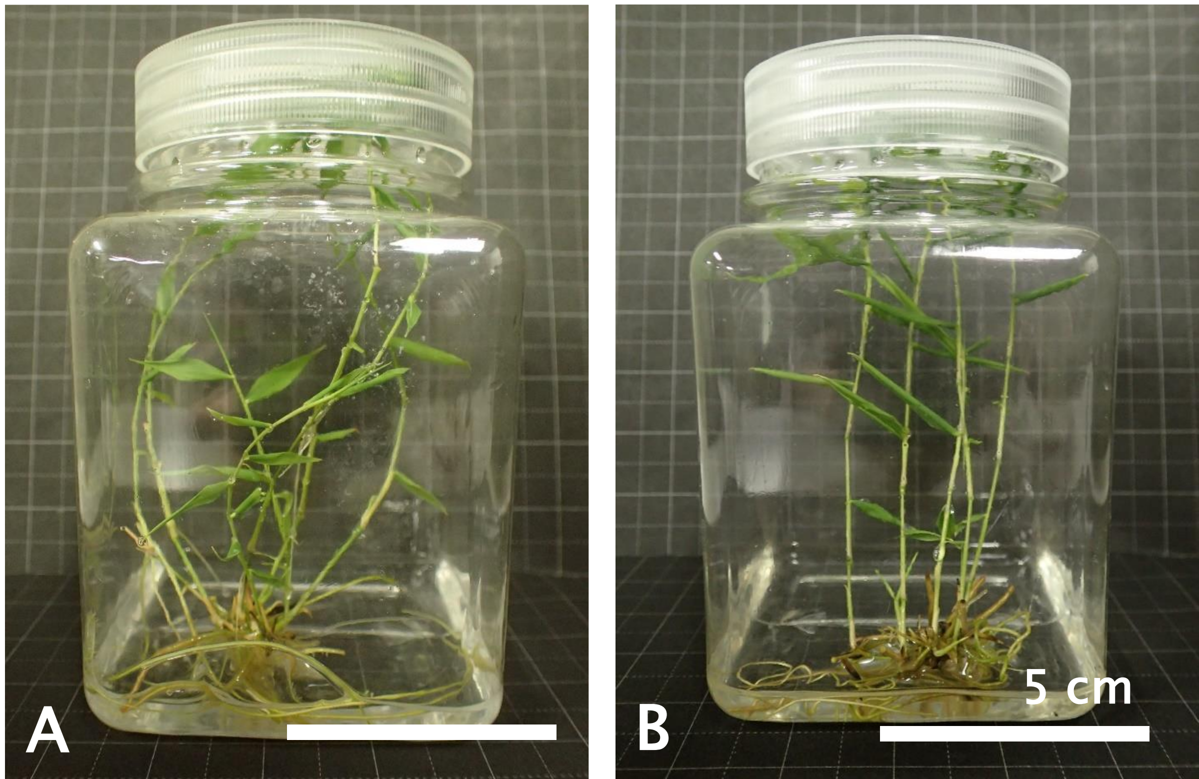


Figure 1.1. Typical *in vitro* culture stocks of Bamboo. Sympodial type, *B. multiplex* (A) and monopodial type, *P. meyeri* (B). Scale bar = 5 cm.

1.3. Results

Bamboo nodes showed wide range of variation while cultured on solid and liquid medium (**Table 1.1, Figure 1.2**). Growth performance of cultured nodes were summarized as shown in **Table1.1**. Among the five growth parameters, three important growth indexes were presented as graphical representation for easy comparison for both species (**Figure 1.3 and 1.4**). In all characters, liquid medium showed better growth for bamboo node but in case of *P. meyeri,* weight increasement as statistically significant difference and in case of *B. multiplex* all characters showed statistically significant difference in solid and liquid medium (**Table 1.1, Figure 1.3, 1.4**).

Table 1.1. Effect of solid and liquid medium conditions on growth performance of bamboo node cultures.

Species / medium condition	Growth performance				
	Plant height (cm)	No of shoots/ explant	No. of roots/ Explant	Length of root (cm)	Weight incensement (mg)
Bm / Solid	4.22 ± 0.95	1.2 ± 0.12	1.4 ± 0.47	5.84 ± 1.11	96 ± 0.03
Bm / Liquid	9.9 ± 1.16	1.2 ± 0.15	4.6 ± 0.59	8.26 ± 1.39	338 ± 0.04
p-value	0.00139*	0.5	0.011866*	0.165642	0.003522*
Pm / Solid	4.95 ± 1.17	1 ± 0.21	2.1 ± 0.45	2.1 ± 1.19	44 ± 0.42
Pm / Liquid	7.6 ± 3.00	1.6 ± 0.40	2.4 ± 0.75	8 ± 1.27	191 ± 0.38
p-value	0.216171	0.086002	0.85559	0.044966*	0.011815*

Growth performance was summarized after 30 days of culture.

Data represent the average values ± SD from 5 explants.

Data for each column were analyzed by Student-t test. * $P < 0.05$

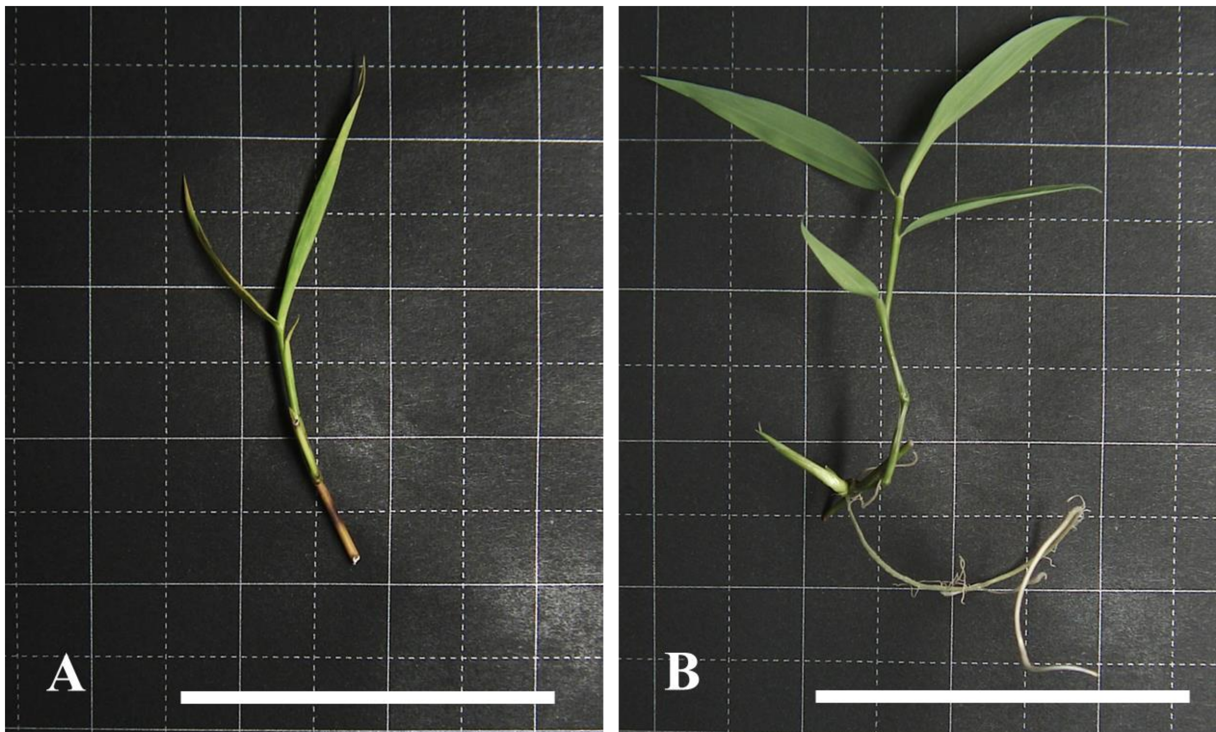


Figure 1.2. Effects of solid (A) and liquid (B) medium on growth of *P. meyeri* bamboo nodes. Scale bar = 5 cm.

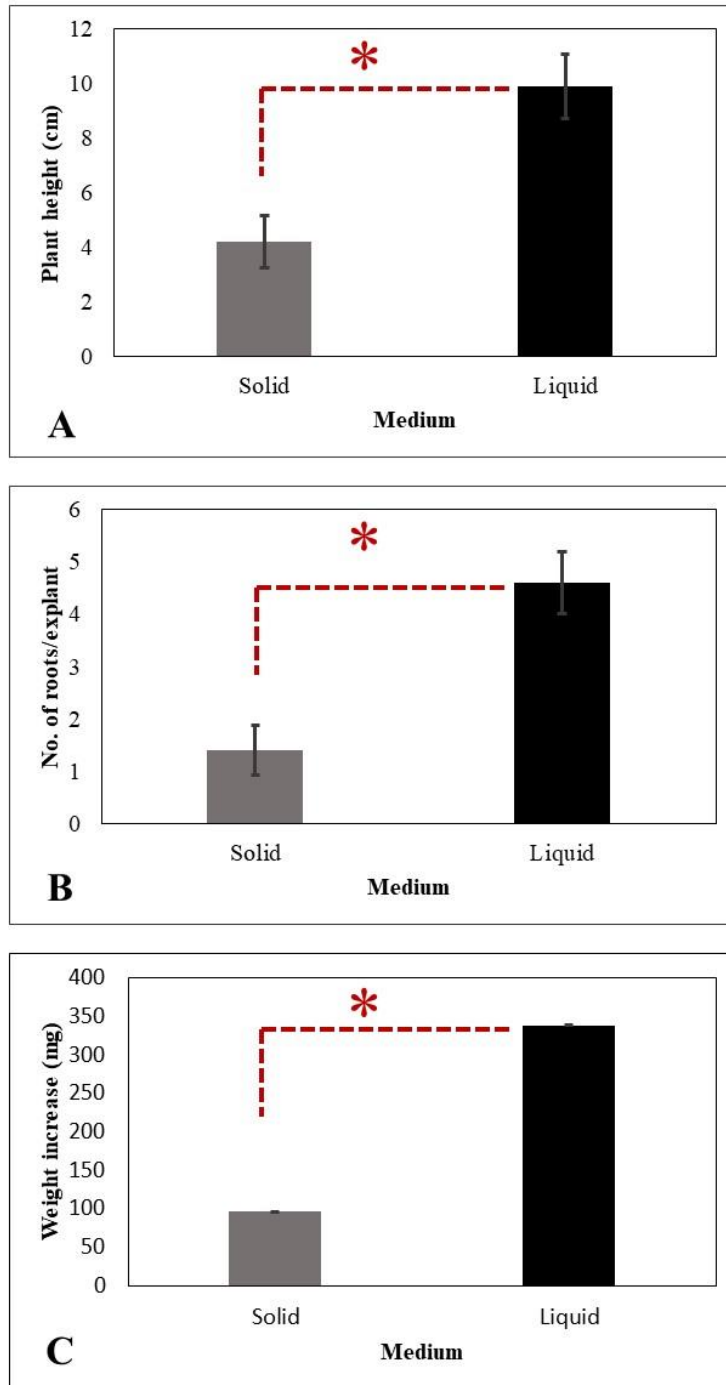


Figure 1.3. Graphical representation of effects of solid and liquid medium on growth parameters of *B. multiplex*. A. Plant height, B. No. of roots/plant and C. Weight increasement. (* $P<0.05$).

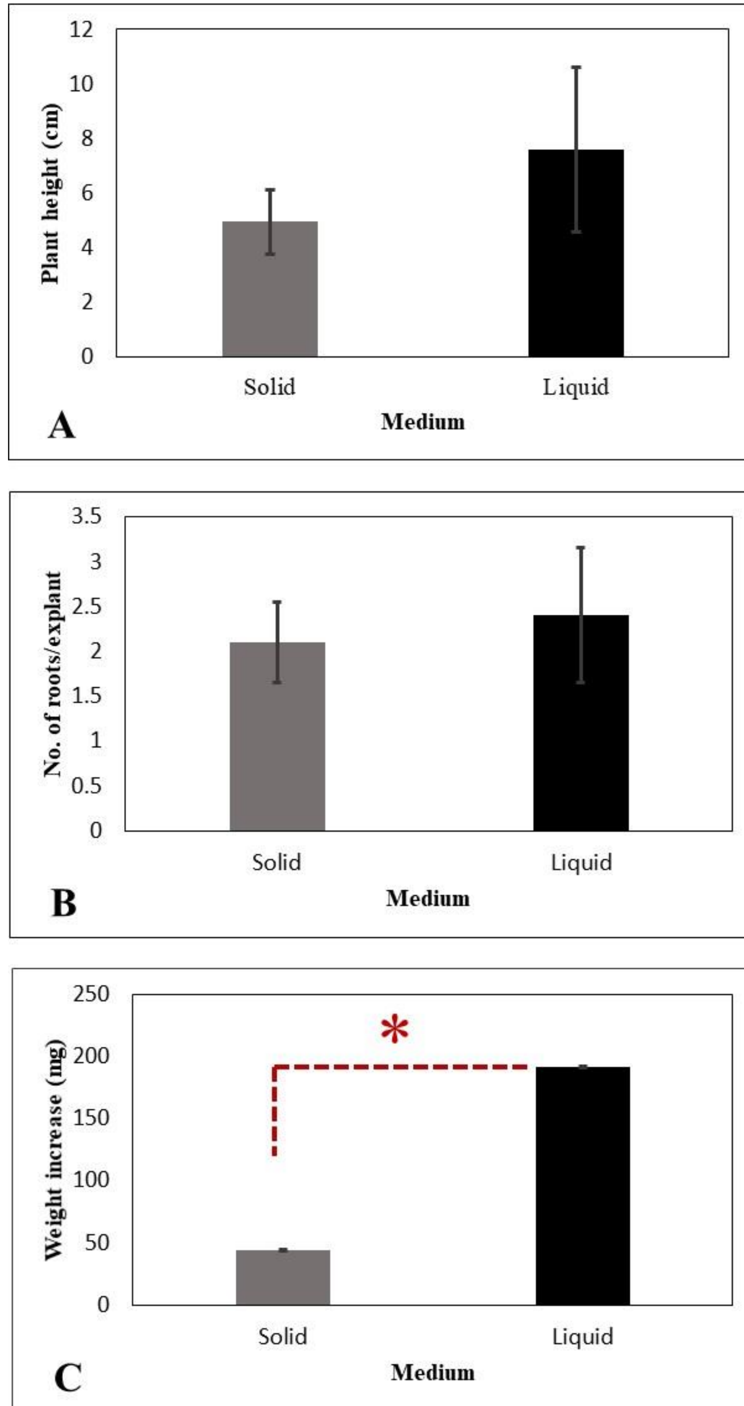


Figure 1.4. Graphical representation of effects of solid and liquid medium on growth parameters of *P. meyeri*. A. Plant height, B. No. of roots/plant and C. Weight increasement. (* P<0.05).

1.4. Discussions

Suitability of liquid medium condition for *in vitro* bamboo tissue culture had been mentioned especially in three major bamboo genera (*Bambusa*, *Dendrocalamus*, *Phyllostachys*) (Sood *et al.* 2002, Ogita *et al.* 2008, Das and Pal 2005, Negi and Saxena 2011). In this present research, my focus was to compare some selected important growth parameters in solid and liquid MS medium. Because, a key subject of this study is culture environment setting. During preliminary test experiments, I have noticed a wide range of variation in growth characters of bamboo while culturing in to solid and liquid MS media.

In general, agar or gellan gum solidified medium is used for tissue culture of plants, however, several workers have reported higher rates of shoot multiplication and improved growth in liquid medium in comparison to semi-solid medium (Sood *et al.* 2002, Das and Pal 2005, Shirin and Rana 2007, Ogita *et al.* 2008). Sood *et al.* (2002) tested the effect of agar-solidified medium and liquid medium on shoot multiplication and root formation was investigated in *Dendrocalamus hamiltonii* and suggested that a liquid culture condition is more suitable for tissue culture of this bamboo species. Negi and Saxena (2011) used MS liquid medium for *in vitro* micropropagation of himalayan

weeping bamboo, *Bambusa balcooa* Roxb. By using my node culture system, I had checked the effects of solid and liquid medium on growth of bamboo node cultures of Bm and Pm and found that better growth performance could be seen in liquid medium condition (**Table 1.1**). This protocol was highly applicable to other bamboo species, such as *B. glaucescens*/Bg, *B. oldhamii*/Bo, *Pleioblastus simonii/*Ps, *Sasa Kurilensis*/Sk, related model grass species (rice, barley and *Brachypodium distachyon*), and some dicot plants (strawberry, dokudami) (data not shown).

Based on the result in Chapter I, I have selected liquid MS medium as the base medium condition for my further experiments.

Chapter II

Establishment of small-scale liquid culture environment to investigate morphological and histochemical responses of *in vitro* shoot apical meristem and root apical meristem of bamboo

2.1. Introduction

The concept for Plan-Do-Check-Act (PDCA) cycle in plant tissue culture (Ogita 2015) is very important idea for this study. Generally, researchers should logically consider the following requirements, such as explant selection, medium preparation, and culture environment setting in the P and D cycles. Based on a careful monitoring of size, shape and color variations in the target cells and tissues by macro- and microscopic observations, I can recognize the specific pattern of growth promotion and/or inhibition during the culture of the target explant. These inputs are thought highly useful and interesting for optimization of a protocol especially in the C and A cycles.

There are several reports related to bamboo tissue cultures using nodal segment as explant (Mudoi *et al.* 2013). However, no study describes the maturity of nodal segment and criteria for selection of explant and apical meristem abundancy. The node

portions of these culture stocks with apical and intercalary meristems were considered as excellent models for the morphological and histochemical response control of the shoot apical meristem (SAM) and root apical meristem (RAM) of bamboo. General tissue culture techniques for *in vitro* micropropagation of bamboos through enhanced axillary branching using node explants have been well reviewed, e.g. by Singh *et al.* (2013). Factors affecting success in micropropagation of bamboos such as medium, plant growth regulators, medium pH, carbon source, propagule size, and culture duration were summarized in this report. However, there is no detailed description of a versatile methodology to regulate *in vitro* growth of bamboo nodes.

Since the obtained shoots of a 1-month-old culture box showed growth variation, it had been distinguished shoot types based on plant height, i.e., short (less than 5 cm), medium (ca. 5–10 cm), and tall (more than 10 cm) and used for further experiments with 2 concepts. First, based on the preliminary result in Chapter I, I used a 6-well microplate containing 2 mL of a liquid medium per well which provide a SLCE for optimization of culture protocol of bamboo within a short period. This culture system will be a unique tool for careful monitoring of the SAM and RAM during a culture period. Second concept of this study is autofluorescence measurement technique. In the present studies,

I specified color variation of explants as the relative fluorescent intensity by checking the autofluorescence property of whole shoots under a LED 365 nm illumination with RGB (red, green, and blue) digital imaging analysis using ImageJ software. The detailed autofluorescence properties of the outward of culms and nodes tissues also identified using an inverted fluorescent microscope under B- and U-excitation lights with RGB and HSB (hue, saturation, and brightness) digital imaging analysis. The aim of Chapter II is to review a simple but versatile node culture system in a liquid culture environment to better understand unique biological properties of its vegetative growth and to, thus, reveal the relation between color variation in the outward regions of culm and node tissues and their suitability as explants.

2.2. Materials and methods

2.2.1. Plant material

Nodes of 11 different bamboo species that belong to seven major bamboo genera were cultivated using planter boxes in a greenhouse: *Bambusa*—*B. multiplex* Raeush (Bm), *B. glaucescens* f. Horaikomachi (Bg), *B. oldhamii* Munro (Bo); *Dendrocalamus*—

D. giganteus Munro (Dg); *Phyllostachys*—*P. bambusoides* Sied. Et Zucc (Pb), *P. nigra* Munro var. Henonis (Pn), *P. pubescens* Mazel ex J. Houz (Pp); *Tetragonocalamus*—*T. angulatus* (Munro) Nakai (Ta); *Chimonobambusa*—*C. marmorea* (Mitford) Makino (Cm); *Pleioblastus*—*P. simonii* (Carriere) Nakai (Ps); and *Sasa*—*S. kurilensis* Makino et Shibata (Sk). Fresh nodes were collected from the branches of these bamboo plants and cultured *in vitro* according to a method previously reported (Ogita *et al.* 2008). *In vitro* node culture stocks of Bm and Pm were also used to understand the sequential developmental processes of SAM and RAM of node explants in the following experiments. Briefly, half strength MS (Murashige and Skoog 1962) liquid medium containing 30 g/L sucrose was prepared as a standard medium unless otherwise specified. The pH of the medium was adjusted to 5.7 before autoclaving. All the cultures were maintained at 25°C, with a 16 h photoperiod under a fluorescent illumination (65 μmol m^{-2} s^{-1}).

2.2.2. Node culture protocol and its applications

I demonstrated a versatile node culture protocol using 2 mL of a liquid half

strength MS medium per well of a 6-well microplate which provided a small-scale liquid culture environment (SLCE), see **Figure 2.1**. One node segment (1.5 -2.0 cm in length) of each bamboo plants was put in a well of the SLCE. Mature dormant nodes from greenhouse grown bamboo branches and the first node (the base node next to a rhizome tissue) from the *in vitro* node cultures of Pm and Bm were used in the experiments unless otherwise specified. Three types of node portion, the first node, middle nodes (upper nodes next to the 1st node) and the top meristem were independently cultured in the SLCE to define a bent for explants. Effects of plant growth regulators, such as benzyl adenine (BA) and thidiazuron (TDZ), on induction of *in vitro* SAM development, and effects of 2, 4-dichlorophenoxy acetic acid (2,4-D) on the promotion of *in vitro* RAM development were also investigated after a short period in the SLCE.

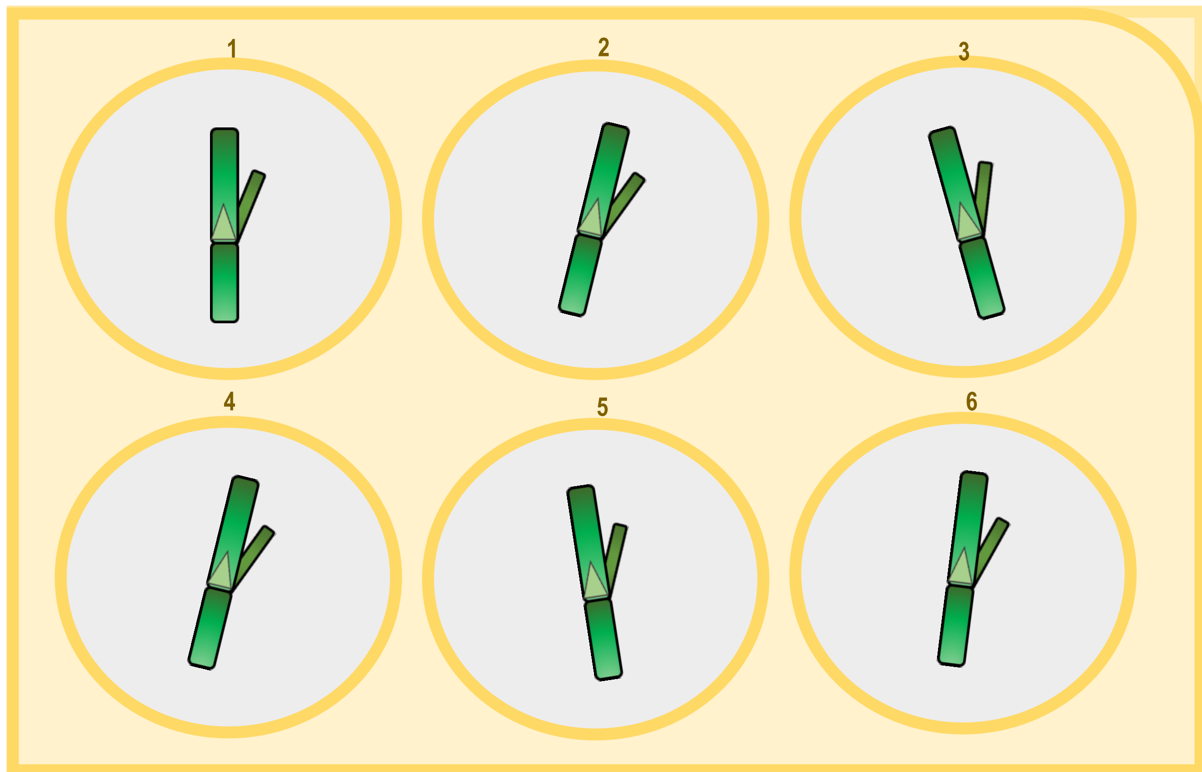


Figure 2.1. The image of node culture via small-scale liquid culture environment (SLCE) in a 6-well culture plate.

2.2.3. Wiesner staining and imaging analysis

In order to know the maturity of culms of *in vitro* shoots, lignin deposition around vascular bundles was monitored. Terminal portion of an internode is the matured part of the tissue. So, below internode portion of each nodes has been sectioned transversely (**Figure 2.2**) and stained with Wiesner's reagent (Phloroglucinol 0.5g,

100% EtOH 25ml and HCl 12.5 mL) and after 5 minutes observed the reaction as shown in **Figure 2.3**. Finally measure the red color stain in internode portion from digital image analysis through ImageJ software.

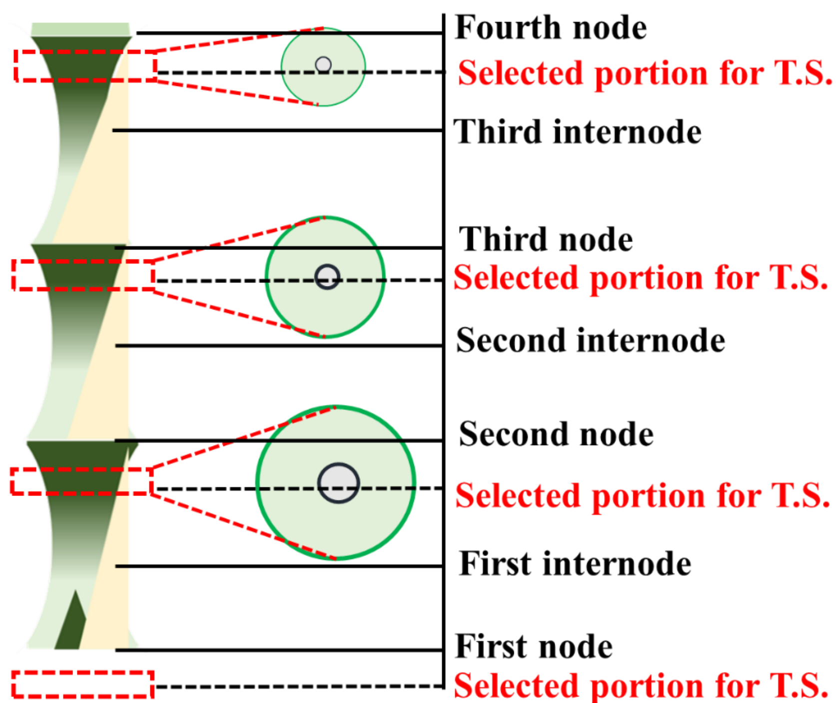


Figure 2.2. Selected portions in internode for transverse sections (T. S.) for Wiesner staining.

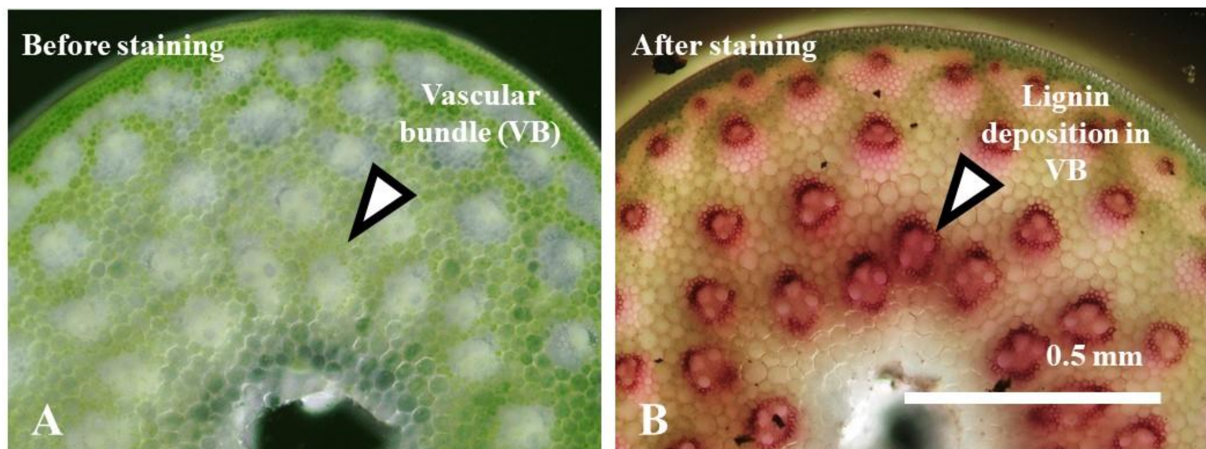


Figure 2.3. Reaction of Wiesner staining in internode of bamboo.

2.2.4. Observations and digital imaging analysis

Dormant and active lateral buds of 11 bamboo species were sectioned in longitudinally and stained with Sytox green (SG) for monitoring mitotic activity and counter stained with Safranin (SF) for detection of the inward of SAM region according to the method (Ogita *et al*. 2012) with a minor modification.

In order to evaluate growth performance of explants, whole images of *in vitro* cultured shoots were captured with a digital camera system (UV CUBE, LC science Co., Ltd.) under a bright-field and LED 365 nm illumination without staining. Autofluorescence property of the obtained images were evaluated by the RGB (red, green, and blue) digital imaging analysis using ImageJ software. A stereo microscope

(SZ40, Olympus) was also used to monitor the morphological characteristics of the target tissue. The detailed autofluorescence properties of the outward of culms and nodes tissues were observed using an inverted cell culture microscope (CKX53, Olympus) under B- and U-excitation lights (B; Band pass (BP) filter, 460–495; Barrier (BA) filter, 510IF; Dichroic mirror (DM), 505, U-FUW; BP filter, 340–390; BA filter, 420IF; DM, 410) with RGB and HSB (hue, saturation, and brightness) digital imaging analysis. HSB color space in ImageJ software is thought the same as HSV (hue, saturation, and value) color space.

Lignified tissues detected by the Wiesner reaction were also observed under an inverted microscope and the obtained images were analyzed by ImageJ software as described above.

2.3. Results

2.3.1. Histochemical analysis of mitotic activity in node portions of 11 bamboo species

As shown in **Figure 2.4 and 2.5**, the dormant lateral buds of all bamboo nodes resumed expanding and elongating within 7 days in the SLCE. The mitotic activity was calculated by a digital imaging analysis and found that it rose up to 1.2- to 3.8-fold in SG/SF ratio in 7-day-culture (**Table 2.1**).

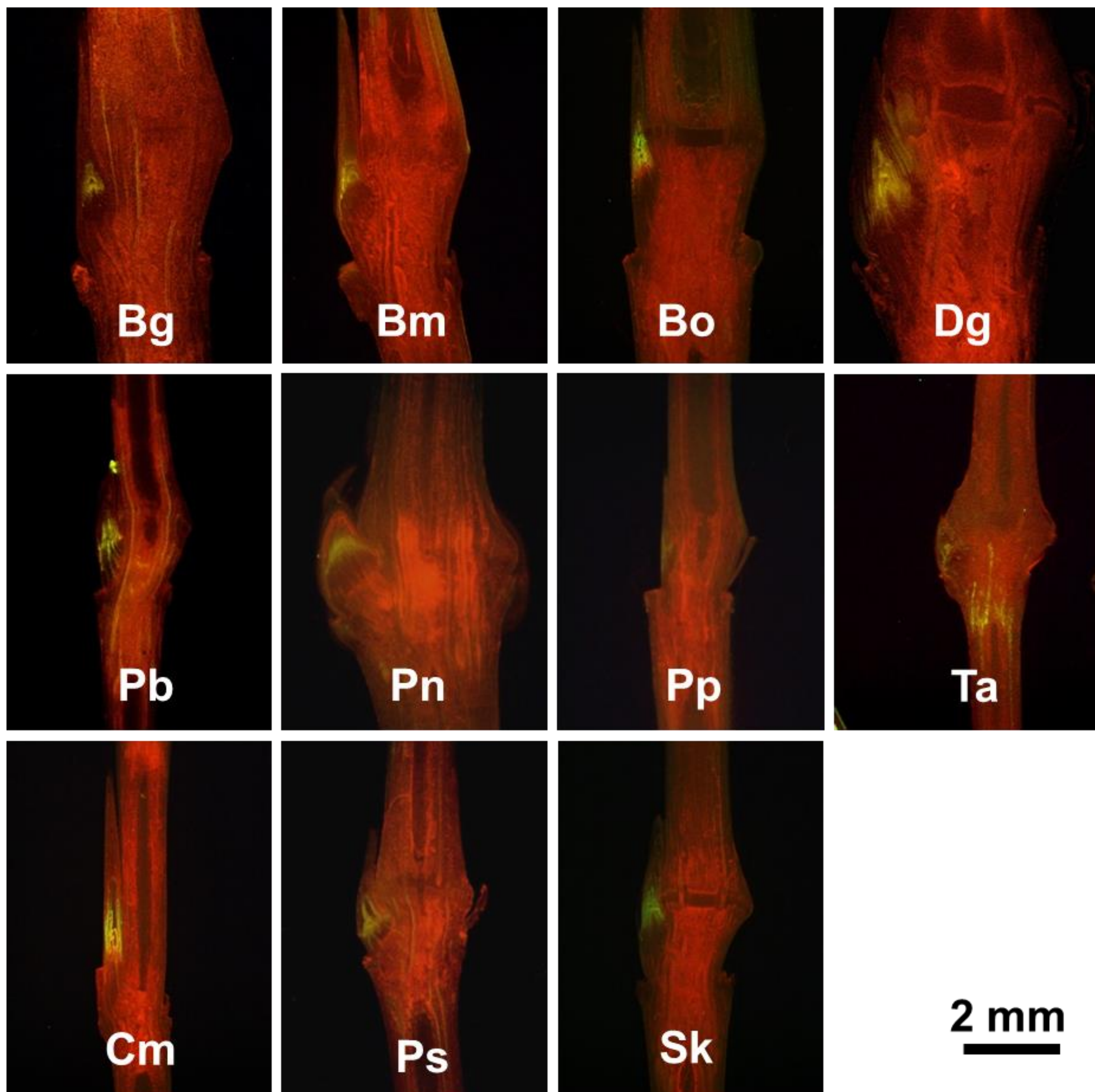


Figure 2.4. Histochemical analysis of mitotic activity in node portions of 11 bamboo species, dormant stage (0 day). Longitudinal sections of the lateral buds were stained with Sytox green (SG, yellow) and counter stained with Safranin (SF, red). Relative activity was calculated as the ratio of stained area (SG/SF) in **Table 2.1**.

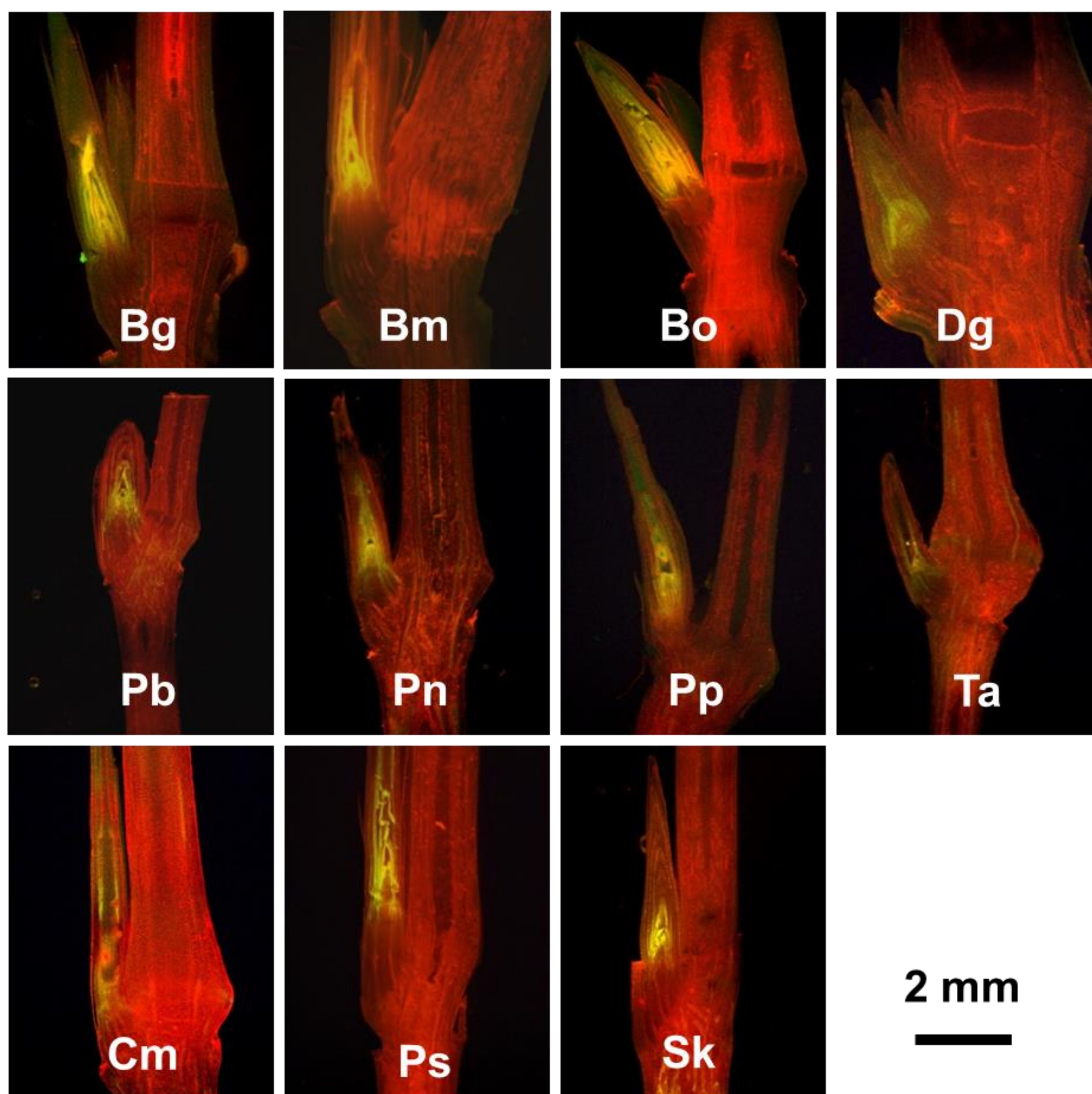


Figure 2.5. Histochemical analysis of mitotic activity in node portions of 11 bamboo species, active stage (7 days). Longitudinal sections of the lateral buds were stained with Sytox green (SG, yellow) and counter stained with Safranin (SF, red). Relative activity was calculated as the ratio of stained area (SG/SF) in **Table 2.1**.

Table 2.1. Relative area of Sytox green/Safranin stained portions of different bamboo nodes measurement by Image J Software.

Bamboo species	Relative area SG/Saf*		
	0 day	7 day	Fold
Bg	0.069	0.084	1.22
Bm	0.058	0.083	1.45
Bo	0.079	0.133	1.68
Dg	0.040	0.062	1.57
Pb	0.037	0.069	1.86
Pn	0.036	0.059	1.63
Pp	0.050	0.109	2.17
Ta	0.007	0.025	3.82
Cm	0.040	0.064	1.59
Ps	0.068	0.098	1.44
Sk	0.032	0.056	1.75

*Relative activity was calculated as the ratio of stained area (SG/Saf) from **Figure 2.4 and 2.5** by a digital imaging analysis.

2.3.2. Performances of *in vitro* cultured nodes in the SLCE

In order to review a simple but versatile node culture system in a liquid culture environment to better understand unique biological properties of its vegetative growth and to, thus, reveal the relation between color variation in the outward regions of culm and node tissues and their suitability as explants, I performed following experiments.

2.3.2.1. Morphological features of *in vitro* bamboo shoots

I have systematically maintained *in vitro* node culture stocks of Pm and Bm, as shown in **Figure 1.1.,** according to the method previously reported (Ogita *et al.* 2008). Since a gradual white to green tinge shoots were observed, I investigated the relation between color variation in the outward of culms and nodes tissues and their suitability as explants (**Figure 2.6**). Color variation from pearl to light green under a white light could be seen, especially depending on the position of internode in young shoot of Pn. On the other hand, there were no distinctive visible character to outward of *in vitro* shoot of Pm and Bm under a white light. By checking the autofluorescence property of whole shoots under a LED 365 nm illumination with RGB digital imaging analysis using ImageJ software, I could specify color variation of explants as the relative intensity of

blue value. Positional difference of relative autofluorescence intensity was identified to refer a color scale.

2.3.2.2. Categorization of different type of nodes in *in vitro* bamboo shoots

As shown in **Figure 2.6 and 2.7**, it could distinguish types of shoots according to the plant height, i.e. short (less than 5 cm), medium (ca. 5-10 cm), and tall (more than 10 cm) from a culture box of *in vitro* node culture stock. The number of nodes per shoot varied depending on the size of shoots. Tall shoots having ca. 5 nodes in average will be suitable for a source of explants.

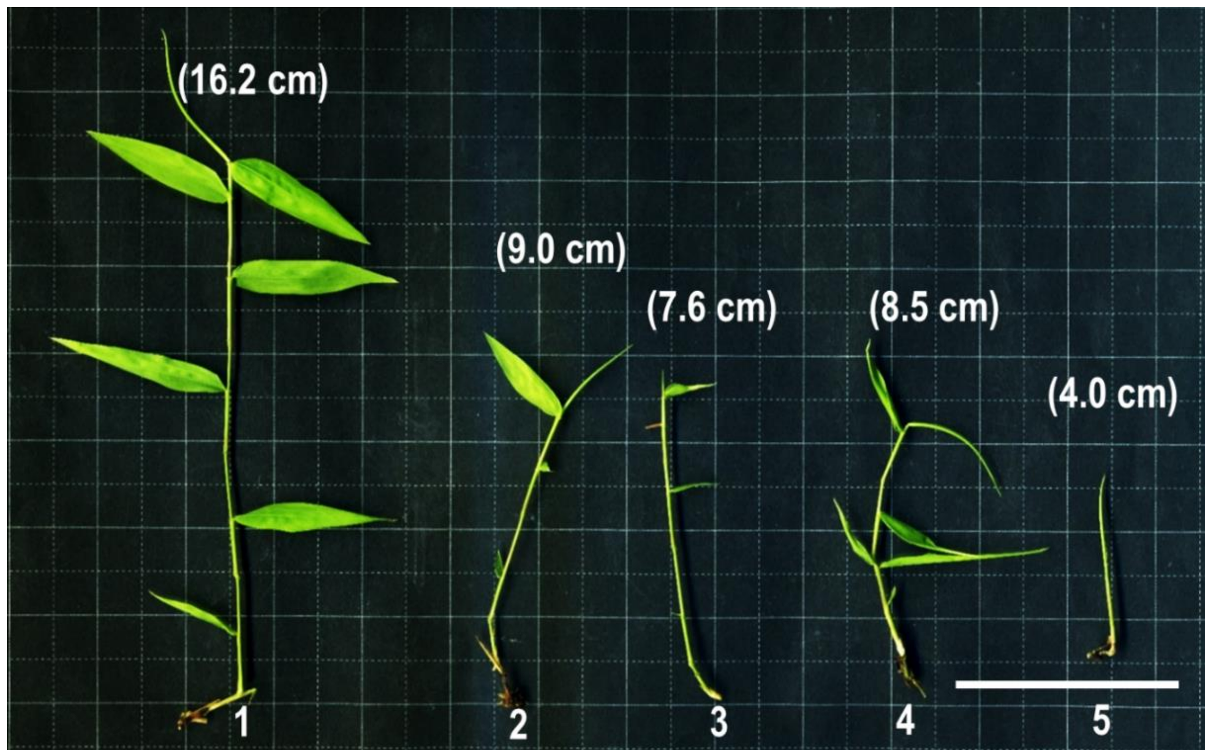
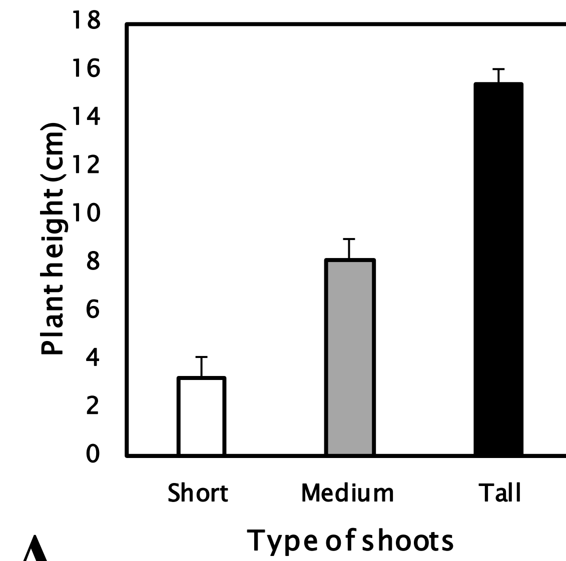


Figure 2.6. Different type of Pm shoots obtained from *in vitro* culture stock. Scale bar = 5 cm.



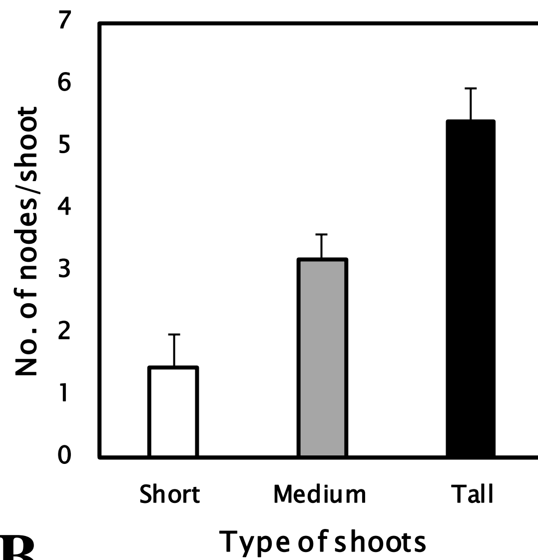


Figure 2.7. Categorization of types of *in vitro* Pm shoots according to A; plant height, and B; number of nodes per shoot. Values was summarized after 30 days of culture. Data represent the average values ± SD from 5 independent stock cultures.

2.3.2.3. Autofluorescence properties of the outward of culms and nodes tissues

Systematically maintained *in vitro* node culture stocks of Bm and Pm, as shown in **Figure 1.1,** were used in this experiment. Since gradual white-to-green tinge shoots were observed, investigation on the relation between color variation in the outward regions of culm and node tissues and their suitability as explants (**Figure 2.8**). Color variation from pearl to light green under a white light could be observed, which is especially dependent on the position of the internode in the young shoot of Pn. On the other hand, there were no distinctive visible characteristics of the outward regions of the *in vitro* shoot of Pm and Bm under a white light. By checking the autofluorescence property of whole shoots under LED 365 nm illumination with RGB digital imaging analysis using ImageJ software, the color variation of explants as the relative intensity of the blue value was specified. To refer a color scale, positional differences of the relative autofluorescence intensity were also identified.

As it already recognized that there is the relation between color variation in the outward of culms and nodes tissues and their suitability for explants (**Figure 2.8**), I tried to identify the detailed autofluorescence properties of the target tissue using an inverted fluorescent microscope under B- and U-excitation lights with RGB and HSB digital

imaging analysis. As shown in **Figure 2.9 and 2.10**, the mature node region, especially near the first node, showed various strong fluorescence intensities. When B-excitation light was adapted, the images from the R and G modes of the RGB analysis (**Figure 2.9**) and the hue and brightness modes of the HSB analysis (**Figure 2.10**) were estimable. When U-excitation light was used, the images from the G and B modes of the RGB analysis (**Figure 2.9**) and the brightness mode of the HSB analysis (**Figure 2.10**) were visible.

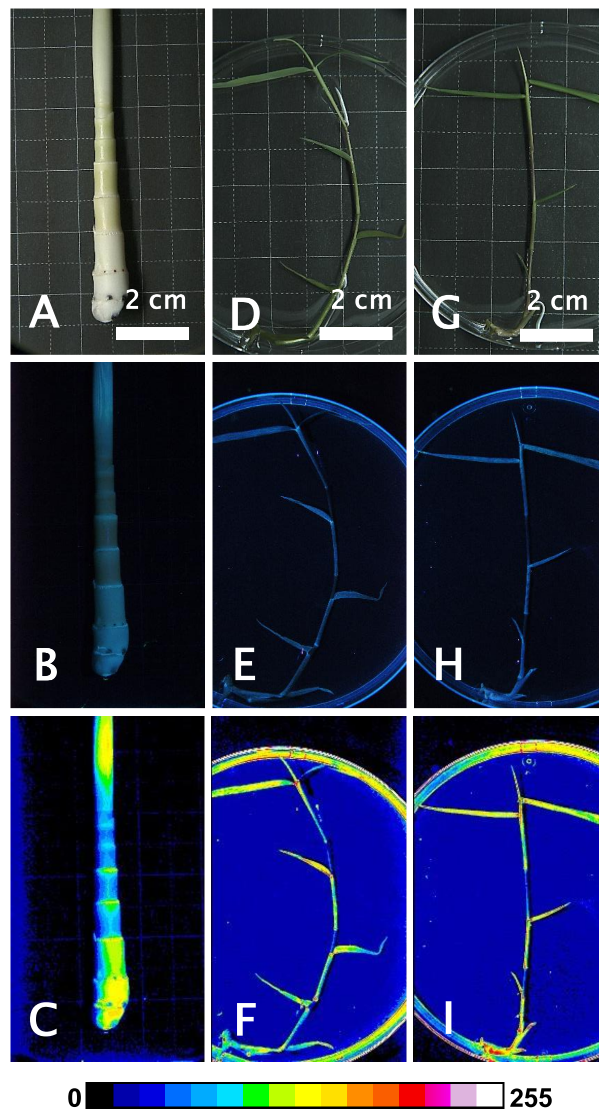


Figure 2.8. Autofluorescence of whole shoots under a LED 365 nm illumination with RGB (red, green, and blue) digital imaging analysis. Young shoot of Pn (A – C), *in vitro* shoot of Pm (D – F), *in vitro* shoot of Bm (G-I). Images in A, D, and G were captured under a white light. B, E, and H are autofluorescence images. C, F, and I are examples of digital imaging analysis in blue mode indicating histochemical differences in outward of tissues.

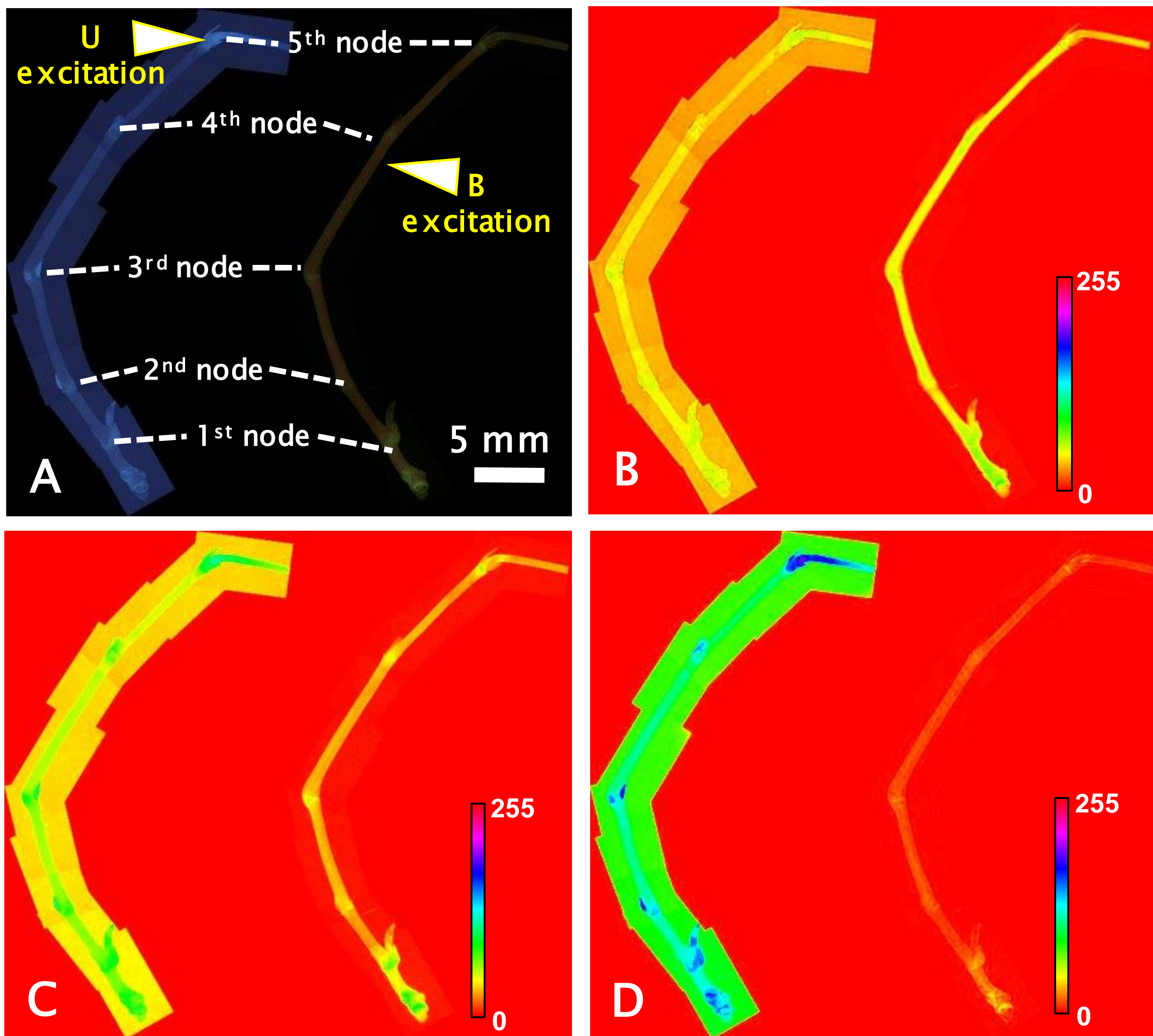


Figure 2.9. Autofluorescence of the outward of culms and nodes tissues under B- and U-excitation lights with RGB digital imaging analysis. A; Autofluroscent image of *in vitro* bamboo shoot under U and B excitation, and B; Red, C; Green, and D; Blue.

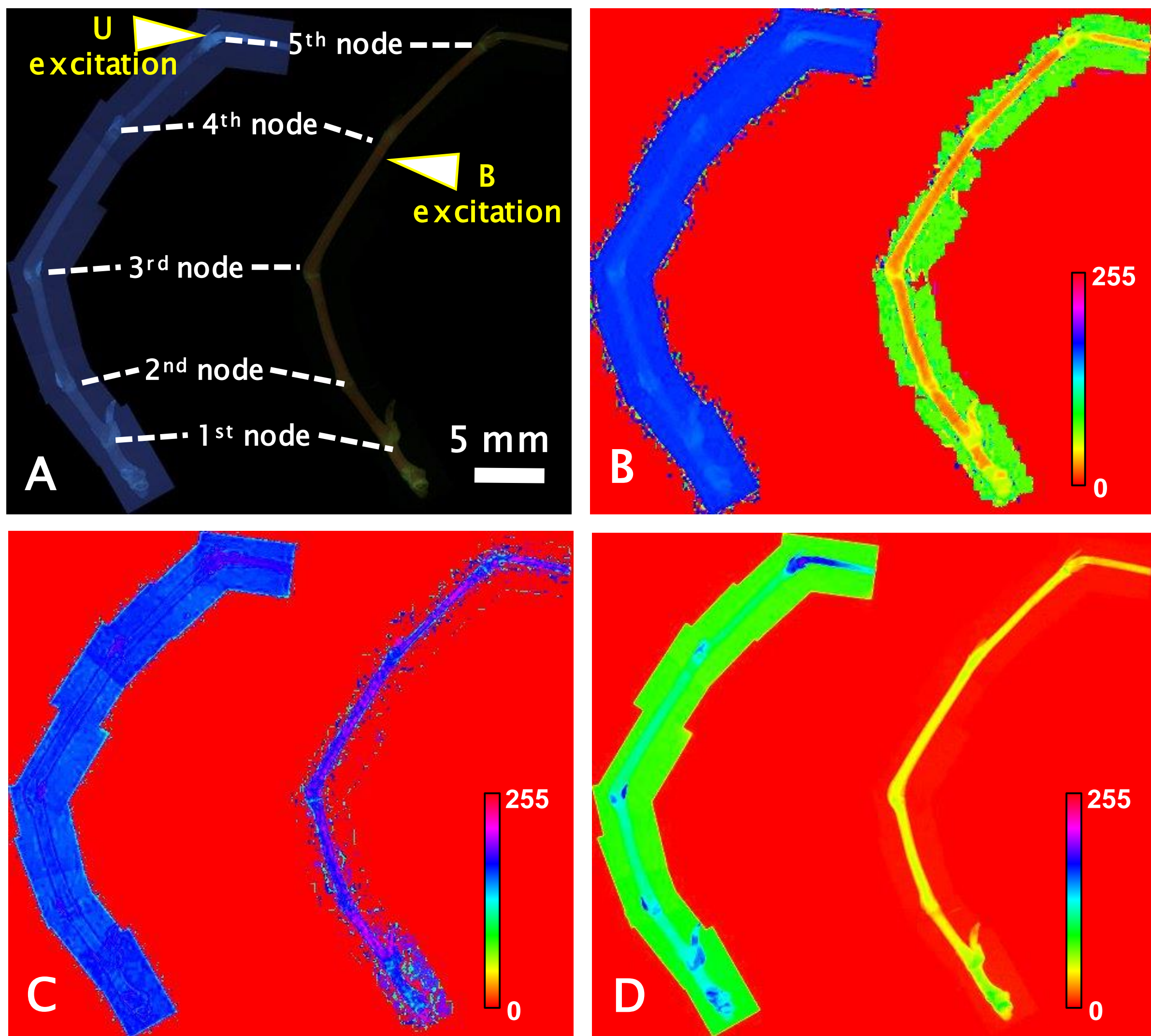


Figure 2.10. Autofluorescence of the outward of culms and nodes tissues under B- and U-excitation lights HSB digital imaging analysis. A; Autofluroscent image of in vitro bamboo shoot under U and B excitation, and B; Hue, C; Saturation, and D; Brightness.

2.3.2.4. Observation of internal morphology of nodes

To see the internal morphology, sheaths were removed and then observed under a stereo microscope. As shown in **Figure 2.11**, lateral bud in the FN was bigger than other buds in MN and TM portions. Longitudinal sections of each nodes portion were observed under a fluorescent microscope. The FN section showed stronger green fluorescence that reflects mature and thick cell wall structure (**Figure 2.11, E**). On the other hand, The MN and TM sections showed more reddish in color that are consist from relatively thin cell wall structure with abundant accumulation of chlorophylls (**Figures 2.11, F and G**). Transverse sections of different internodes (see **Figure 2.3**) showed clear difference in red color deposition (**Figures 2.12 and 2.13**). Each histogram also represented different patterns in terms of distribution of Hue values.

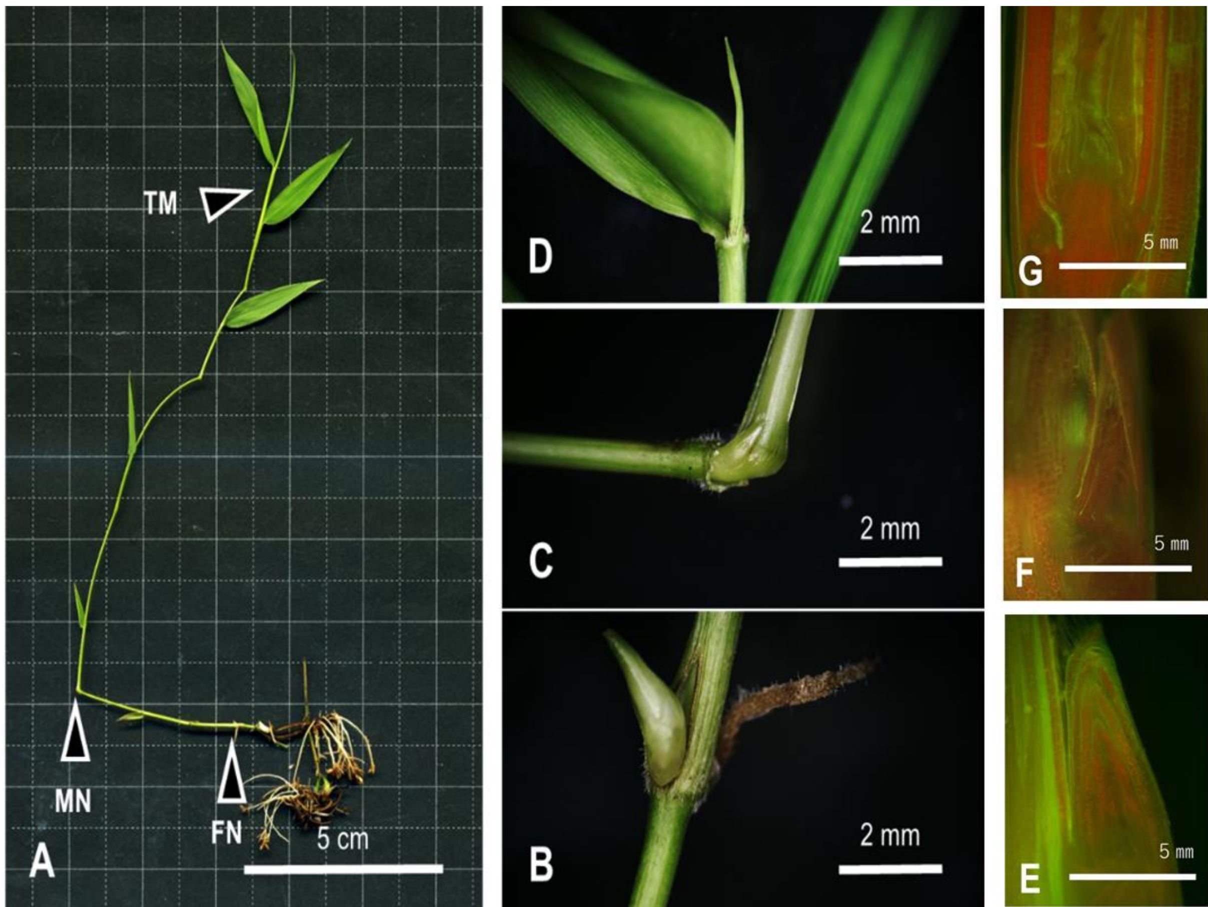


Figure 2.11. The overview of *in vitro* Pm shoot (A) and magnified views of the first node (B), middle node (C), and top meristem (D). Arrowheads in (A) indicate the location of each nodes. Fluorescent images (B-excitation) of FN (E), MN (F), and TM (G).

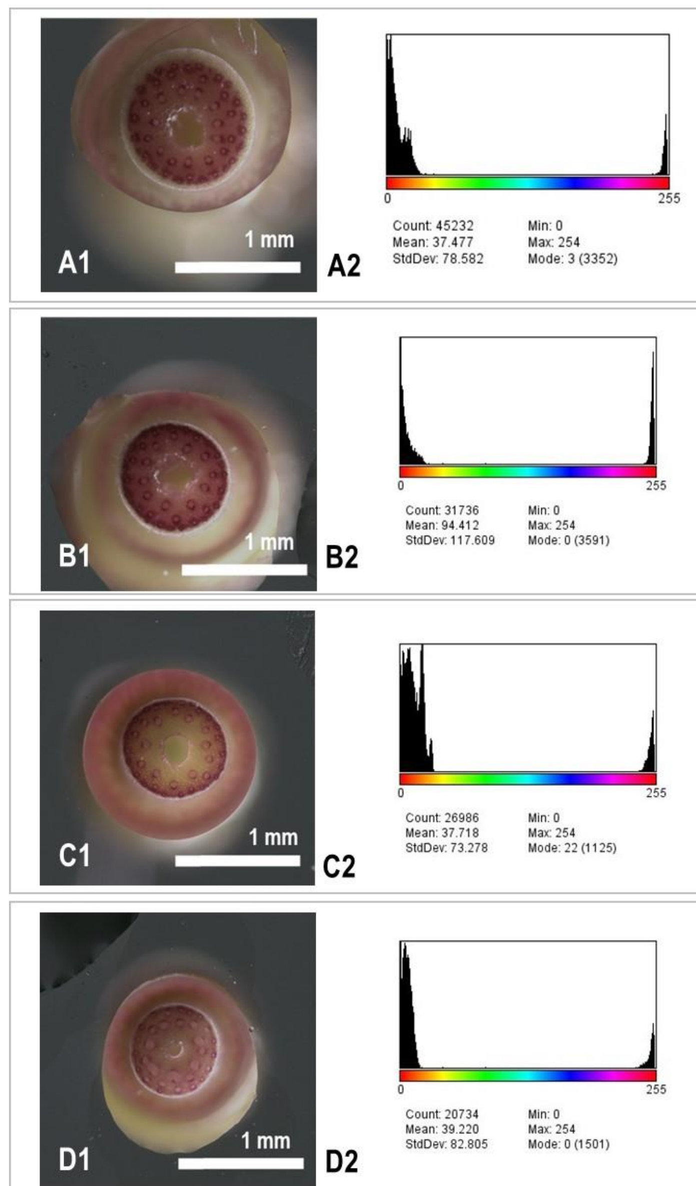


Figure 2.12. Wiesner staining of different internode portions of *B. multiplex* and histogram of the image measured by ImageJ software. A1, B1, C1, D1 are 1st, 2nd, 3rd, 4th are internode sections stained with Wiesner reagent, respectively. A2, B2, C2, D2 are the histograms of 1st, 2nd, 3rd, 4th internode sections, respectively.

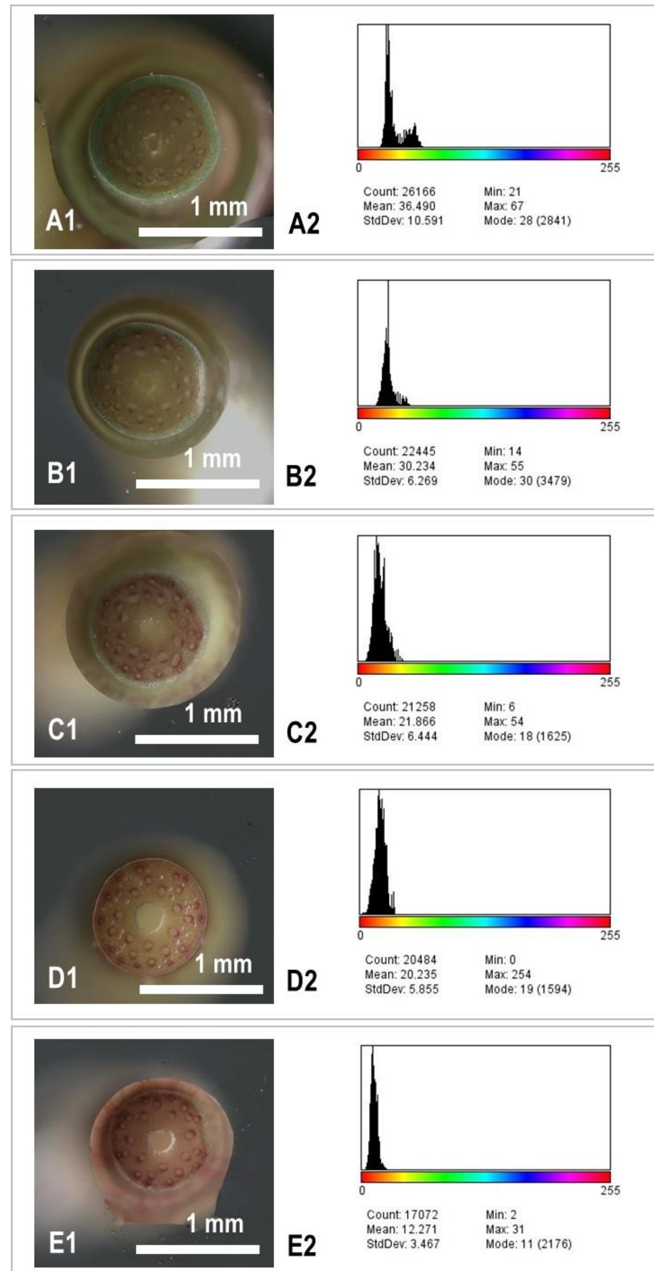


Figure 2.13. Wiesner staining of different internode portions of *P. meyeri* and histogram of the image measured by Image J software. A1, B1, C1, D1 are 1st, 2nd, 3rd, 4th are internode sections stained with Wiesner reagent, respectively. A2, B2, C2, D2 are the histograms of 1st, 2nd, 3rd, 4th internode sections, respectively.

2.3.2.5. Performances of different node portions in *in vitro* culture condition

Three types of node portion, the first node, middle nodes and the top meristem were collected from tall shoots more than ca 10 cm in height and independently cultured in the SLCE to define a bent for explants. The first node showed a better growth performance in terms of weight increment (**Figure 2.14**). Further growth feature of each nodes was also monitored (data not shown) and concluded that the first nodes have superior bent for explants of bamboo node culture. By selecting first nodes as suitable explants, a quick survey for setting a highly efficient culture condition becomes theoretically possible. Without selecting the best node tissues, i.e., first nodes, the resulting responses of a culture tend to vary widely. In the next experiment, I effectively reconfirmed the superior bent of the first node by using a test survey that set a highly efficient culture condition, as follows (see 2.3.3).

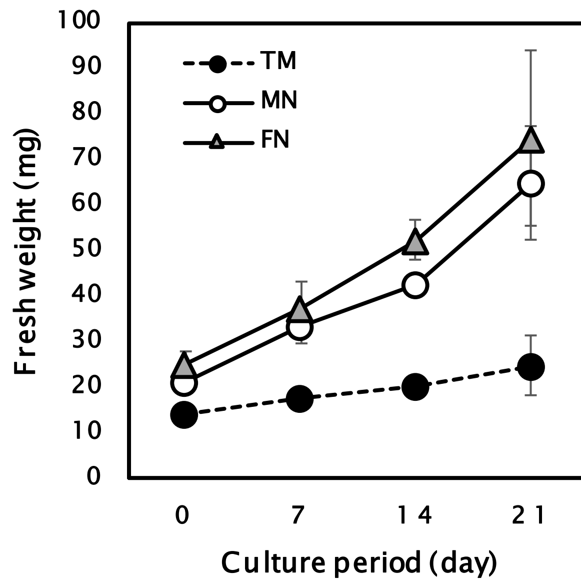


Figure 2.14. Graphical representation of comparison of growth performance of first node, middle node and top meristem in *in vitro* culture condition on the basis of weight increasement at 7, 14 and 21 days of culture.

2.3.3. A test survey of PGRs for setting a highly efficient culture condition

Different concentrations and a combination of cytokinins, i.e., 3 μM of TDZ, and 3 and 10 μM of BA, were used for *in vitro* SAM development, and different concentrations of auxin, i.e., 0.1, 3, and 10 μM of 2,4-D, were also evaluated for whether *in vitro* RAM development was promoted. As expected, clear growth promotion and/or inhibition could be seen in the SLCE, especially in terms of the day to bud break values (**Table 2.2**). To understand the internal morphology, a multiplied shoot in 10 μM BA + 3 μM TDZ condition has been longitudinally sectioned and found that a region of single small shoot (c.a. 5 mm in length) includes 6 nodes at least (**Figures 2.15, C and D**). Because of this unique morphology, 20 ± 3.7 nodes per explant could be seen in a well of the SLCE, where only 4 ± 0.6 nodes/explant were induced in control condition (**Figure 2.16**).

Table 2.2. Effect of PGRs on shoot multiplication in Bamboo (*P. meyeri*).

Plant growth regulator (μM)	Growth performance		
	Days to bud breaking (d)	Length of shoot (cm)	Shoot/root/callus development
0	15.8 ± 2.34	4.1 ± 0.31	Single shoot elongation (control)
TDZ 3	8.00 ± 0.84	2.9 ± 0.32	Single shoot elongation with moderate inhibition
BA 3	5.5 ± 0.55	6.7 ± 0.22	Single shoot elongation with active promotion
BA 10	4.3 ± 0.55	7.1 ± 4.33	Single shoot elongation with active promotion
BA 10 + TDZ 3	3.00 ± 0.45	2.8 ± 0.26	Multiple shoots elongation with moderate inhibition
2,4-D 0.1	7.8 ± 0.82	4.4 ± 0.16	Single shoot elongation as same as control with single root
2,4-D 3	11.17 ± 0.89	3.8 ± 0.08	Single shoot elongation and callusing slight at the base of
2,4-D 10	1.8 ± 3.03	0.15 ± 0.25	Moderate callusing at joint region of node tissue

Growth performances was summarized after 21 days of culture. Data represents the average value ± SD from 5 explants.

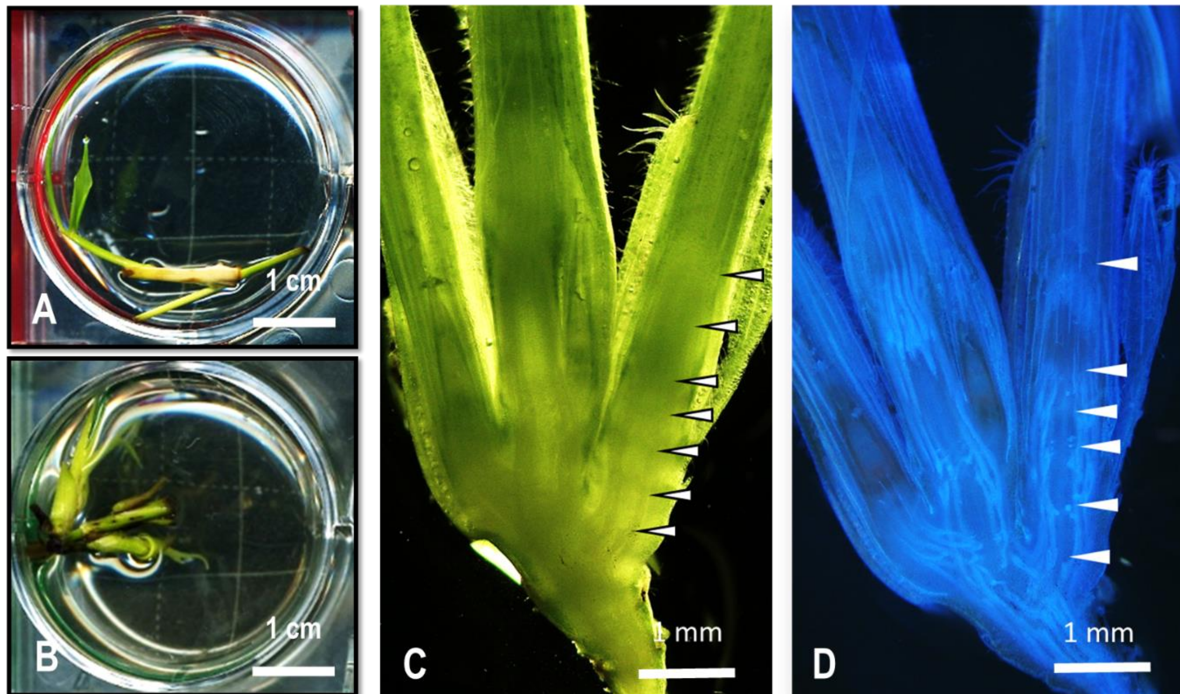


Figure 2.15. Effects of BA and TDZ combination on shoot multiplication in bamboo (*P. meyeri*). Control/ 1/2 MS0 (A). Shoot multiplication in 10 µM BA + 3 µM TDZ (B). L. S. of multiplied shoot under bright field (C) and UV illumination (D) showing the pattern of multiplication from one single node (white arrowheads indicating node portion).

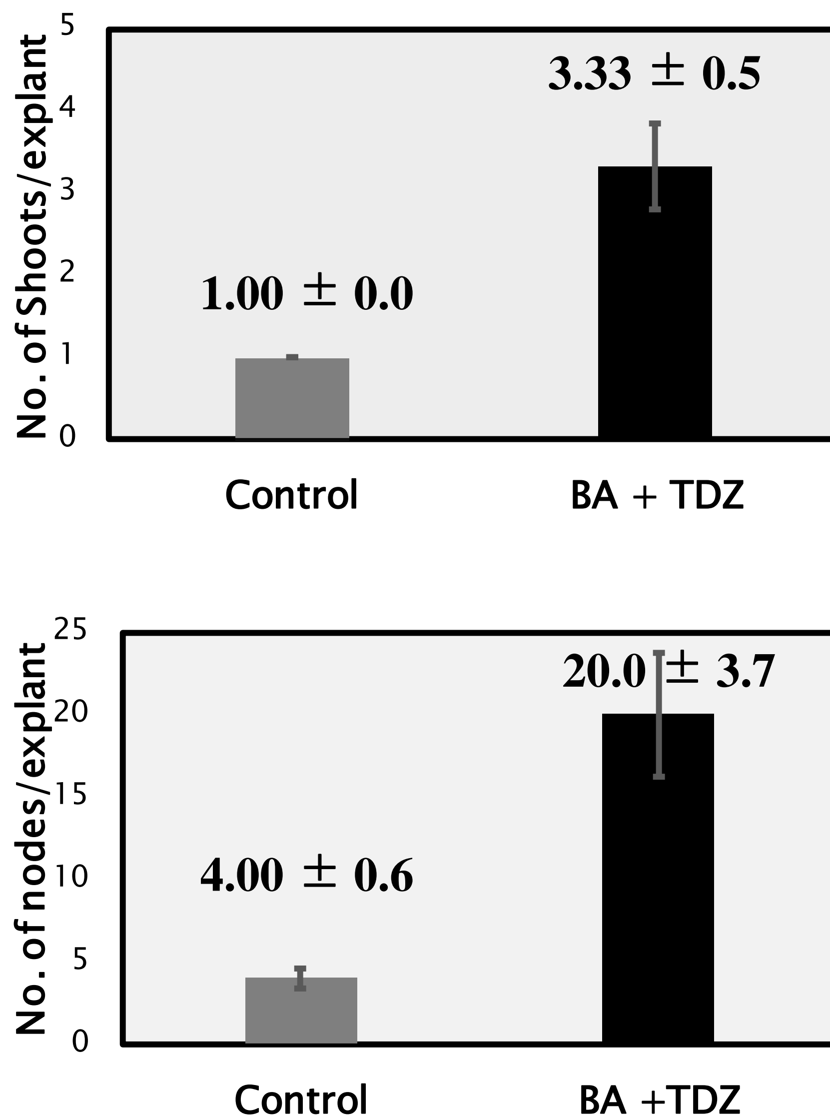


Figure 2.16. Graphical representation of effects of 10 µM BA + 3 µM TDZ on number of shoots/explant (A) and nodes/explant in *P. meyeri* bamboo.

2.4. Discussions

A liquid medium condition has previously been suggested to be suitable for *in vitro* bamboo tissue cultures, especially in three major bamboo genera (*Bambusa*, *Dendrocalamus*, *Phyllostachys*) (Ogita *et al.* 2008, Sood *et al.* 2002, Das and Pal 2005). In the present study, I focused on using a 6-well microplate that contains 2 mL per well of a liquid medium, which provides a small-scale liquid culture environment (SLCE). The underlying concepts of the SLCE are as follows: (1) to establish a simple and versatile liquid assay system to control morphological and histochemical responses of SAM and RAM of the bamboo node within a short period, and (2) to reveal the relation between color variation in the outward regions of culm and node tissues and their suitability as explants using techniques for autofluorescence measurement with a digital imaging analysis.

For the first concept, I evaluated growth performance of nodes from 11 different bamboo species that belong to seven major bamboo genera (*Bambusa*, *Dendrocalamus*, *Phyllostachys*, *Tetragonocalamus*, *Chimonobambusa*, *Pleioblastus,* and *Sasa*), and also investigated growth performances of *in vitro* bamboo nodes. Based on the results obtained, we concluded that the SLCE system using first nodes is the best way to control

in vitro SAM and RAM development of bamboo. As a test assay, I investigated the effects of plant growth regulators—both auxin and cytokinin—since it is well known that these chemicals interact in a complex manner to control many aspects of growth and differentiation (Coenen and Lomax 1997). The auxin–cytokinin interaction in the regulation of plant meristem development is overviewed in terms of biosynthesis, transport, and signaling control (Su *et al.* 2011). As shown in **Table 2.2**, I recognized that the combination of 10 μM of BA and 3 μM of TDZ was effective for *in vitro* SAM development during 3 weeks of culture. The addition of TDZ served as a trigger to induce multiple shoots. There are reports that TDZ is effective for enhancing micropropagation in cereal and grass plants (Kumari *et al.* 2017, Schulze 2007). Moreover, the addition of 2,4-D effectively promoted *in vitro* RAM development. Interestingly, I was capable of monitoring different bud breaking patterns (2–10 days after culture) with various RAM developments through the test assay. The low concentration of 2,4-D (0.1 μM) was effective for shoot and root development, while the high concentration of 10 μM induced early bud breaking with callus formation.

For the second concept, using macro- and microscopic fluorescence observation techniques, as described in the text, I estimated autofluorescence properties in the

outward regions of bamboo node tissues by performing an RGB and HSB digital imaging analysis. The color of an object can be described by several color coordinate systems, referred to as color spaces. One of the most important decisions for imaging analysis is to select a color space, of which the most popular is RGB, often used in video monitors. HSV (HSB in ImageJ) is an alternative representation of the RGB color model designed to be more closely aligned to human vision and perception of color-making attributes (Lazaro *et al.* 2019). As shown in **Figure 2.9, 2.10**, I compared all the images obtained, and decided a suitable combination of B- and U-excitation lights with an RGB and HSB digital imaging analysis. Several staining techniques such as Wiesner reaction, safranin staining, DAPI staining were also helpful to identify histochemical characteristics of bamboo nodes.

To my best knowledge, this is the first report that suggests a versatile node culture method in the SLCE to control morphological and histochemical responses of SAM and RAM in bamboo plants.

In Chapter III, I used this protocol to check the effects of two phenolic compounds, both phloroglucinol (PG) and coumarin (COU), on controlling the SAM

and RAM development in Bm and Pm bamboo plants and other model grass species, i.e. rice, barley and *Brachypodium*.

Chapter III

Effect of phenolic compounds for the promotion of *in vitro* SAM and RAM development in the SLCE system

3.1. Introduction

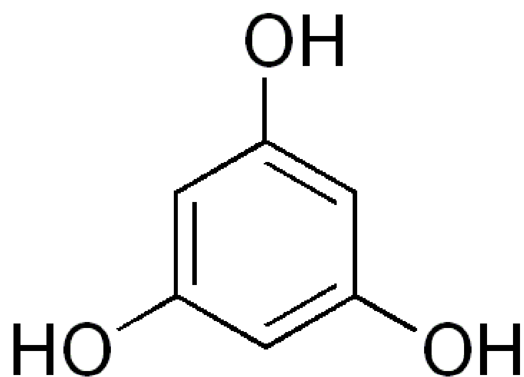
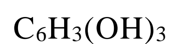
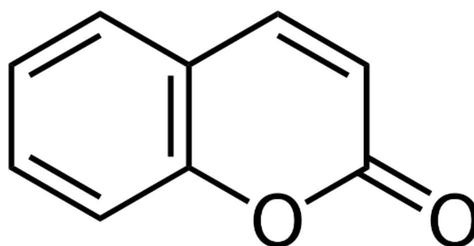
In the PCTOC research, there is a constant need to search new regulatory factors that will lead results in better or more efficient growth *in vitro* (Teixeira da Silva *et al.* 2013). For example, sugar (Sucrose, Maltose, and Trehalose), amino acids (Phenylalanine, Tyrosine, and Glutamine), phenolics (Phologlucinol, Coumarin), etc. used for shoot growth enhancement, root growth promotion in plant cell tissue and organ culture research (Nilanthi and Yang 2014, Bosila *et al.* 2012, Ogita *et al.* 1997, Siwach and Gill 2011, Lupini *et al.* 2014). In this present research, I choose two phenolic compounds, phloroglucinol (PG) and coumarin (COU) to control *in vitro* SAM and RAM development, with the SLCE system (see **Table 3.1** for the detailed chemical properties of these compounds).

Phloroglucinol demonstrates both cytokinin-like and auxin-like activity, much like thidiazuron, and thus has considerable potential for application in a wide range of plant tissue culture studies. There are some reports that PG was effective in tissue

culture for root meristem development (Tan *et al.* 2018, Perez *et al.* 2016, Londe *et al.* 2017, Jani *et al.* 2015). But till now, there is no detailed information of the effect of PG on shoot meristem development in bamboo.

Coumarins are a group of natural products in plants that originate from the general phenylpropanoid pathway. They are often found to accumulate in the root tissues and are involved in plant defense, root development and nitrogen uptake and metabolism (Li and Gao 2011). This phenolic compound affects primary root elongation and lateral root development in *Arabidopsis thaliana* (Lupini *et al.* 2014). Coumarin influenced root morphology and histology, showing a selective effect on maize and *Arabidopsis* root types, inhibiting the primary root elongation and stimulating lateral root formation. Root branching density and root zone distribution confirmed that coumarin may modulate auxin distribution through influx or/and efflux proteins (Lupini *et al.* 2014). However, the exact mechanism of coumarin on root growth has not been clarified yet. A complex network of molecular signaling probably governs coumarin morpho- physiological responses, where auxin transport and/or biosynthesis could play an important role. The details of the effects of phenolic compound on bamboo node and tiller development of some grass species are described below.

Table 3.1. The chemical properties of phloroglucinol and coumarin.

Phloroglucinol (1, 3, 5 trihydroxybenzene)	
Chemical structure	
Chemical formula	 $C_6H_3(OH)_3$
Molecular mass	126.11 $g \cdot mol^{-1}$
Coumarin (1-benzopyran-2-one)	
Chemical structure	
Chemical formula	$C_9H_6O_2$
Molecular mass	146.145 $g \cdot mol^{-1}$

3.2. Materials and methods

3.2.1. Plant material

As described in Chapter II, *in vitro* node culture stocks of Bm and Pm were used to understand the sequential developmental processes of SAM and RAM of first node explants. In addition, 7 days old germinated seedlings of rice (cv. Nipponbare), barley (cv. Wasedori) and *Brachypodium distachyon* (Bd 21) were used in this experiment (**Figure 3.1**). Seeds were germinated in MS medium without growth regulators.

Figure 3.1. Plant materials for grass species. Commercial seeds and 7 days old germinated seedling in MS0 medium. A, C, E are seeds and B, D, F are germinated seedlings of rice, barley and *Brachypodium distachyon*, respectively. Scale bar = 1 cm.

3.2.2. Node culture protocol and its applications

As described in Chapter II, the node culture protocol in the SLCE was applied to investigate the effects of PG and COU. Briefly, one node segment (1.5 -2.0 cm in length) of each bamboo plants was cultured in a well of the SLCE. The first node from the *in vitro* node cultures of Pm and Bm were used in the experiments unless otherwise specified. For the PG experiment, 10 µM BA + 3 µM TDZ which was proved as the best formulation for shoot multiplication in Chapter II was used. For the COU experiment, 0.1 µM 2,4-D which was proved as the best formulation for RAM development in Chapter II was also used. For long time observation, square shaped plastic culture box containing 50 mL medium were used for multiplication pattern checking and rooting experiment. All the cultures were incubated at 25°C, with a 16 h photoperiod under a fluorescent illumination (65 µmol m^{-2} s^{-1}).

3.2.3. Detailed assay protocols

3.2.3.1. Effect of PG on early bud initiation and increase the rate of multiplication in bamboo

Different concentrations of PG (5, 50 and 500 µM PG) were used to find best

concentration for early bud breaking and 500 μM PG proved to be the best for early bud initiation. This concentration caused bud initiation within 24 hours. After 14 days of cultures, moderate browning and some abnormal growth characters such as growth inhibition and shoot-leaf curling were found. That's why, early initiated buds were transferred in to shoot multiplication medium without PG.

3.2.3.2. Effect of PG in combination with BA and TDZ on shoot multiplication

Explants treated and non-treated with PG cultured on 10 μM BA + 3 μM TDZ to know the effect of PG on increase the rate of multiplication in bamboo.

3.2.3.3. Effect of COU singly or in combination with 2,4-D for root meristem development

5, 50 and 500 μM COU has been applied on first node portion of *P. meyeri* and *B. multiplex* to find best concentration for RAM development.

3.2.3.4. Root meristem development of PG treated multiplied brunches

To check the rooting capacity of those multiplied branch (in 10 μM BA + 3

TDZ), has been proved better shoot multiplication from Chapter II) of shoots 5 μM COU has been tested singly or in combination with 0.1 μM 2,4-D (has been proved better for rooting from Chapter II).

3.2.3.5. Application of best formulation of SAM and RAM development through PG and COU in other model grass species

Best formulations (see 3.2.3.2.) for shoot multiplication (10 μM BA + 3 μM TDZ, PG treated) and 5 μM COU for RAM development (see 3.2.3.3.) has been applied on other grass species, especially rice, barley and *Brachypodium*.

3.2.4. Observation and collection of data

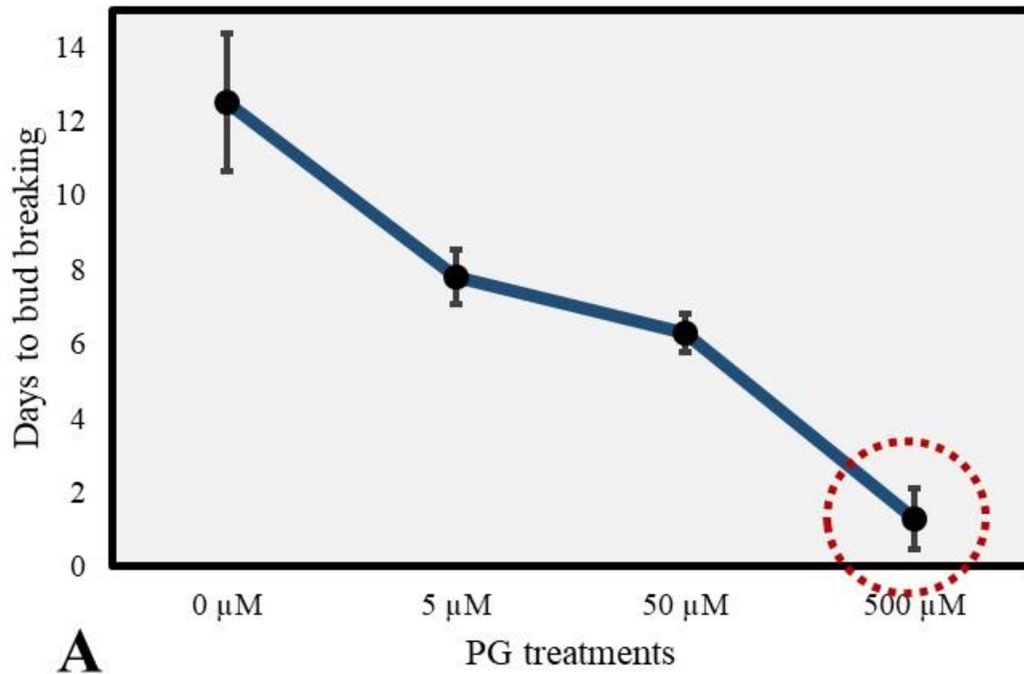
As described in Chapter II, observation of *in vitro* SAM and RAM development has been done.

3.3. Results

3.3.1. Effect of PG on early bud initiation and increase the rate of multiplication in bamboo

3.3.1.1. Effect of PG for early bud initiation

By using the SLCE system with a careful observation, I could identify the tendency of bud breaking as shown in **Figure 3.2**. The highest PG treatment, 500 μM, was the best condition to induce early bud breaking in Bm and Pm nodes. However, after 2 weeks of culture, the explants in 500 μM PG treatment turn brown and showed abnormal growth (**Figure 3.3**). In this period, RAM development was also visible. In case of *B. multiplex,* rooting elongation were noticed in 50 μM and in 500 μM rooting with lateral root formation (LR). However, abnormal growth characters such as stem-leaf curling and callusing were also recognized at the base (**Figure 3.3, red arrowheads**).



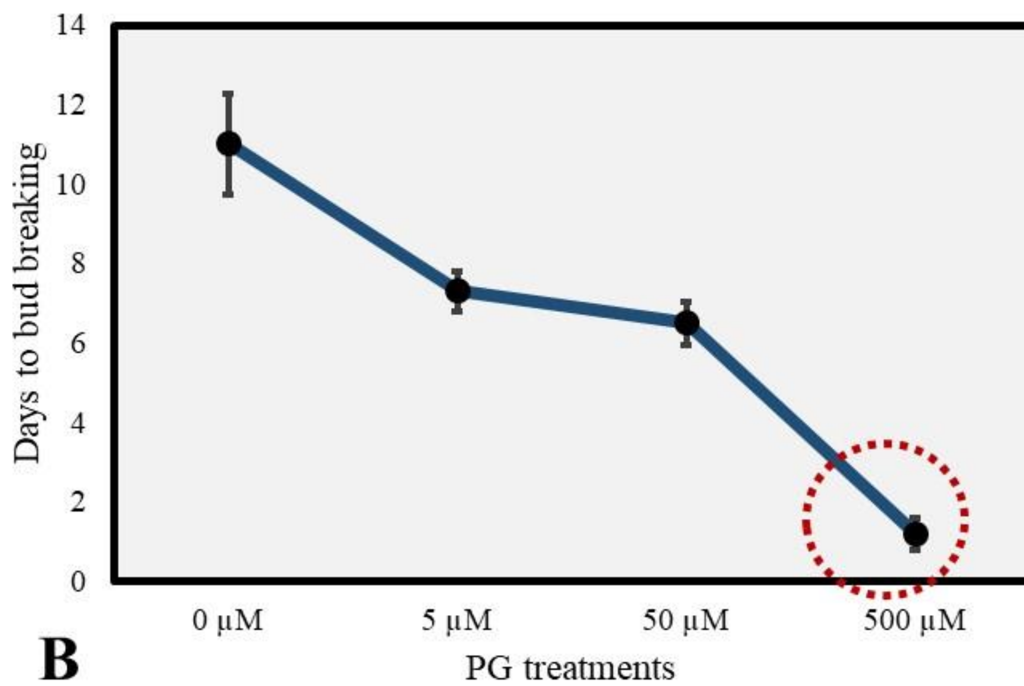


Figure 3.2. Bud breaking tendency during PG treatment culture in *B. multiplex* (A) and *P. meyeri* (B).

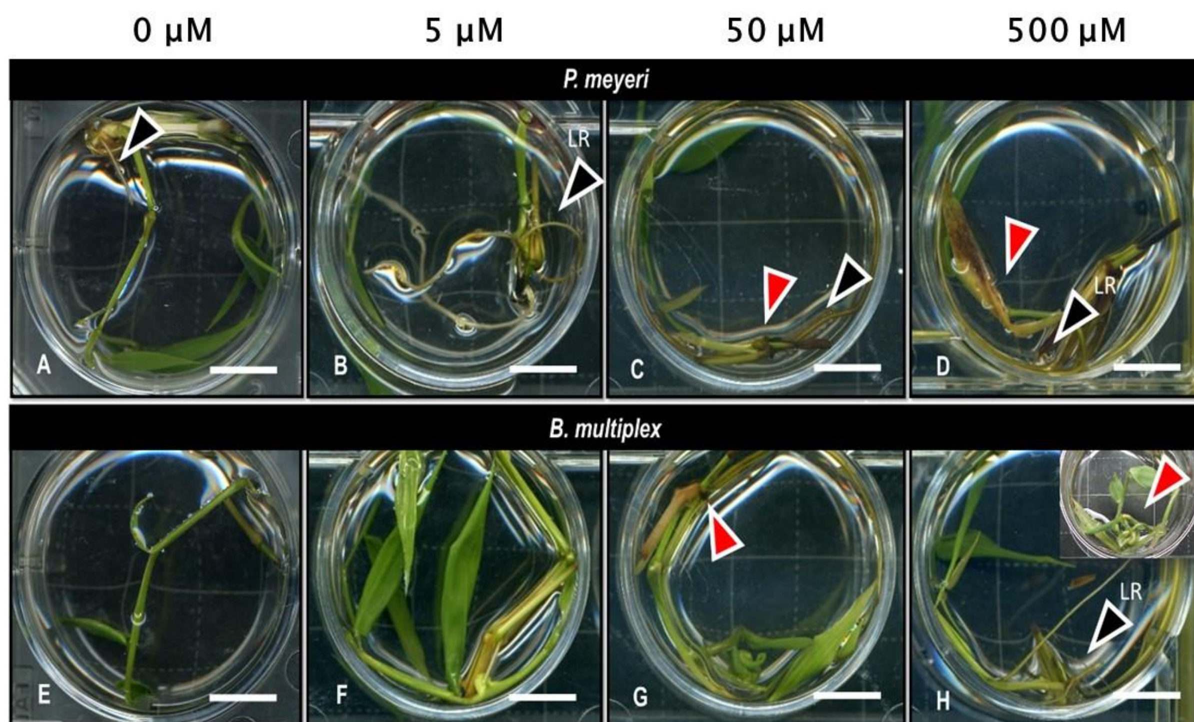


Figure 3.3. Effect of PG on bamboo node culture of *P. meyeri* and *B. multiplex* after 3 weeks of culture. A-D. PG 0 µM, 5 µM, 50 µM, 500 µM for *P. meyeri*. E-H, same concentrations for PG for *B. multiplex*. Scale bar = 1 cm. Black colored arrowheads represent the positive RAM growth but red arrowheads show abnormal growth characters.

3.3.1.2. Effect of PG in combination with BA and TDZ on shoot multiplication

Since continuous supply of PG showed a potential to bud breaking within 1 day, but negative side effects such as browning and stem- leaf curling were also visible. So, attempted to rescue the PG treated nodes to a shoot multiplication medium containing 10 µM BA + 3 µM TDZ. As a result, by treating PG there were 500.5 ± 123 nodes/ explant, where in non-treated and control were 20 ± 3.74 and 4 ± 0.63 respectively (**Figure 3.4**).

I also attempted to rescue the PG treated nodes at different time durations (24 h/ 48 h/ 72 h) to a shoot multiplication medium containing BA 10 µM + TDZ 3 µM. As a result, different growth performance could be seen as shown in **Figure 3.5**. Briefly, shoot multiplication pattern could be seen in the SLCE, yellow-whitish and partially necrotic shoots appeared from the longer PG treatment conditions. After that, these treated nodes were transferred to a large scale culture environment, i.e. a culture box containing 50 mL medium, to see further shoot development. Highest number of shoots/node (96.00 ± 1.00) was found in explants treated with 500 µM PG 48 h. The leaf and stem color looked whitish green (**Table 3.2, Figure 3.5).** 24 h PG treated explants also showed higher growth performance, i.e. number of Shoots/node (74.33 ±

1.5), and formed shoots looked light green color and healthy**.** 72 h PG treated explant hardly browned and did not show normal growth performance. Shoot branching pattern of node explant in 10 μM BA + 3 μM TDZ treated with 500 μM PG was monitored under a florescent microscope as shown in **Figure 3.7**. Based on this image, I made a comparison model of aerial branching pattern of bamboo with axes of consecutive order as shown in **Figure 3.8**.

From the above mentioned characteristics, I concluded that 24 h and 48 h PG treatment could be used for high frequent shoot multiplication with activation of SAM development.

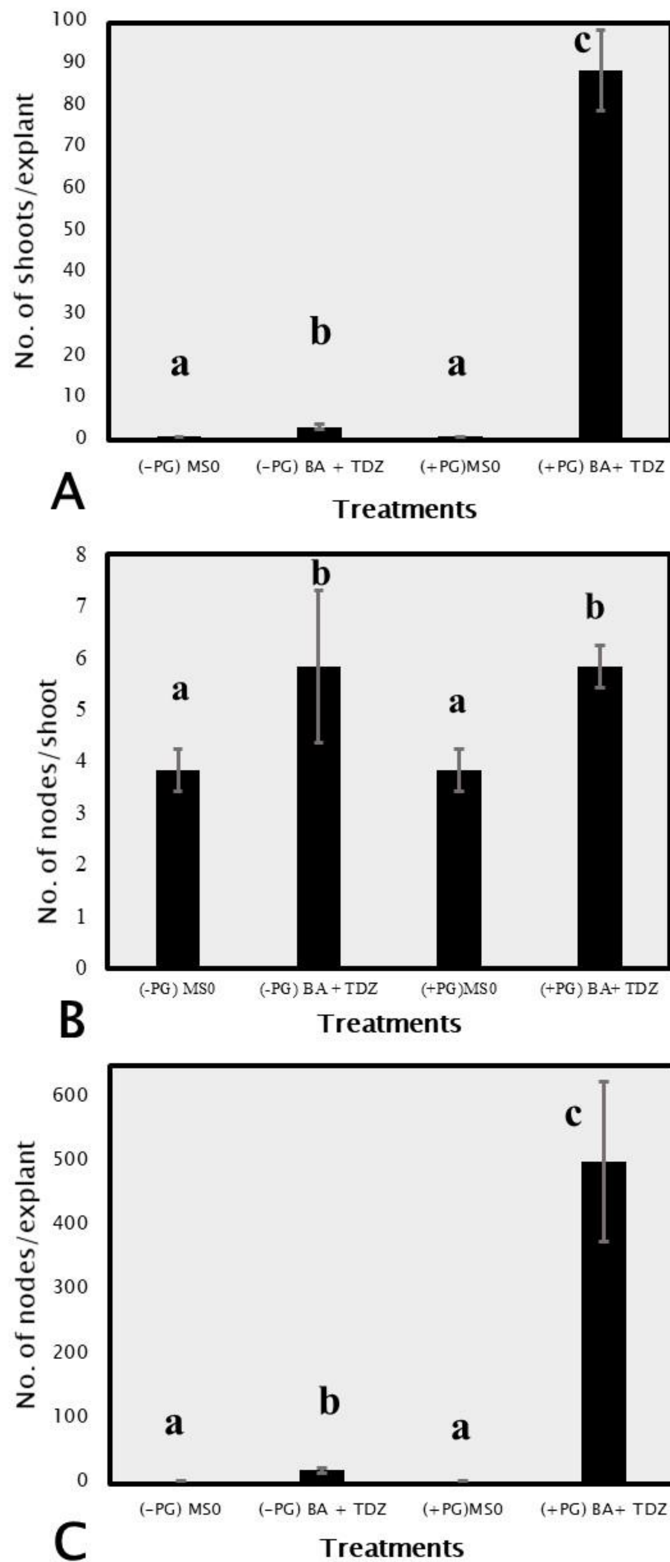


Figure 3.4. Graphical representation of different parameters related to shoot multiplication in Bamboo *(P. meyeri*) using ± PG (at 28 DAC).

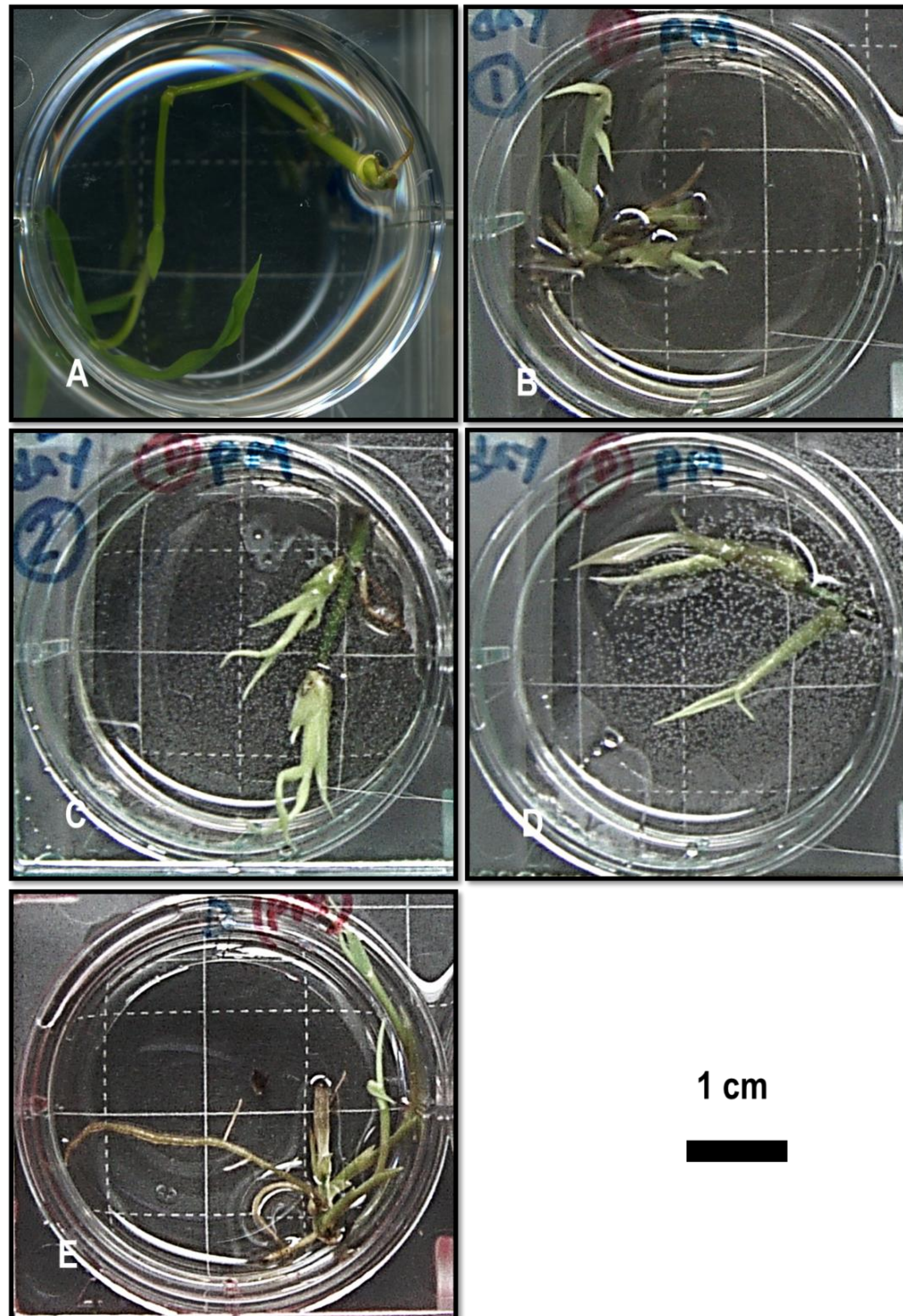


Figure 3.5. Selection of perfect time for rescue of buds for shoot multiplication (10 μM BA + 3 μM TDZ) of bamboo *(P. meyeri*). A. Control (MS0). B, C, and D represent rescued nodes after 24 h, 48 h, and 72 h PG treatment respectively. E. Non rescued from 500 μM PG at 2 weeks of culture.

Table 3.2. Effect of PG pre-treatment times on shoot multiplication in bamboo.

Treatment duration (h)	Growth characters			
	No of shoots/ node		Weight increase	Growth characters
	7 DAC	28 DAC		
Control	1 ± 0.0	1 ± 0.0	15-fold	Single shoot elongation/ dark greenish
24 h	7.67 ± 0.58	74.33 ± 1.5	64-fold	Multiple shoot/ light green
48 h	5.67 ± 1.15	96 ± 1.00	117-fold	Multiple shoot/ white green
72 h	3.33 ± 0.58	55 ± 1.00	165-fold	Multiple shoot/ yellow green
Non- rescued	1 ± 0.00	1.33 ± 0.58	17-fold	Single shoot elongation/ lateral root/ explant browning
F value (*)	64.33*	5948.67*		

* Significant at 5 % level. Mean ± SD has been used in table.

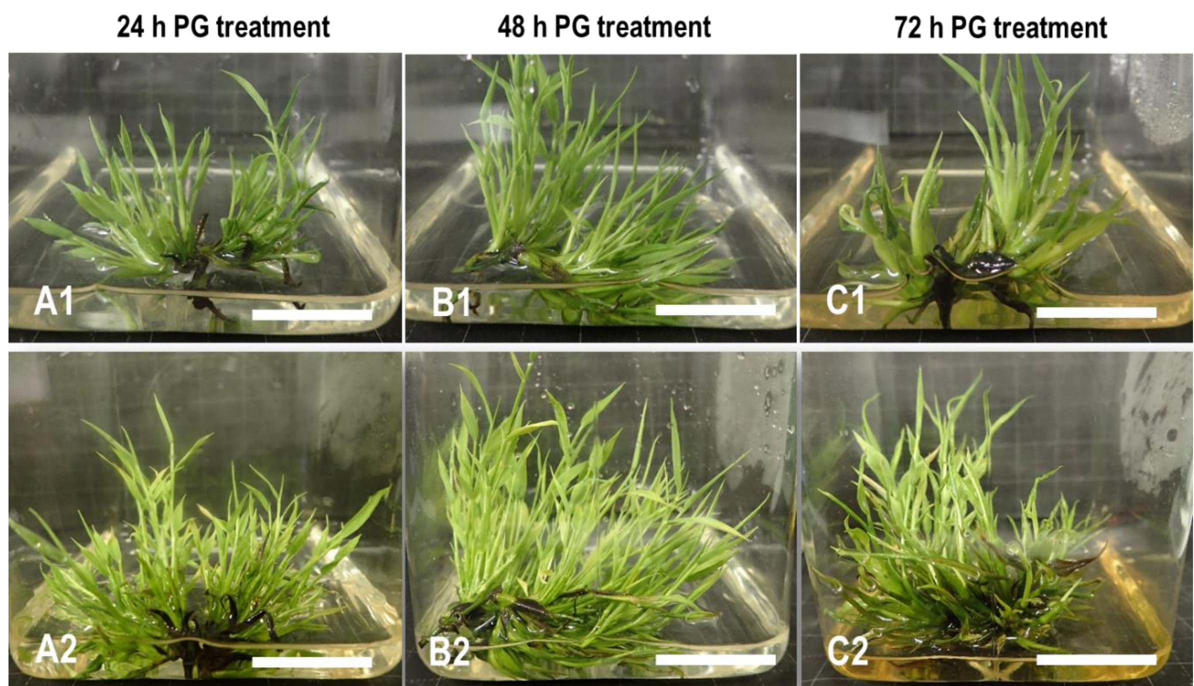


Figure 3.6. Effect of duration of time of 500 μM PG on bamboo (*P. meyeri*). 24 h, 48 h, 72 h PG treated bamboo nodes A1, B1 and C1 respectively after 28 days of culture in a large scale culture environment. And same explants A2, B2, and C2 after 35 days of culture in a largescale culture environment, respectively. Scale bar = 3 cm.

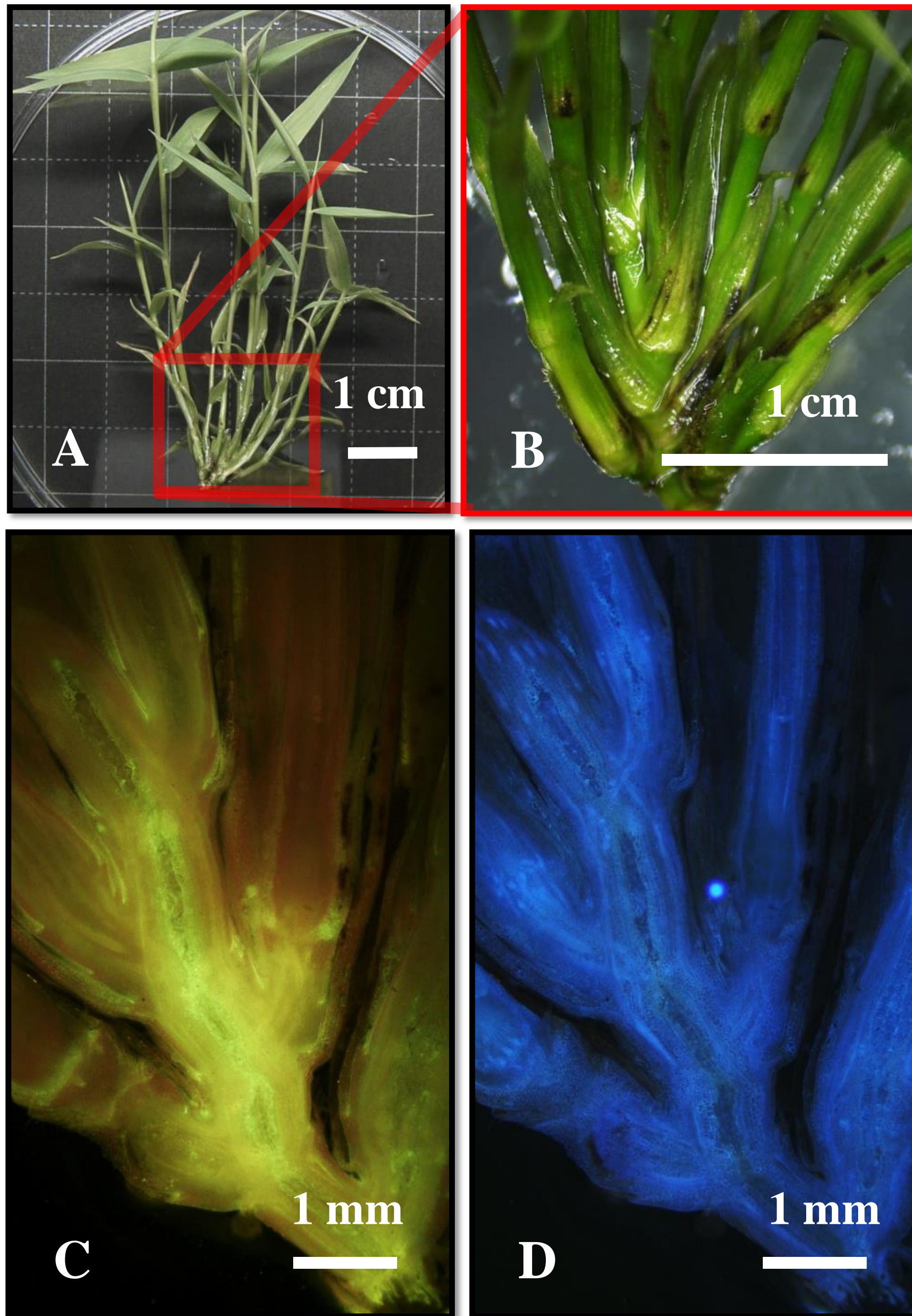


Figure 3.7. Shoot branching pattern of bamboo in 10 µM BA + 3 µM TDZ treated with 500 µM PG. A. Bunch of bamboo shoot separated from main multiplied bushy structure. B. Magnified view of the base portion of multiplied node. C-D. L.S. of shoot showing pattern of multiplication, auto fluorescent image (C. B excitation and D. UV light).

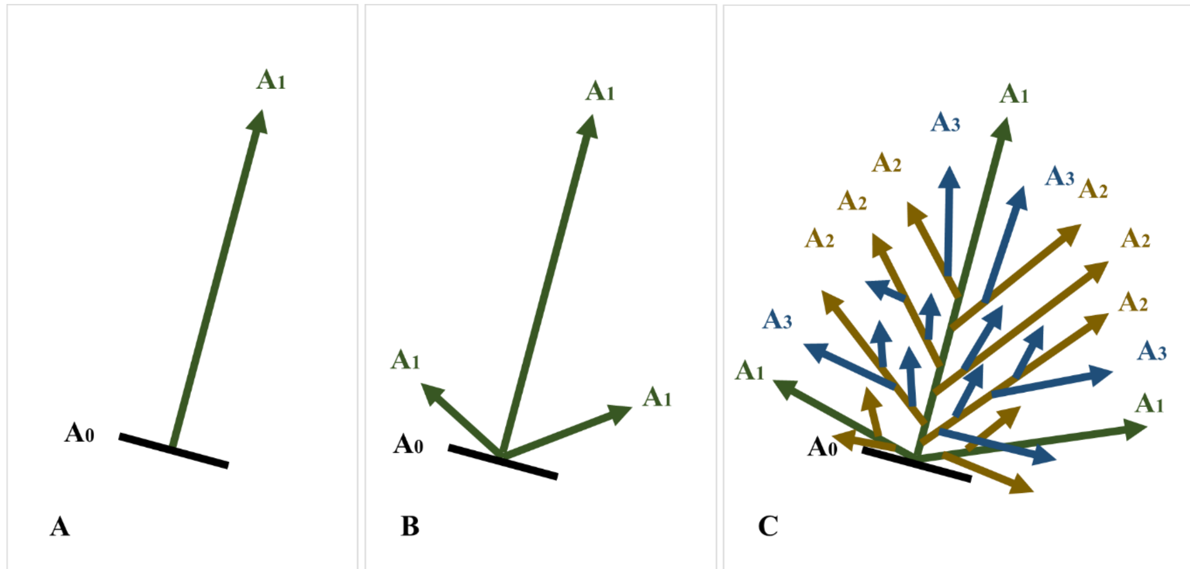


Figure 3.8. Comparison of aerial branching pattern of bamboo with axes of consecutive order (A1, A2, A3) in ± PG. A. MS0, B. 10 μM BA + 3 μM TDZ (-PG) and C. 10 μM BA + 3 μM TDZ (+PG).

3.3.2. Effect of COU as an effective promoter for RAM development in bamboo

3.3.2.1. Root meristem in control condition

In order to understand the occurrence and distribution of root meristems in field growing bamboo, a small shoot from greenhouse growing *P. nigra* (**Figure 3.9, A**) was collected, remove the sheath, and observed under a stereo microscope. Several distinct root meristems were visible in upper portion of node joint region as shown in **Figure 3.9, B-C**. In *in vitro* growing bamboo, I recognized the existence of single lateral bud (SAM) and single RAM in the upper portion of node joint region (**Figure 3.10, A**). Histological characteristic of the RAM could be seen under an inverted fluorescent microscope as shown in **Figures 3.10, B-E**). I applied this observation technique to the following experiment.

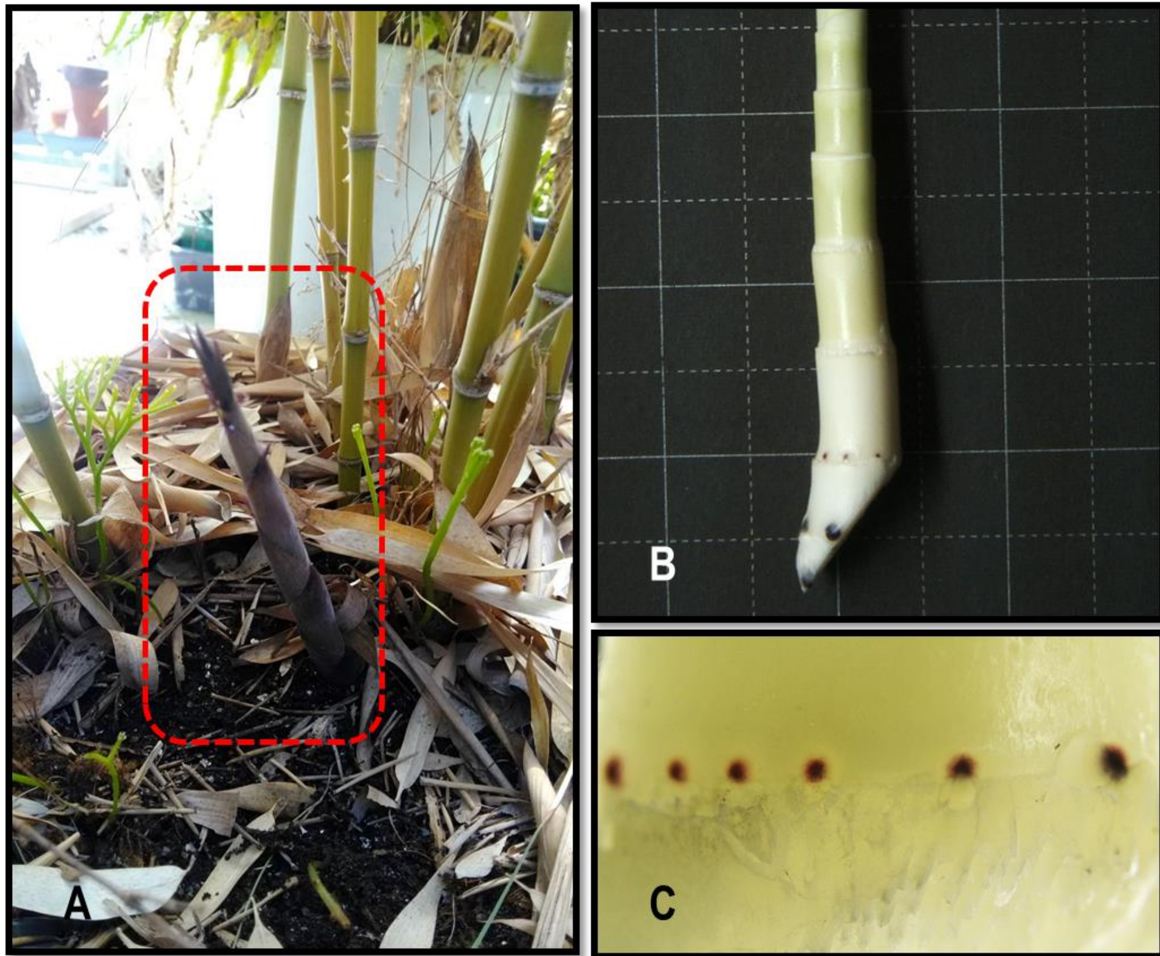


Figure 3.9. Position of root meristem in field growing bamboo shoot. Root meristem in field growing *P. nigra* bamboo (A). B. Field growing shoot after removing the sheath showing the position of root meristem, magnified view of node portion showing multiple root meristems (C).

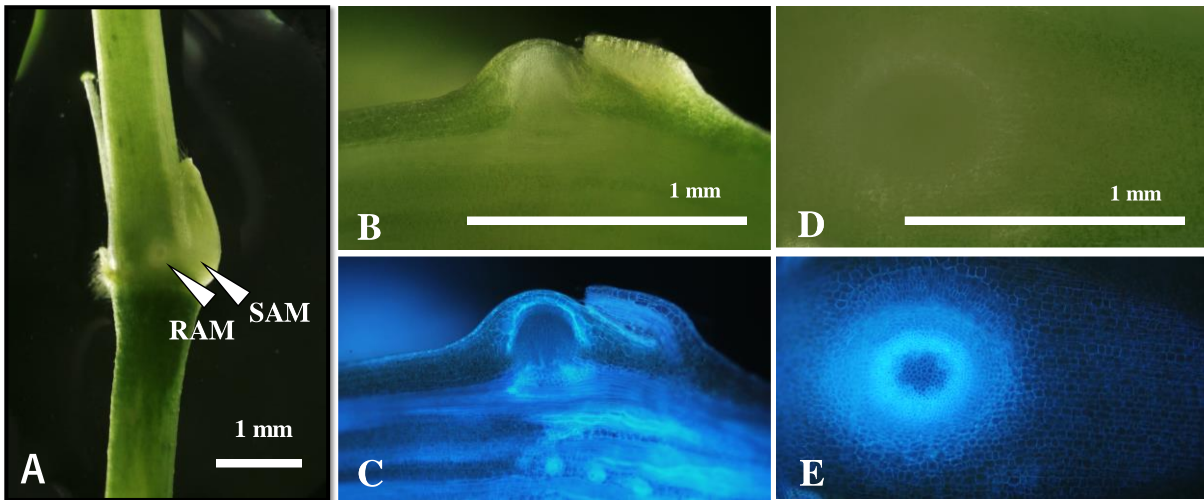


Figure 3.10. Position of root meristem in *in vitro* growing bamboo (*P. meyeri*). A. *In vitro* growing node showing single shoot and root meristem, B-C. L.S. of root meristem under bright field and UV light of fluorescent microscope respectively. D-E. Thin L.S. from the surface of skin showing vascular bundle of root under bright field and UV light of fluorescent microscope respectively. DAPI staining method has been done to see the target parts under fluorescent microscope.

3.3.2.2. Histological observation of the effect of COU on RAM development

Effect of COU on RAM development by using fluorescent microscopy has been observed. As shown in **Figure 3.11, A1-D2**, 5 μM of COU promoted early development of dormant root meristems in node portion within 1- 3 days of culture. Development of RAM was clearly visualized with DAPI staining. So, to check a suitable concentration of COU by using the SLCE method are as follows.

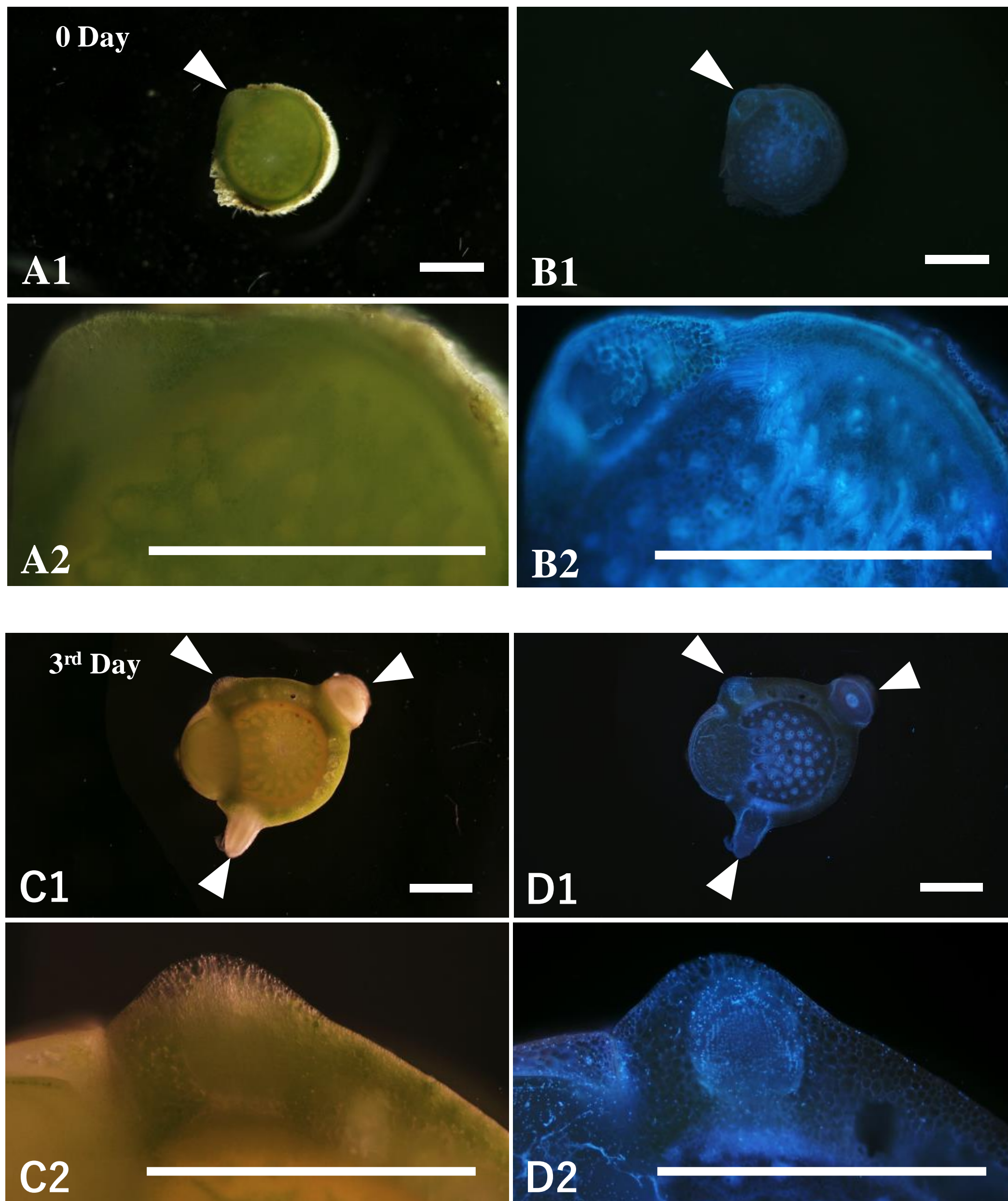


Figure 3.11. Multiple root meristem development using 5 µM COU within 3 days of culture. T. S. of node showing single root meristem under bright field (A1-A2) and UV light (B1-B2) of fluorescent microscope at 0 day. T.S. of node portion showing multiple root meristem emerged (C1- C2) under bright field and D1-D2 under UV light of florescent microscope (stained with DAPI) within 3 days of culture on to MS medium

fortified with 5 μM COU. Scale bar = 1 mm.

3.3.2.3. Effect of COU for RAM development in bamboo using the SLCE method

In order to investigate the effect of COU for RAM development, I applied the established SLCE method as described in the Chapter II. In this experiment, different concentrations of COU, i.e. 0, 5, 50, and 500 μM, were added in a well alone (**see Figure 3. 12**) or in combination with 0.1 μM 2,4-D (**see Figure 3.13**) and evaluated its effect.

In overall evaluation, higher concentrations of COU (50 μM and 500 μM) showed abnormalities such as browning and slight callusing. On the other hand, 5 μM COU singly or in combination with 0.1 μM 2,4-D showed multiple root meristem development. Days to rooting and rooting percentage also indicated the promotive effect of 5 μM COU (**Table 3.3**). Highest rooting efficiency, five root forming shoots out of six tested shoots, with early RAM development was recognized in the COU condition.

To understand the long-term effect of COU, explants has been transferred to plastic culture box contained 50 ml of the same medium conditions. As shown in **Figure 3.13**, multiple rooting with lateral root formation was observed in the COU conditions. 5 μM COU singly showed higher number of roots with frequent lateral rooting also will

be clearer after 5 months of culture (**Figure 3.14**).

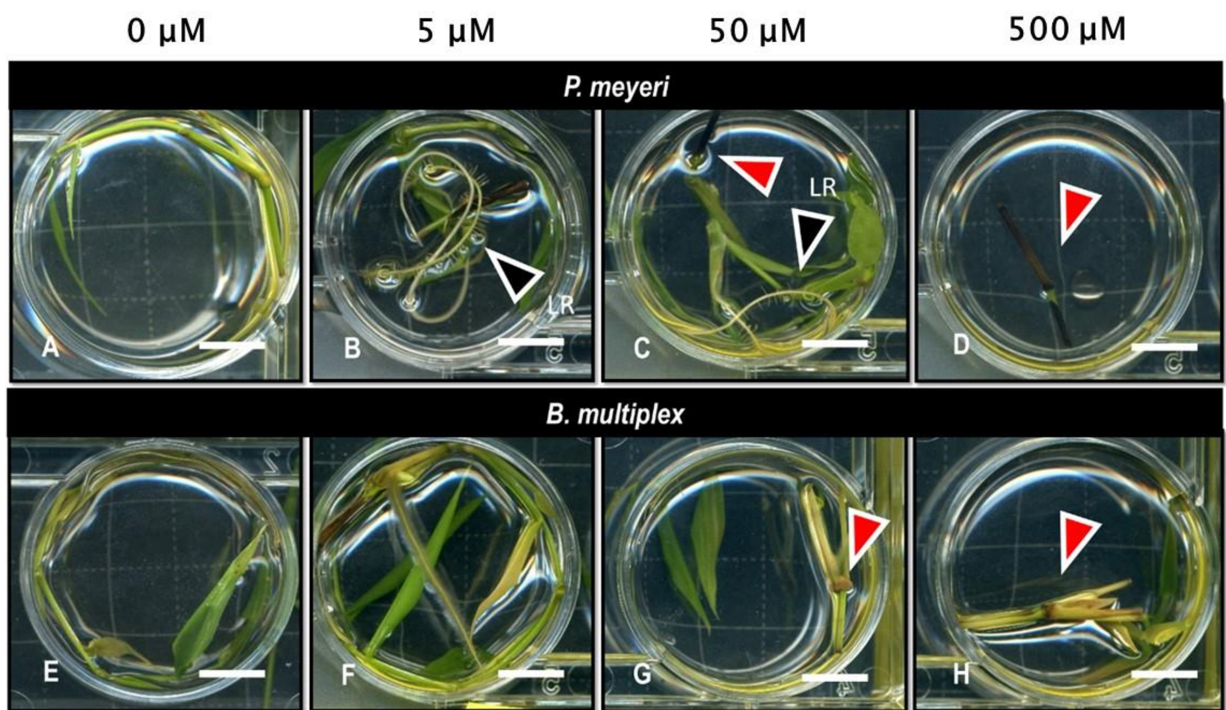


Figure 3.12. Effect of COU on bamboo node culture of *P. meyeri* and *B. multiplex* after 3 weeks of culture. A-D. 0 μM, 5 μM, 50 μM, 500 μM COU for *P. meyeri*. E-H, same concentrations in *B. multiplex*. Scale bar = 1 cm. Black colored arrowheads showed the positive growth characters and red arrowheads showed abnormal growth characters.

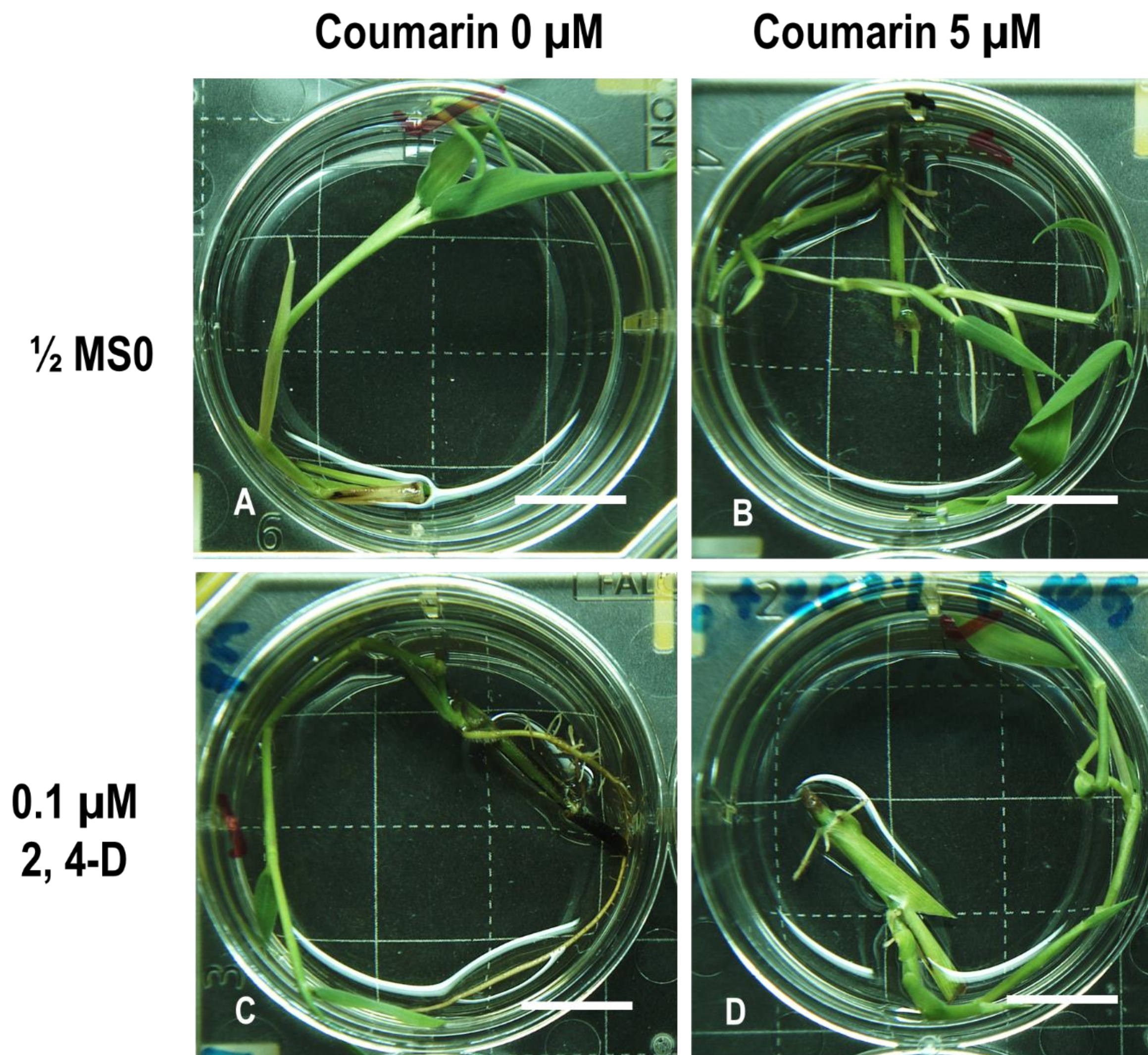


Figure 3.13. Effect of COU singly or in combination with 2,4-D in *P. meyeri*. A; 0 µM COU and B; 5 µM COU. C; 0.1 µM 2,4-D and D; 0.1 µM 2,4-D + 5 µM COU. Scale bar = 1 cm.

Table 3.3. Effect of COU on days to rooting and root formation in bamboo.

Treatment (μM)	Growth performance	
	Days to rooting (d) Mean ± SD	Number of root forming shoots/no. of shoot tested
Control (1/2 MS0)	Not detected	Not detected
5 μM COU	1.0 ± 0.63	5/6
0.1 μM 2,4-D	8.5 ±7.2	4/6
5 μM COU + 0.1 μM 2,4-D	1± 0.89	4/6

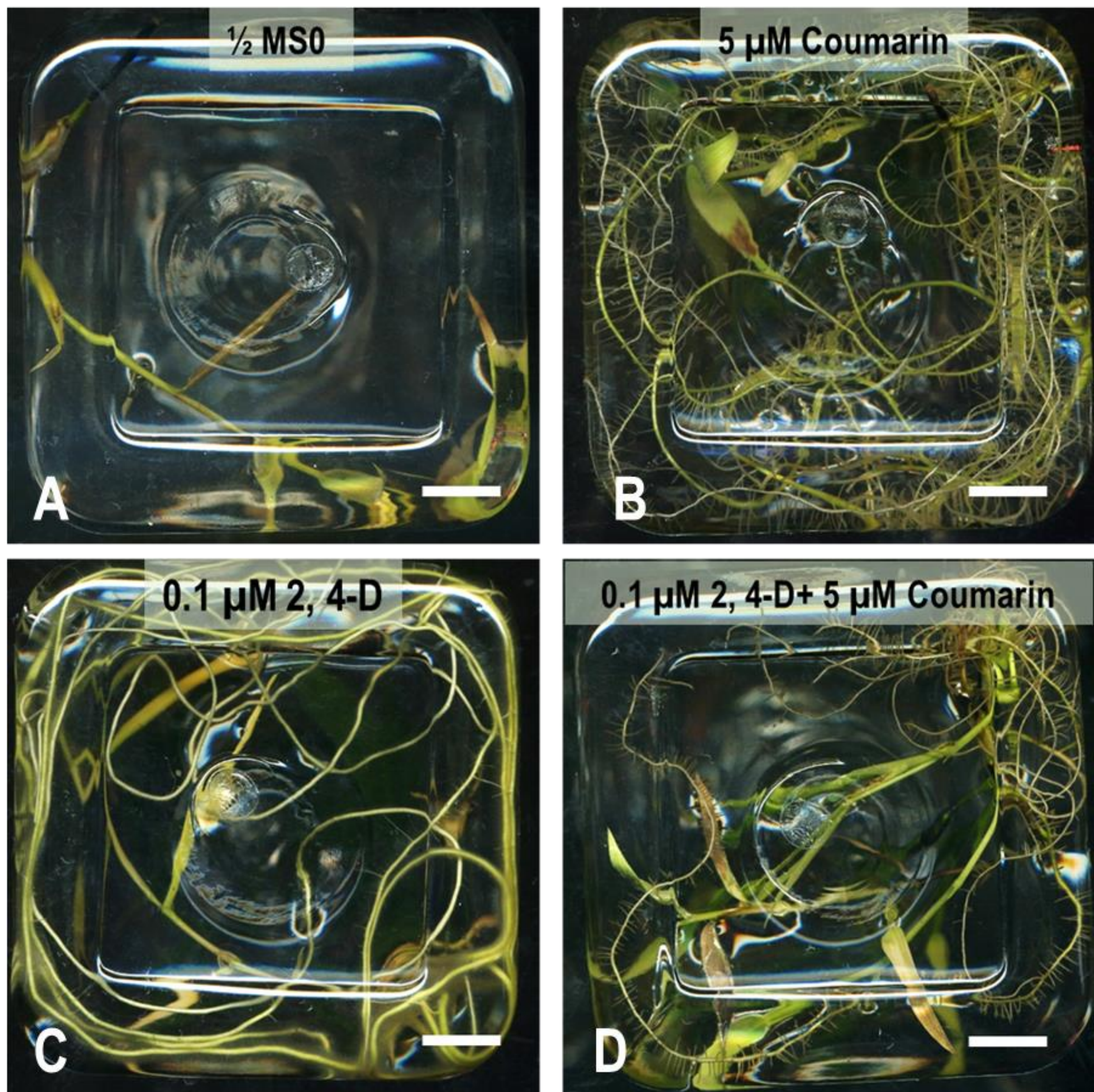


Figure 3.14. Effect of 5 µM COU singly or in combination with 2,4-D after 5 months. A; Control (1/2 MS0), B; 5 µM COU, C; 0.1 µM 2,4-D and D; 0.1 µM 2,4-D + 5 µM COU. Scale bar = 1 cm.

3.3.3. Establishment of a new protocol for *in vitro* SAM and RAM development in bamboo by adapting PG and COU treatments

Finally, I combined two regulatory factors, both PG and COU, to control *in vitro* SAM and RAM development in bamboo. Briefly, once multiplied shoots were produced by the PG treatment and its rescue culture program (see 3.3.1), a bunch of shoots was transferred to the COU containing medium (see3.3.2). Compared with the control condition, this new protocol showed a great capacity to multiply bamboo plants (**Figure 3.15**). To understand the long-term effect of a new protocol, all cultures tested were observed during 8 weeks of culture period. As shown in **Figure 3.16**, multiple rooting with lateral root formation was observed in the COU conditions.

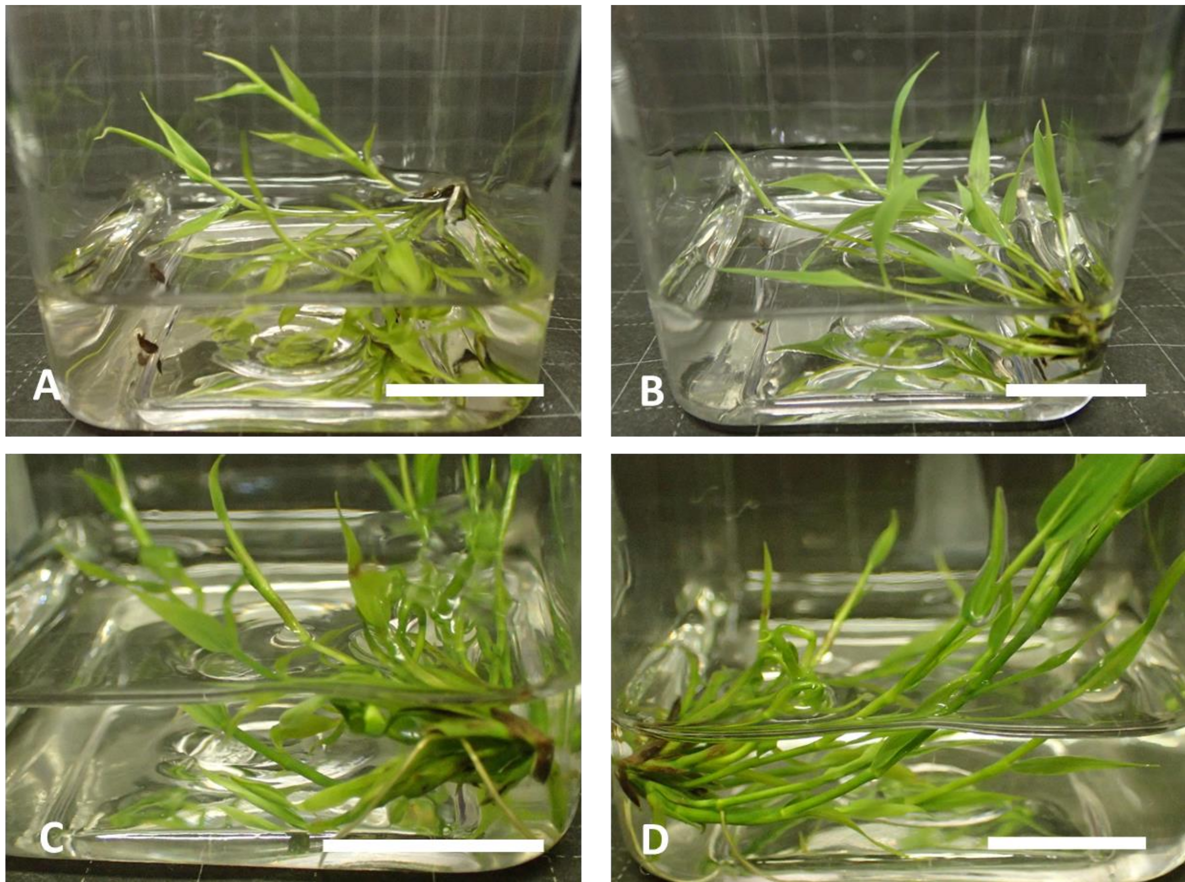


Figure 3.15. Effect of COU on PG treated multiplied brunch of bamboo. A; Control (1/2 MS0), B; 0.1 µM 2,4-D, C; 5 µM COU and D; 5 µM COU + 0.1 µM 2,4-D at 4 weeks. Scale bar = 2 cm.

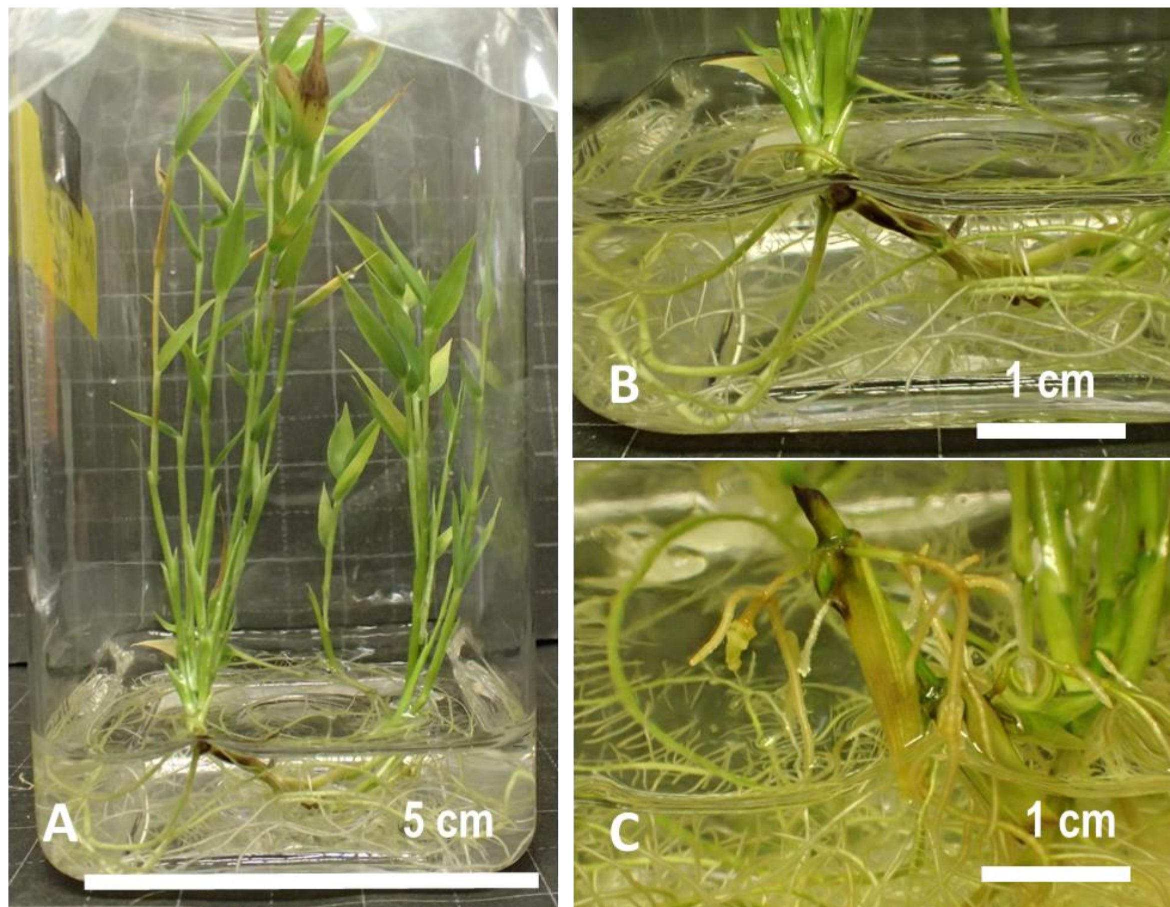


Figure 3.16. Detailed view of multiple root meristem development in COU on PG treated multiplied shoots after 8 weeks of culture. A; 5 µM COU containing *in vitro* culture box of bamboo and B; magnified view of node portion showing multiple rooting with lateral roots. C; Magnified view of node portion showing multiple rooting with callusing tips of lateral roots in 5 µM COU + 0.1 µM 2,4-D.

3.3.4. Application of a new protocol for *in vitro* SAM and RAM development in other model grass specie

The new protocol for *in vitro* bamboo SAM and RAM development (see 3.3.3.) was applied to other grass species, especially rice, barley and *Brachypodium* as follows.

3.3.4.1. Effect of PG on model grass species

Phloroglucinol 24 h treatment has been applied in rice, barley and *Brachypodium* and high frequency multiplication found (**Figure 3.17, D, E and F**). Where in control only single shoot has been elongated without multiplication (**Figure 3.17. A, B and C**).

3.3.4.2. Effect of COU on model grass species

A. Effect of different concentrations of COU on tiller development of rice and barley: Firstly, 0, 5, 50, 500 μM COU has been used to select the best formulation of rooting in bamboo. After selecting the best formulation, we applied that formulation on other grass species, especially rice, barley and *Brachypodium.* Lower concentration of COU 5 μM showed multiple root meristem development in rice and barley and 50 μM,

500 μM showed same result as bamboo (**Figure 3.19**).

B. Effect of COU on rice and *Brachypodium* plantlets: Developed tillers of rice and *Brachypodium* has been cultured on 5 μM COU ± 0.1 μM 2,4-D and here also difference between COU and 2,4-D containing medium has been noticed. 5 μM COU showed multiple roots with thin lateral root (**Figure 3.19, 3.20, A-B**) but in presence of 2,4-D roots became thicker than control and COU condition (**Figure 3.19, C-D**).

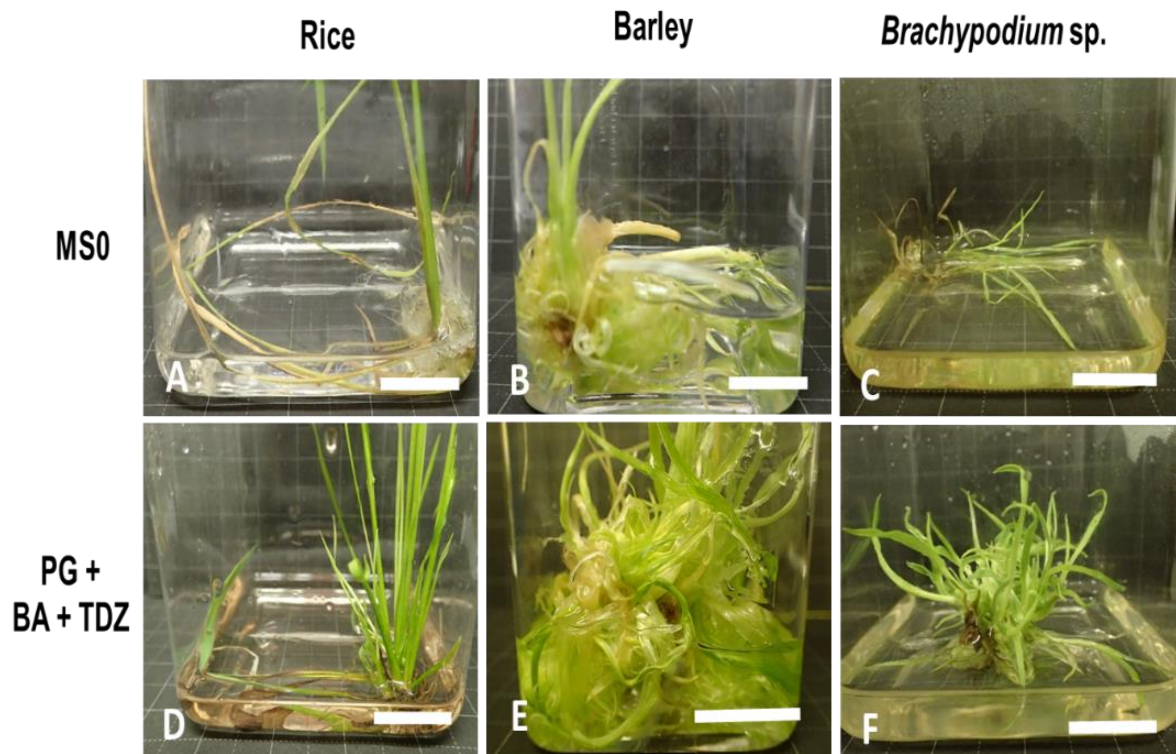


Figure 3.17. Shoot multiplication in PG + 10 µM BA + 3 µM TDZ in Rice, Barley and *Brachypodium* (C, F). A, B, C; are control conditions and D, E, F; are PG + 10 µM BA + 3 µM TDZ conditions of rice, barley and *Brachypodium,* respectively. Scale bar = 2 cm.

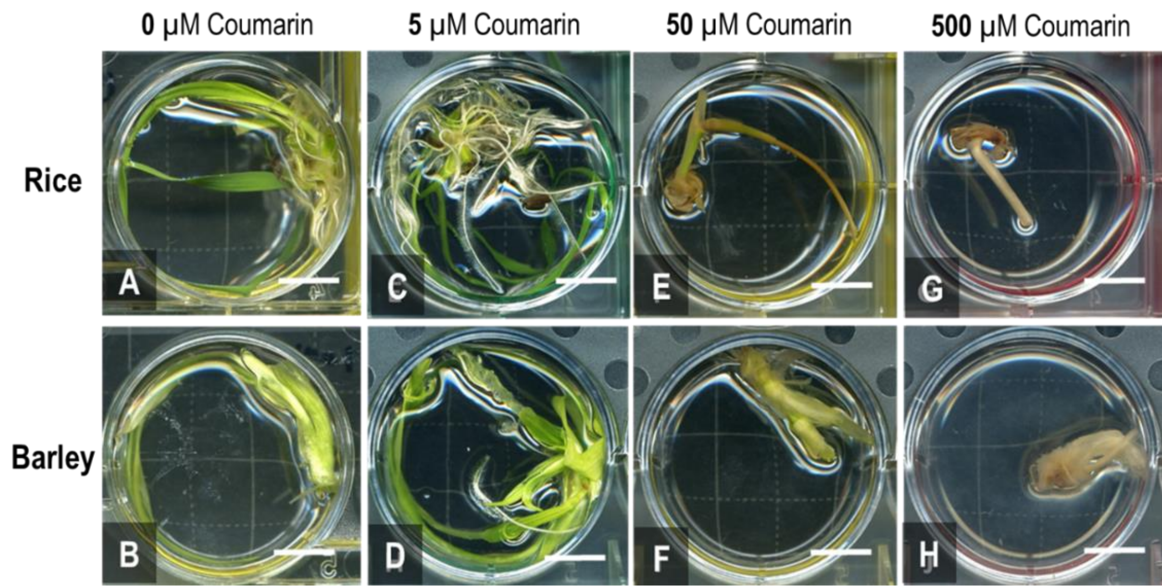


Figure 3.18. Effect of COU on seedling development of rice and barley. A, C, E, G; 0 µM, 5 µM, 50 µM and 500 µM, respectively in rice. B, D, F, H.; 0 µM, 5 µM, 50 µM and 500 µM respectively for barley. Scale bar = 1 cm.

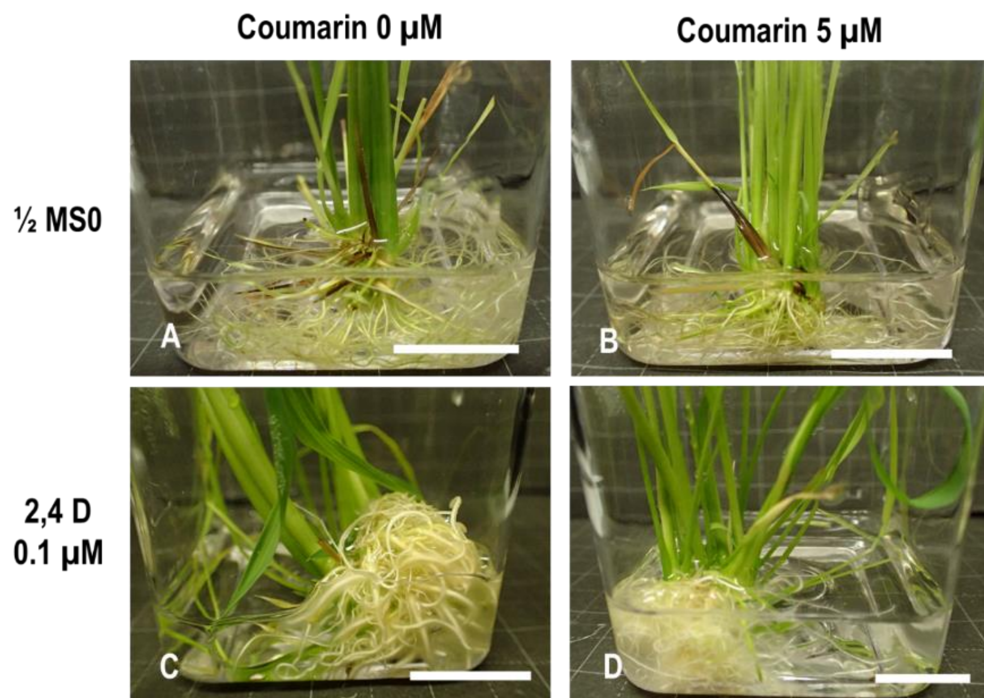


Figure 3.19. Effect of COU singly or in combination with 2,4-D on rice seedling. A; Control (1/2 MS0), B; 5 µM COU, C; 0.1 µM 2,4-D and D; 5 µM COU + 0.1 µM 2,4-D. Scale bar = 2 cm.

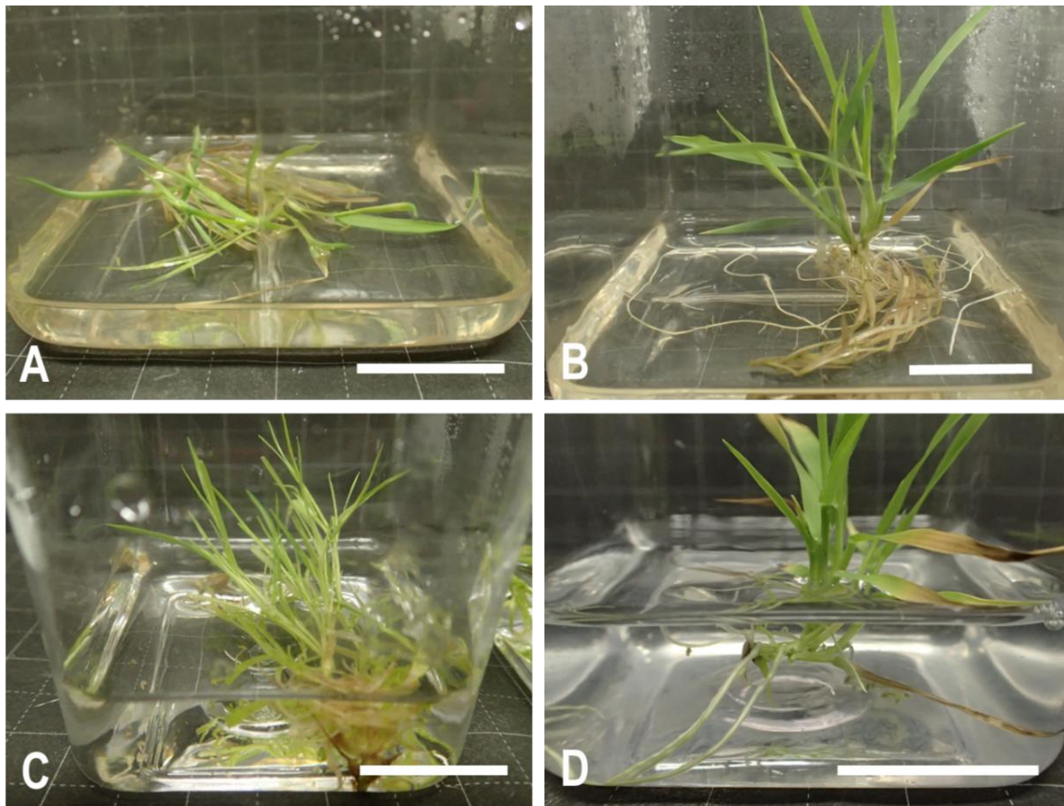


Figure 3.20. Effect of COU singly or in combination with 2,4-D on *Brachypodium* seedling. A; Control (1/2 MS0), B; 5 μM COU, C; 0.1 μM 2,4-D and D; 5 μM COU + 0.1 μM 2,4-D. Scale bar = 2 cm.

3.3.4.3. Comparison of effects of different PGRs and phenolic compound in bamboo, rice and *B. distachyon*

Effects of three different promising treatments (10 μM BA + TDZ 3 μM, PG pretreatment + 10 μM BA + 3 μM TDZ and MS + 5 μM COU) in bamboo, rice and *Brachypodium* has been compared (**Table 3.4**). In case of 10 μM BA + 3 μM TDZ, shoot multiplication sign has been noticed within 7 days of culture for all species and number of shoots increased with time. After 21 days of culture bamboo shoot growth became slow and number of shoot and shoot length not increased in case of bamboo but rice and *Brachypodium* showed increasement of shoot length and also have higher number of shoot than bamboo. On the other hand, when the node of bamboo treated with PG for 1-2 days and rescued in 10 μM BA + 3 μM TDZ medium, 30 times higher number of shoot multiplication has been found in bamboo with shoot branching. In rice and *Brachypodium,* first one week seems like inhibition of growth but after that shoot number has been increased than non-treated condition.

In case of COU treatment, multiple RAM developed within 1-3 days of culture for all species. Number of roots has been increased with time with lateral root formation. After three weeks of culture *in vitro* flower has been occurred in *Brachypodium* but rice

and bamboo also showed stopping leaf like structure, but flower formation don’t notice.

After six weeks of culture *in vitro* spikelet died.

Table 3.4. Comparison of effects of different PGRs and phenolic compound in bamboo, rice and *Brachypodium*.

Treatments	Species	Days of culture: 7	14	21	28	35	42	59
PGRs (10 µM BA + 3 µM TDZ)	BAM	Shoot multiplication occurred within 1 week	Number of shoot increased with time	Shoot growth is very slow				
	RIC	Shoot multiplication occurred within 1 week	Number of shoot increased with time	High rate of multiplication with bushy structure				
	BRA	Shoot multiplication occurred within 1 week	Number of shoot increased with time	High rate of multiplication with bushy structure				
PG treatment (500 µM PG + 10 µM BA + 3 µM TDZ)	BAM	Bud breaking within 1 DAC and sign of multiplication understandable		High rate of multiplication with bushy structure				
	RIC	Looks inhibition of SAM and RAM development	Become normal of SAM and RAM development and multiplication occurred			High rate of multiplication with bushy structure		
	BRA	Looks inhibition of SAM and RAM development	Become normal of SAM and RAM development and multiplication occurred			High rate of multiplication with bushy structure		
COU treatment (5 µM COU)	BAM	Multiple RAM developed within 3 DAC	Multiple RAM elongated with lateral rooting ***Stopping leaves, symbol of flowering has been occurred**					
	RIC	Multiple RAM developed within 3 DAC	Multiple RAM elongated with lateral rooting ***Stopping leaves, symbol of flowering has been occurred**					
	BRA	Multiple RAM developed within 3 DAC	Multiple RAM elongated with lateral rooting *** In vitro flowering/ spikelet formation**					Spikelet start dying

Here, BAM= bamboo (*P. meyeri*), RIC= rice and BRA= *Brachypodium*.

3.4. Discussions

In the present chapter, investigations on the effects of phenolics, such as phloroglucinol (PG) and coumarin (COU), on SAM and RAM development are described. The pretreatment of nodes with 500 µM PG for 24–48 h greatly enhanced the breaking of dormant buds. However, continuous PG treatment at a higher concentration induced intense browning of the tissue and the medium. For this reason, the pretreated node was immediately transferred into the MS medium supplemented with 10 µM BA and 3 µM TDZ. After rescue, the PG-treated node could produce 30-times higher number of shoots than that produced under the control condition (**Table 3.2, Figure 3.6**). COU (5 µM) was a promising trigger for enhanced development of dormant RAM. Interestingly, these simple but unique procedures were also very effective in controlling the development of other important grass species, such as rice and *Brachypodium*.

Teixeira da Silva *et al.* (2013) reported PG increases shoot formation when added to rooting media together with auxin, it further could stimulates rooting, most likely because PG and its homologues act as auxin synergists or auxin protectors. There are some reports on PG in tissue culture for root meristem development purpose (Tan *et al*. 2018, Perez *et al*. 2016, Londe *et al*. 2017, Jani *et al*. 2015). But till present there is

no reports on PG used for shoot meristem development on bamboo. PG is not well-known to many plant tissue culture scientists. Although PG is often used as a supplement to other plant growth regulators (PGRs) *in vitro*, it has rarely been the focus of tissue culture or developmental studies simply because its true effect has usually been masked by the presence of other, more commonly used PGRs. Various phenols are typically added to tissue culture media mainly to enhance callus growth, form adventitious shoots more effectively, improve rooting, and increase the rate of shoot proliferation in certain shoot cultures. Most plant responses to phenols involve a synergism with auxins, particularly indole-3-acetic acid (IAA), so it is likely that the mode of action is dependent on the regulation of internal IAA levels. Oxidative catabolism of IAA is the chemical modification of the indole nucleus or side chain that results in the loss of auxin activity (Teixeira da Silva *et al.* 2013).

In this present experiment, 5, 50 and 500 µM of PG has been applied and 500 µM proved to be the positive enhancer of bud breaking within 24 h (**Figure 3.2**). Due to continuous supply of higher concentration of phenolic compound it caused some abnormal growth characteristics (**Figure 3.3**). So, early initiated buds have been rescued to 10 µM BA + 3 µM TDZ condition that previously proved as a suitable treatment for

shoot multiplication. Frequency of multiplication and shoot number also changed dramatically in comparison with PG non - treated 10 μM BA + 3 μM TDZ derived explant. Same result also found in rice, barley and *Brachypodium*. PG treated node in 10 μM BA + 3 μM TDZ showed shoot multiplication 30 times higher number of shoots from a single node with well-developed root meristems than non-treated. On the other hand, non-treated only has 3 shoots from the single node within same time duration (Chapter II). As a result, by treating PG there were 500.5 ± 123 nodes/ explant, where in non-treated and control were 20 ± 3.74 and 4 ± 0.63 respectively (**Figure 3.4**). Another important finding, 10 μM BA + 3 μM TDZ (-PG) proved to be the suitable for shoot multiplication, but when it will pretreated (24 h-48 h) with PG it could not only multiply 30 times higher number of shoots, but also provided as a positive modulator of shoot branching (**Figure 3.6**). Shoot branching is a major determinant of plant architecture and is highly regulated by endogenous and environmental cues (Umehara *et al.* 2008). Two classes of hormones, auxin and cytokinin, have long been known to have an important involvement in controlling shoot branching (Umehara *et al.* 2008). Umehara *et al.* (2008) and Gomez- Gomez-Roldan *et al.* (2008) suggest that endogenous strigolactones or related compounds inhibit shoot branching in plants. But PG as a

precursor in the lignin biosynthesis pathway, to effectively control hyper-hydricity through the process of lignification, thus maximizing the multiplication rate of woody species and other species that are difficult to propagate (Teixeira da Silva *et al.* 2013). By application of Strigolactone and mutant study could be helpful to understand the molecular mechanism of PG for apical meristem development and branching in plant.

Another objectives of this present chapter was, regulation of the development of these dormant root meristems by using COU. In general, *in vitro* rooting in a target plant is required two-step regulation such as 1) root meristem formation on shoots by adding auxin, 2) elongation of roots. It takes a long time, e.g. more than 1-2 month(s) to develop a high efficient tissue culture protocol. In my present project, improved node culture system of bamboo plant and found that there are dormant root meristems in mature node portion of *in vitro* bamboo shoots. That's why, different 5 µM, 50 µM and 500 µM of COU alone or in combination with 2,4-D were tested and 5 µM of COU alone in showed to be the best trigger for early development of dormant root meristems in node portion within 1- 3 days of culture with highest rooting percentage and elongation of multiple roots in SLCE. But effects were little bit different in *P. meyeri* and *B. multiplex.* 50 µM, 500 µM showed different abnormalities on both species but these

concentrations formed lateral rooting in *P. meyeri*, but for *B. multiplex* 5 µM created single root elongation and 50 µM created callusing at base of node. And 500 µM caused explant browning and growth inhibition for both bamboo species. In rice and *Brachypodium*, 5 µM COU proved to be the positive enhancer of root meristem development. 5 µM COU also proved to be effective for lateral rooting or root branching in these grass species and when this concentration of COU combination with 2,4-D it caused callusing at the tip of the root meristem. There are some reports on COU for rooting in bamboo and other species (Sood *et al.* 1991, Mishra *et al.* 2008) but till present there is no reports that can perfectly describe the role of COU of RAM development on bamboo and other model grass species. COU can cause inhibitory affects to rice root system also has been reported by Mahmood *et al.* (2013) and Salvador *et al*. (2013). Lupini *et al.* (2014) reported, COU not only restored the lateral roots number to control value in presence of auxin transport inhibitors but also the mitotic sites number, suggesting that it is able to overcome the inhibitory effect of both auxin transport inhibitors positively acting on lateral root initiation and emergence of lateral root primordia.

Conclusion

Based on my thesis research on "Studies on versatile methods to control the development of shoot and root apical meristems of bamboo and some model grass species through plant cell tissue and organ culture techniques", it could be concluded as follows:

I focused on controlling morphological and histochemical responses of the shoot apical meristem (SAM) and root apical meristem (RAM) of bamboo node by using a simple and versatile liquid culture system. First, nodes of 11 different bamboo species that belong to seven major bamboo genera (*Bambusa*, *Dendrocalamus*, *Phyllostachys*, *Tetragonocalamus*, *Chimonobambusa*, *Pleioblastus,* and *Sasa*) were cultured using 2 mL per well of a liquid medium in a 6-well microplate to form a small-scale liquid culture environment (SLCE). The dormant lateral buds of all bamboo nodes resumed expanding and elongating has been noticed by using Sytox green- safranin staining and mitotic activity was calculated using a digital imaging analysis, which showed an increase of up to 1.2- to 3.8-fold in terms of the SG/SF ratio after 7 days in the culture in SLCE. Moreover, I used *in vitro* node cultures of two typical bamboo species, the sympodial type *B. multiplex* and monopodial type *P. meyeri* and noted the following: (1) since

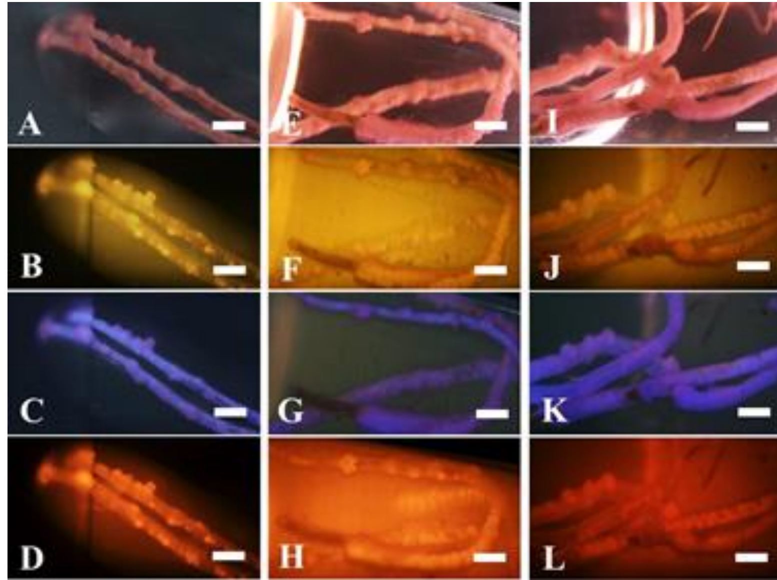
gradual white-to-green tinge shoots were observed, we investigated the relation between color variation in the outer regions of culm and node tissues and their suitability as explants. By checking the autofluorescence property of whole shoots under LED 365 nm illumination with an RGB (red, green, and blue) digital imaging analysis using ImageJ software, we specified the color variation of explants as the relative intensity of the blue value. (2) Since the obtained shoots of a 1-month-old culture box showed growth variation, I distinguished shoot types based on plant height, i.e., short (less than 5 cm), medium (ca. 5–10 cm), and tall (more than 10 cm). Tall shoots that have ca. 5 nodes on average were suitable for explant. (3) Difference in lignin deposition pattern around vascular bundle was observed by using Wiesner staining and measured the red staining regions by the digital image analysis. (4) Three types of node portions—the first node (the base node near a rhizome tissue), middle nodes (upper nodes near the 1st node), and the top meristem—were independently cultured in the SLCE, and it was found that the first node showed the best growth performance. (5) By culturing the first node in the SLCE system, I performed a quick survey during the 3 weeks in the culture and found that a combination of 10 μM BA and 3 μM TDZ was effective for *in vitro* SAM development, while the addition of 2,4-D was effective for promoting *in vitro*

RAM development. (6) The detailed autofluorescence properties of the outer regions of culm and node tissues were also identified using an inverted fluorescent microscope under B- and U-excitation lights with RGB and HSB (hue, saturation, and brightness) digital imaging analysis. (7) By applying phenolic compounds, phloroglucinol (PG) and coumarin (COU) with the SLCE system, I could establish the following new protocols to control SAM and RAM development. Briefly, the pretreatment step in 500 μM PG in 24 hours is essential to promote bud breaking of first nodes. The pretreated first nodes showed a great growth performance in terms of *in vitro* SAM development. Addition of 5 μM COU is also effective to promote an active *in vitro* RAM development within a short period. Furthermore, these new protocols were versatile enough to be adjusted to control SAM and RAM development in rice, barley and *Brachypodium.*

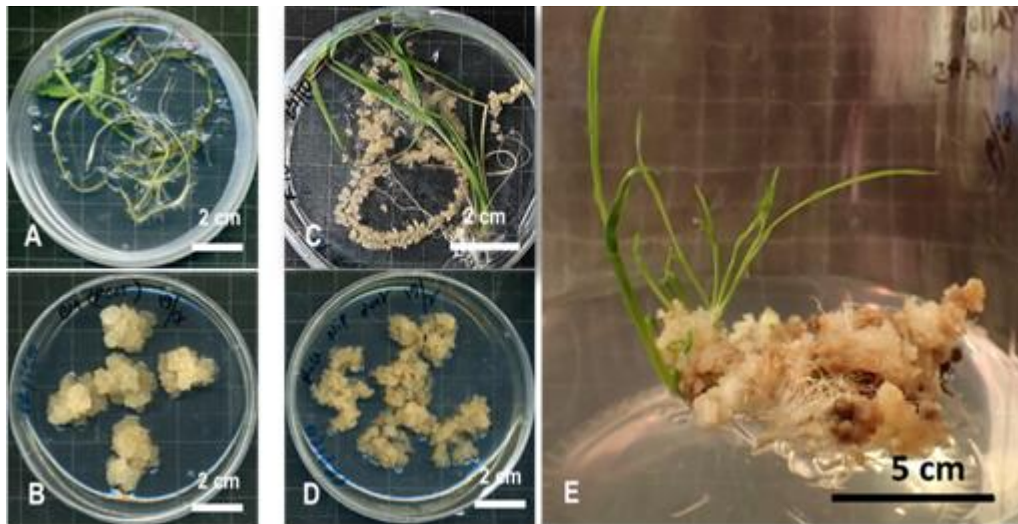
Perspectives

As perspectives of the new findings of my study, I introduce the following tips though these are still preliminary results. I have checked activation pattern of *in vitro* RAM development by using young rice seedlings. Rice seedlings were cultured in the SLCE system with 3 μM, 10 μM and 30 μM of 2,4-D and constantly observed under an inverted

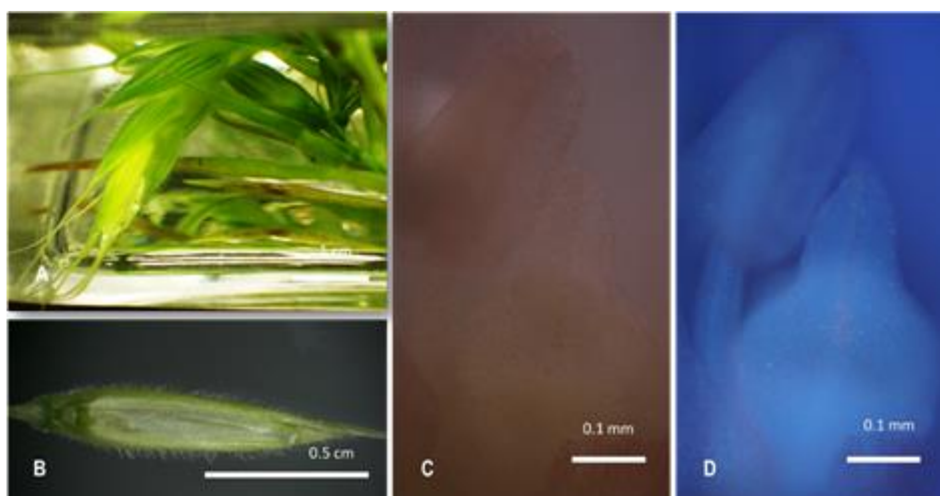
microscope. Interestingly, within 7 days of culture, a large number of nodular structures in the inward of root tissue could be seen. By staining with safranin, I could identify the formation of lateral root primordia under a fluorescent microscopy (**Perspective Figure 1**). One application of this growth capacity is to establish an efficient induction of organogenesis from root derived callus as shown in **Perspective Figure 2**. Rice, bamboo and *B. distachyon* seedlings were cultured according to the method described above tasted in MS medium fortified with 10 µM BA + 3 µM TDZ and 10 µM KIN + 5 µM NAA. 10 µM KIN + 5 µM NAA created shoot regeneration in rice (**Perspective Figure 2, E**). Other species also showed positive responses in terms of organogenic callus induction (data not shown). These techniques will use for further researches in the field of stem cell biology and molecular biology. Possibility of *in vitro* flowering has been realized from the COU experiment as shown in **Perspective Figure 3**. With further detailed analysis in relation to regulation mechanism of *in vitro* flowering, this technique will be a successful application to open a new window for cell manipulation and genetic transformation of bamboos and other grass species.



Perspective Figure 1. Fluorescent microscopy of lateral root tissues of rice cultured on 3 μM (A-D), 10 μM (E-H) and 30 μM (I-L) of 2,4-D. Safranin staining has been applied to identify root primordia formation. Bright field (A, E, I), B-excitation light (B, F, J), U-excitation light (C, G, K) and G-excitation light (D, H, L). Scale bar = 1 mm.



Perspective Figure 2. Organogenic callus induction from lateral RAM and shoot regeneration. Lateral root primordia induction in loot tissues of *B. multiplex* (A) and *O. sativa* cv. Nipponbare (C). One-month old callus of *B. multiplex* (B) and *Oryza sativa* cv. Nipponbare (D) in modified MS (680 mg/L KH_2PO_4) medium + 10 µM Picloram. Shoot regeneration from callus of *O. sativa* (E) in MS + 10 µM KIN + 5 µM NAA.



Perspective Figure 3. *In vitro* flowering of *B. distachyon* in MS medium with COU treatment. *In vitro* plantlet with Spikelet in MS liquid medium (A) and morphological characteristics of single flower under stereo microscope (B). Florescent microscopy of spikelet stained with Safranin under bright field (C) and under U-excitation light (D), showing an anther and an ovule.

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List of Publications

1. Ara, Most Tanziman; Nomura, Taiji; Kato, Yasuo; Ogita, Shinjiro (2020) A versatile liquid culture method to control the development of shoot and root apical meristems of *in vitro* bamboo plants. *American Journal of Plant Sciences*. **11**: 262–275.

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