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Full Length Research Paper

Induction of somatic embryogenesis and plant regeneration in the reed grass (*Phragmites communis* Trin.)

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An *in vitro* culture system for the large-scale propagation of *Phragmites communis* Trin. (reed) was established by optimizing culture conditions for callus induction and differentiation together with plant propagation using regenerated plantlets. Callus was induced from stem segments with callus induction medium containing auxin: 4-fluorophenoxyacetic acid (4-FA) or 2,4-dichlorophenoxy acetic acid (2,4-D). A high frequency of callus induction was observed at relatively low concentrations (0.5 and 1 mg L^{-1}) of both 2,4-D or 4-FA. However, high concentrations (3, 4 and 5 mg L^{-1}) of either auxin suppressed callus induction. When applied for the first time, 1.0 mg L^{-1} 4-FA markedly improved the frequency of callus induction (up to 93%). The callus was then transferred to MS medium supplemented with 1.0 mg L^{-1} 2,4-D to promote the formation of embryogenic calli. The calli were grown in MS supplemented with different concentrations of 2,4-D and naphthaleneacetic acid (NAA) alone or in combination with benzyladenine (BA). After seven weeks of culture, the regeneration efficiency was determined for the calli maintained for the 45 differentiation media formulations. The highest regeneration capacity was obtained from the medium containing 0.05 mg L^{-1} NAA and 2 mg L^{-1} BA, and the combination of 0.2 mg L^{-1} NAA and 2 mg L^{-1} BA. Propagation of the regenerated plantlets was also examined in medium containing different sucrose concentrations; this experiment found that 60 g L^{-1} sucrose showed the best growth rate. These improved regeneration and propagation systems could be used for bioreactor-based mass propagation or an *in vitro* culture system, and would be useful for transformation in *Phragmites communis* Trin.

Key words: *Phragmites communis* Trin, callus, regeneration, propagation, *in vitro* culture, 2,4-dichlorophenoxy acetic acid, 4-fluorophenoxyacetic acid.

INTRODUCTION

Reed grass (*Phragmites communis* Trin.) is commonly found worldwide due to its adaptability under diverse environmental conditions such as salinity, drought and heavy metal pollution (Hronec and Hajduk, 1998). This plant has important ecological functions because it is a perennial, fast growing grass with high-yield potential that provides a habitat for many wildlife species. With recent attention to environmental problem, natural purification of wastewater using wetlands and aquatic macrophytes is in the limelight as an alternative to conventional wastewater treatment. Especially, reed grass is one of the most important and abundant species among the aquatic macrophytes used for wastewater purification, and it has been applied for reducing pollution in industrial, domestic and agricultural wastewater (Kern and Idler, 1999; Wood

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et al., 2007; Zhao et al., 2008, 2009). Macrophyte varieties, especially reeds, can also be used for riverside environment enhancement applications.

Increased attention focused on the use of food crops such as maize, cassava and sorghum as biofuels has led to food shortages and increased food prices. Biomass resources that do not involve food-based biofuels would ease these problems. Reed plants may serve as a potential bioenergy source because they are globally ubiquitous, have a high-yield potential and can be cultivated without fertilizer, irrigation or pesticides (Sathitsuksanoh et al., 2009).

All these uses of reeds require the large-scale regeneration and propagation system of these plants. Currently, the propagation of wild reeds is mainly accomplished by spreading rhizomes, a technique that is not suitable for mass propagation. Micropropagation via plant tissue culture is an alternative way to produce large numbers of plant in a small space within a short time throughout the year in a controlled environment. A number of studies have published the *in vitro* propagation of reeds via tissue culture since *in vitro* reed tissue culturing was first reported (Sangwan and Gorenflot, 1975). In these previous studies, reed propagation mainly involved the differentiation of somatic embryos derived from various reed tissues such as stem nodes (Máthé et al., 2000; Yang et al., 2003), seeds (Cui et al., 2002; Lee et al., 2006; Straub et al., 1988; Wang et al., 2001) and immature inflorescence (Lauzer et al., 2000). In these studies, 2,4-dichlorophenoxy acetic acid (2,4-D) was commonly used to induce embryogenic callus formation and plant regeneration by shoot differentiation through the regulation of synthetic growth regulators. However, callus induction and maintenance, plantlet development after shoot formation and plantlet-based propagation system are as important as shoot differentiation from calli for continuous mass propagation throughout the year. This study describes the induction and maintenance of calli using 2,4-D and 4-fluorophenoxyacetic acid (4-FA), and the establishment of a plantlet-based regeneration and propagation system in the reed grass.

MATERIALS AND METHODS

Plant material and callus induction

Reed plantlets (*P. Communis* Trin. 'Daedong') regenerated from callus stock and grown *in vitro* were used in this study. Stem segments with node excised from axenic plants were used for culturing to induce callus formation. For callus induction, nodal stem segments approximately 1 cm long were cultured on MS medium (Murashige and Skoog, 1962) containing 30 mg L^{-1} sucrose and 3 g L^{-1} Gelrite supplemented with growth regulator (2,4-D and 4-FA). The pH of the medium was adjusted to 5.8 before autoclaving at 120°C for 10 min. To investigate the effects of 2,4-D and 4-FA on callus induction, stem segments were incubated on MS medium containing 0, 0.5, 1, 2, 3, 4 or 5 mg L^{-1} of 2,4-D or 4-FA in the dark at 25°C for six weeks. Subculturing was carried out every three weeks. Each plate contained 10 explants and 10 plates were used for each concentration of 2,4-D and 4-FA. The frequency of callus formation was determined after six weeks of culture. The calli were then excised from the explants and transferred to MS medium supplemented with 1 mg L^{-1} 2,4-D. The calli were then incubated in the dark at 25°C and subcultured every three weeks until they were used for the differentiation test.

Plant regeneration and transfer to soil

Plant regeneration was investigated in the presence or absence of a growth regulator. The calli were incubated on MS medium containing 30 mg L^{-1} sucrose and 3 g L^{-1} Gelrite supplemented with 2,4-D (0, 0.05, 0.1, 0.5 or 1.0 mg L^{-1}) and NAA acid (0, 0.05, 0.1, 0.2 or 0.5 mg L^{-1}) either alone or in combination with benzyladenine (BA) (0, 0.5, 1.0, 1.5, or 2.0 mg L^{-1}). In total, 45 different medium compositions were tested. The calli were incubated at 25°C with a 16 h photoperiod and subcultured every three weeks. After about four weeks of culture, green somatic embryos were observed. After seven weeks of culture, the number of calli forming shoots and the number of shoots formed from callus were counted to determine the regeneration efficiency. Regenerated plantlets were transferred to magenta vessels containing MS medium without any growth regulator. After seven weeks of culture, regenerated plants approximately 10 cm in height were transplanted into pots containing gardening soil. The pots were placed in a growth room maintained to acclimate the plant at 24 ± 1°C with a 16 h light/8 h dark cycle for two weeks prior to transfer to the greenhouse.

Propagation using regenerated plantlets

Propagation was carried out using MS medium in the presence or absence of gelling agents (agar and gelrite) containing varying concentrations of sucrose (0, 10, 20, 30, 40, 50, 60, 70 or 80 g L^{-1}). MS medium containing solid MS medium for propagation contained 3 g L^{-1} Gelrite or 8 g L^{-1} agar, and the pH of the medium was adjusted to 5.8 before autoclaving at 120°C for 10 min. Stem segments with nodes were excised from regenerated plants and cultured on these media. Plantlets were propagated at 25°C with a 16 h photoperiod. During the culture, shoots and roots were successfully formed from the nodal segments and developed into whole plants. After six weeks in culture, plant height and fresh weight were measured and used to determine the best conditions for reed plant propagation and growth of reed grass.

Statistical analysis

Callus induction rate, regeneration result and the growth data were statistically analyzed using Duncan's multiple range test using SPSS for window version 18.0.

RESULTS AND DISCUSSION

For an efficient callus induction, stem segments with nodes excised from *P. communis* Trin. 'Daedong' were incubated in MS basal medium supplemented with different concentrations of 2,4-D or 4-FA, and callus formation was examined (Figure 1 and Table 1). Callus formation from stem tissue was induced by both 2,4-D and 4-FA in a concentration-dependent manner (Figure 1). Stem segment explants cultured on auxin-free medium, regenerated shoots and roots via adventitious

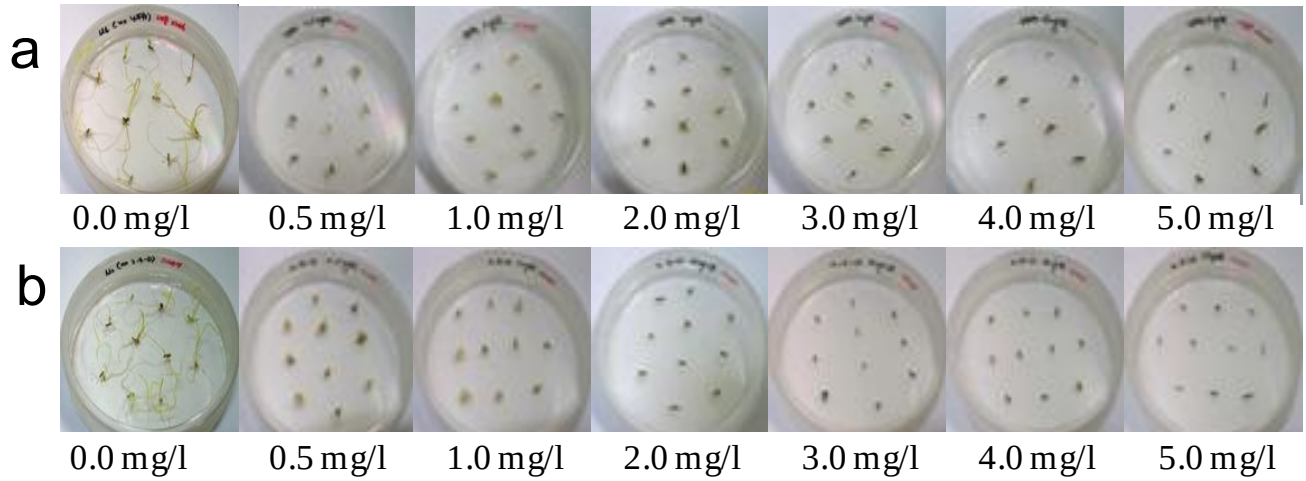

Figure 1. Effect of different concentrations of 2,4-D and 4-FA on callus formation from stem tissue of *P. communis* Trin.'Daedong'. a, 4-FA treatment; b, 2,4-D treatment.

Table 1. Effect of 2,4 D and 4-FA on callus formation from nodal stem tissue of *P. communis* 'Daedong' in MS medium for six weeks.

Parameter	Number of inoculated explant	Number of explants with callus	Efficiency of callus formation (%)
2,4-D (mg L^{-1})			
0.5	10	7.2 ± 1.3b[a]	72
1.0	10	7.1 ± 1.0[b]	71
4-FA (mg L^{-1})			
0.5	10	8.8 ± 0.8[a]	88
1.0	10	9.3 ± 0.7[a]	93

Values represent the mean ± S.D; [a]Mean separation within columns by Duncan's multiple range test, at 0.05 level.

buds on the surface of the stem segments. However, these plantlets eventually died from lack of light due to the fact that they were incubated in the dark, a condition which was required for callus induction and growth. Effective callus induction was observed when stem tissue was incubated on medium containing the auxin. A high frequency of callus induction in stem explants was observed with relatively low concentrations of both auxins (0.5 and 1 mg L^{-1}), but higher concentrations suppressed callus induction.

The appearance of the formed calli differed depending on the auxin on the medium. Friable, light-yellowish and large calli formed in the presence of 2,4-D, whereas yellow, hard, compact and large calli were produced using 4-FA. Calli formed using 2,4-D resulted in a higher frequency of plant regeneration as compared to those formed using 4-FA. However, callus formation efficiency and calli yield of the explants was significantly increased in the presence of 4-FA as compared to 2,4-D (Table 1). Furthermore, hard and compact calli formed in the presence of 4-FA were converted to friable and light-yellowish calli suitable for plant regeneration when the calli were transferred to MS medium containing 1 mg L^{-1} 2,4-D.

The effect of 2,4-D on callus induction in various plant tissues has been reported by many researchers; previous studies also used 2,4-D for inducing callus formation in reed tissues (Cui et al., 2002; Lauzer et al., 2000; Lee et al., 2006; Máthé et al., 2000; Yang et al., 2003). In reed plants, only 2,4-D was used alone or in combination with other plant growth regulators to induce callus formation since *in vitro* reed tissue culturing was introduced (Sangwan and Gorenflot, 1975). In our study, callus induction from reed explants was also observed at a high frequency in the presence of 0.5 and 1 mg L^{-1} 2,4-D, and approximately 70% of the explants produced calli (Table 1).

In comparison, this study is the first report that introduces 4-FA for promoting callus formation in reed plants. Our results show that this compound markedly

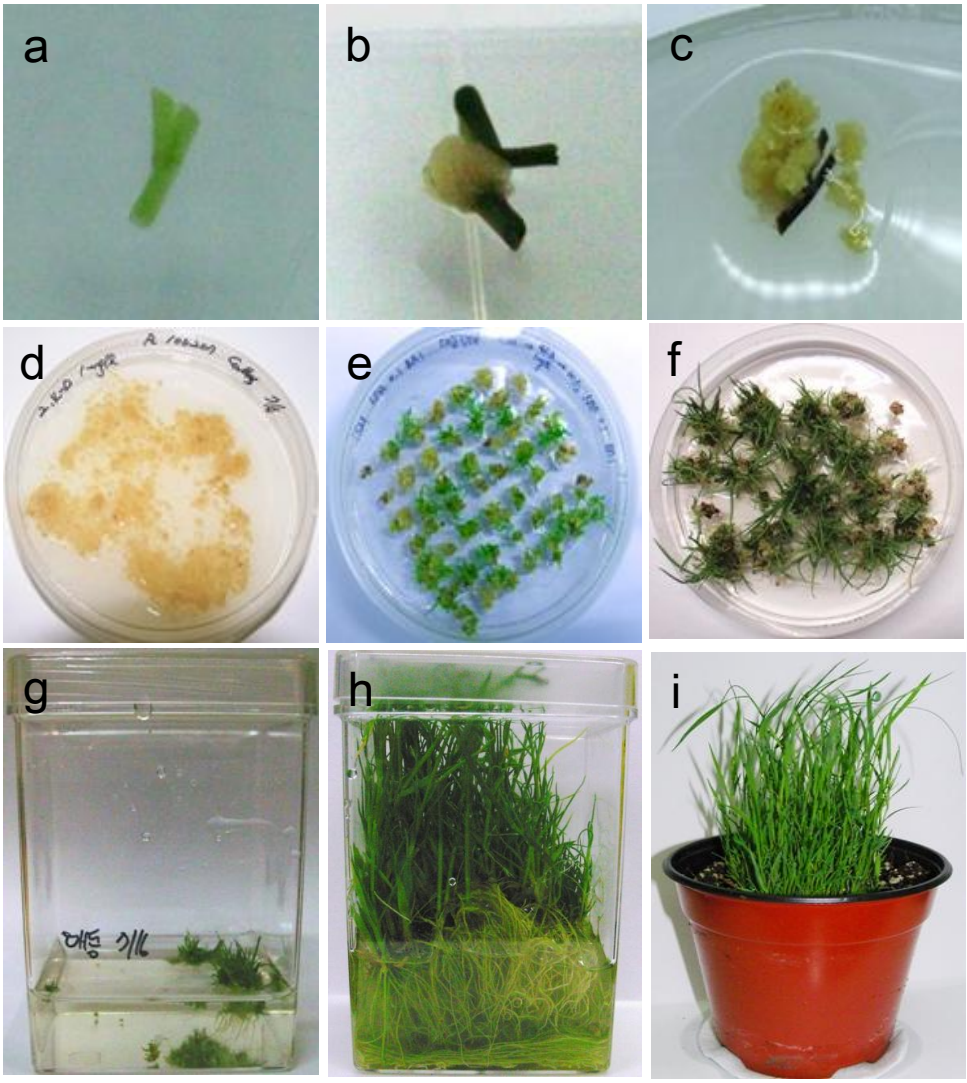

Figure 2. *P. communis* Trin.'Daedong' regeneration. **A,** Stem nodal segment grown in callus induction medium; **B,** callus formation from an explant after three weeks of culture; **C,** callus formation after eight weeks of culture; **D,** embryogenic callus formation and maintenance in 2,4-D medium; **E and F,** regeneration of plants grown from callus in the regeneration medium; **G and H,** the growth of regenerated plants in the growth medium; **I,** plant appearance after been transplanted to soil.

improved the callus induction efficiency from a reed explant up to 93% (Table 1). 4-FA has been mainly used for inducing callus formation in sweet potato plants, although its influence on callus induction has been tested in several species including rice (Yasuda et al., 1990), sugarcane (Brisibe et al., 1994) and onions (Tanikawa et al., 1998). In rice, 4-FA was found to be superior in inducing callus formation and maintaining regeneration abilities as compared to 2,4-D (Yasuda et al. 1990). Although, the regeneration ability of calli produced in the presence of 4-FA was lower than those developed in medium containing 2,4-D in this study, this ability was recovered when the calli were subculture in medium supplemented with 2,4-D. Thus, the combination of callus induction using 4-FA, which can be used to obtain a large number of calli, and subculturing on 2,4-D medium appeared to be suitable for the regeneration of reed plant needed for mass propagation.

Figure 2 shows callus induction and plant regeneration from reed stem explants. Calli were induced on the abaxial surface of the explants; these were initially white and translucent, and then became yellow when grown in MS medium supplemented with 1 mg L^{-1} 4-FA (Figure 2a to c). After six weeks of culture, calli were excised from the explants and transferred to MS medium supplemented with 1 mg L^{-1} 2,4-D. During callus culture in the 2,4-D medium, the hard and compact calli produced from the medium supplemented with 4-FA turned into friable and bright yellow calli (Figure 2d).

MS medium with 1 mg L^{-1} 2,4-D was used for callus

maintenance and growth in this study. In some previous studies, MS medium supplemented with 2,4-D was used for reed callus maintenance and growth (Cui et al., 2002; Lauzer et al., 2000; Yang et al., 2003). Cui et al. (2002) reported that the optimal concentrations of 2,4-D for maintaining embryogenic calli were 0.5 to 1 mg L^{-1} for dune reeds and 2 to 3 mg L^{-1} for swamp reeds. Furthermore, Lauzer et al. (2000) showed that MS medium containing 1 mg L^{-1} 2,4-D was effective for promoting the formation of embryogenic callus. This medium was effective for embryogenic calli proliferation but not regeneration. Embryogenic calli grown in the maintenance medium retained a high regeneration potential for at least six months, but their regeneration was markedly decreased after long-term subculturing (that is, >1 year; data not shown). Moreover, albino plantlets were often produced from the regeneration of older reed calli.

Embryogenic calli were transferred to the regeneration medium for shoot and root regeneration. After about two to three weeks of culture in the regeneration medium, green somatic embryos were observed on the surface of the calli and successfully developed to plantlets (Figure 2e to h). Previous studies found that low concentrations of auxin leads to the formation of somatic embryos (Lee et al., 2006; Yang et al., 2003). However, the addition of auxin negatively affects reed plantlet regeneration (Wang et al., 2001). Thus, hormone-free MS medium is often used as a regeneration medium in reed (Cui et al., 2002; Lauzer et al., 2000; Máthé et al., 2000; Straub et al., 1988).

The combination of auxins with cytokinines is effective for plant regeneration; higher concentrations of cytokinin and lower auxin levels are commonly used as excellent stimulators of shoot and root development. In this study, the effects of various hormone combinations on the regeneration potential of reed calli were investigated (Figure 3 and Table 2). Shoot formation was strongly inhibited by 2,4-D, regardless of the concentration used (Figure 3). No plantlets regenerated from calli grown on media supplemented with 2,4-D alone or in combination BA. This negative effect of 2,4-D on plant regeneration was previously described (Lauzer et al., 2000); the authors reported that calli maintained on a medium containing 2,4-D never develop into plantlets even if the embryos and calli turn green. It appears that 2,4-D is unsuitable as a supplement in regeneration medium for the formation of shoots and roots at least during reed regeneration. When grown on the hormone-free MS medium, embryogenic calli developed into plantlets via somatic embryogenesis (Figure 3). The addition of BA alone or in combination with NAA could be used for generating shoots (Figure 3). A high number of calli that formed shoots was observed in the presence of MS medium containing 1 mg L^{-1} BA, 0.2 mg L^{-1} NAA and 2 mg L^{-1} BA, as well as in hormone-free MS medium (Table 2). Furthermore, shoot formation per callus was greatest on MS medium supplemented with 0.05 mg L^{-1} NAA and 2 mg L^{-1} BA, with an average of 14.6 plantlets regenerated from the calli. In this study, the highest regeneration capacity was found among plantlets grown on the medium containing 0.05 mg L^{-1} NAA and 2 mg L^{-1} BA. The combination of 0.2 mg L^{-1} NAA and 2 mg L^{-1} BA also showed excellent plant regeneration efficiency (Table 2).

Reed plants can be propagated through *in vitro* culture of nodal stem segments with axillary primordial buds. The effects of sucrose concentration and gelling agents on plantlet growth were investigated in this propagation system (Figure 4). Plant growth was significantly affected by the different kinds of gelling agents. Liquid cultures lacking gelling agents were superior to solid cultures using agar or Gelrite as a gelling agent for increasing plant growth (Figure 4). Among the solid media tested, the overall growth and fresh weight were higher among plantlets grown in Gelrite than the ones maintained in agar. Lee et al. (2006) reported a difference in the effect of gelling agents (agar and phytagel) on plant regeneration and growth of plantlets during *in vitro* reed culture. They hypothesized that this resulted from the degree of hyperhydricity induced by different gelling agents. In our study, the reed plants also grew best in liquid culture. These results emphasize the importance of medium selection.

Sucrose in the medium also affected plant height and fresh weight, especially with the plants grown in liquid medium (Figure 4). In liquid cultures, plant growth markedly increased together with sucrose contents, but growth was reduced in sucrose concentrations greater than 60 g L^{-1}. As the principle carbohydrate source, sucrose affects callus formation and plant regeneration (Chen, 1978; Chen et al., 1998; Khan et al., 2006; Shahnewaz and Bari, 2004). Since sucrose acts as an osmotic agent as well as a carbon source, its influence is likely related to shoot and root formation from stems with nodes and early growth of reed plants. In this study, liquid medium without gelling agents and supplemented with 60 g L^{-1} sucrose had the best growth of the reeds, and may be useful for mass propagation using bioreactors or *in vitro* culture systems.

In conclusion, we developed an efficient regeneration system from nodal stem-derived calli and a propagation system using regenerated plantlets for the large-scale production of reeds, an environmentally important wetland plant species. The addition of 4-FA to the plant culturing media markedly improved callus formation efficiency, while 2,4-D was suitable for the maintenance and growth of the calli. The regeneration efficiency was best in the presence of the medium containing 0.05 mg L^{-1} NAA and 2 mg L^{-1} BA, or with a combination of 0.2 mg L^{-1} NAA and 2 mg L^{-1} BA. For propagation of the regenerated plantlets, liquid medium without gelling agents and supplemented with 60 g L^{-1} sucrose produced the highest growth rate. These regeneration and

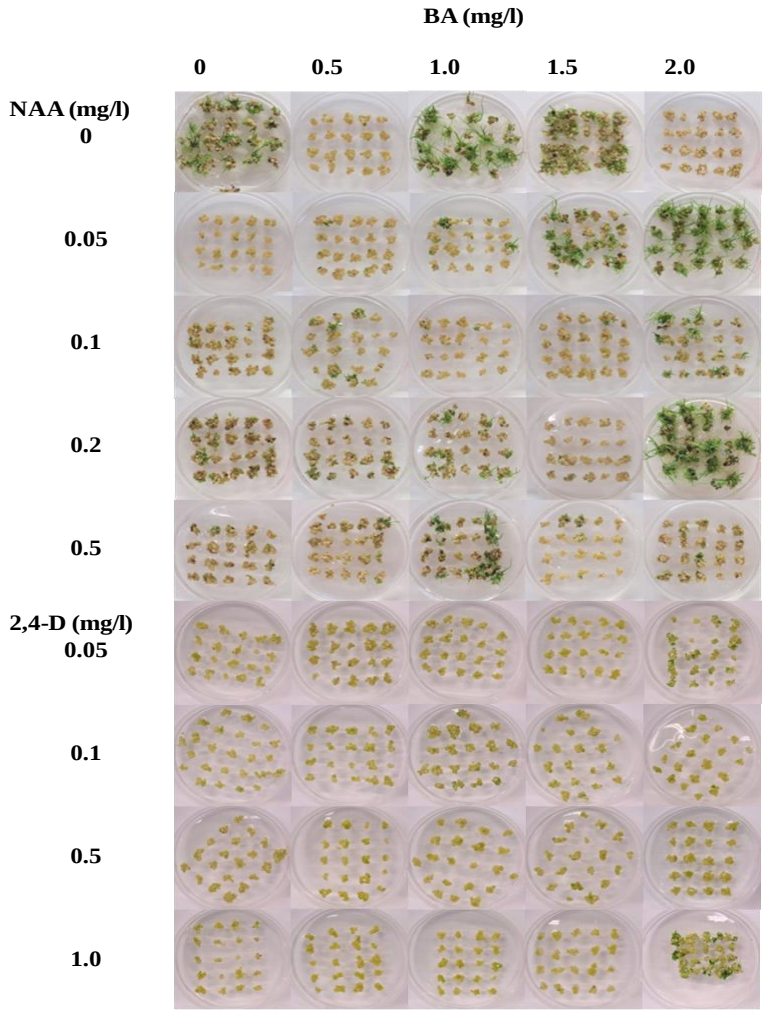

Figure 3. Effect of various plant growth regulators on regeneration from the calli. Data were recorded after seven weeks of culture.

Table 2. Effect of different combinations of BA and NAA on shoot regeneration from calli derived from nodal stem explants of *P. communis* Trin. 'Daedong'.

NAA (mg L^{-1})	BA (mg L^{-1})				
	0	0.5	1	1.5	2
Number of shoots from calli					
0	17.7ab[a]	-[b]	17.0abc	13.7b-e	-
0.05	-	11.0d-i	6.0jkl	13.0c-f	14.7a-d
0.1	-	8.3g-l	8.7f-k	4.0l	14.0b-e
0.2	11.7d-h	7.0i-l	10.0e-j	5.0kl	18.7a
0.5	6.0jkl	-	12.7d-g	7.7h-l	10.3d-j
Number of shoots formed per callus					
0	9.8bcd	-	11.2abc	7.6b-e	-
0.05	-	2.6fg	3.5efg	11.4abc	14.6a
0.1	-	2.5fg	4.3efg	1.2g	7.2c-f
0.2	5.3d-g	1.6g	2.4g	3.3efg	11.2abc
0.5	2.0g	-	10.3abc	12.1ab	11.6abc

Values represent the mean; [a]those followed by the same letter are not significantly different at the 0.05 level of confidence using Duncan's multiple range test; [b]not detected.

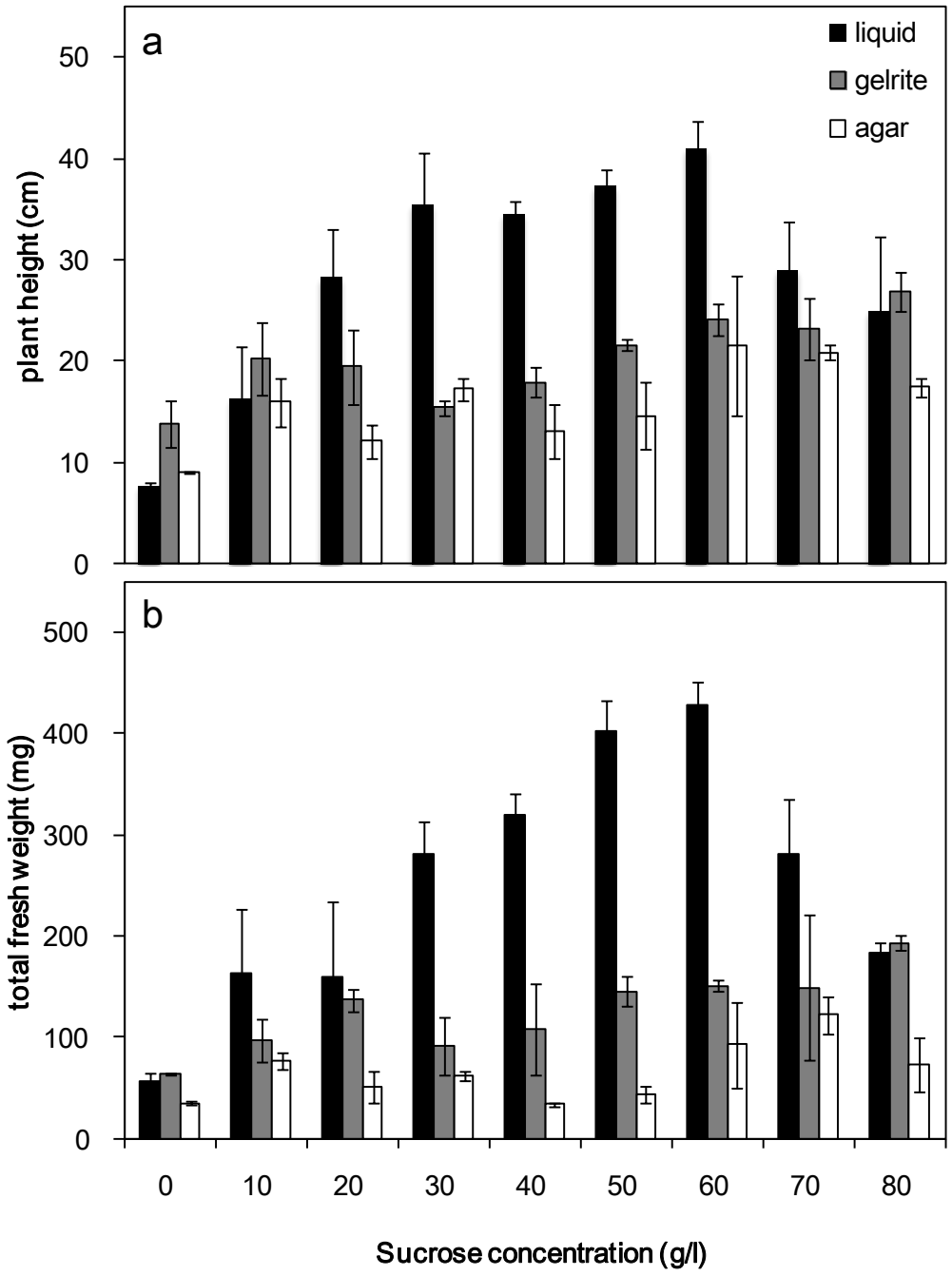

Figure 4. Effects of different sucrose concentrations and the different types of gelling agents on reed growth. Stem nodal segments were excised from regenerated plants and cultured in each medium for seven weeks. Plant height (A) and total fresh weight (B) were then measured.

propagation systems could be used for the mass production of reed plants that could be used for a wide variety of purposes such as a biofuel source or for the management of ecosystem.

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