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Effects of Sucrose and pH on Shoot Multiplication of Indian olive (*Elaeocarpus robustus* Roxb.)

Indian olive (*Elaeocarpus robustus* Roxb.)のシュート増殖に及ぼすスクロース濃度及びpHの影響

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Abbreviations: BA, 6-benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; MMS, modified MS (Murashige and Skoog [12]) medium; NAA, α-naphthaleneacetic acid.

Summary

The internode-derived calli of *Elaeocarpus robustus* were cultured on the shoot regenerating medium, modified MS medium supplemented with 4.4 μM BA and 0.5 μM NAA, with different concentrations of sucrose and pH levels for adventitious shoot regeneration. The results showed that the sucrose concentration at 3% was most suitable for efficient shoot multiplication and pH 5.8 was mostly effective. In the medium supplemented with 3% sucrose at pH 5.8, the best results were obtained in the frequency of shoot regeneration (90%), number of shoots per culture (6.2 shoots), and average length of shoots per culture (5.3 cm). The higher and lower sucrose concentrations and pH levels were less effective for shoot development in this species.
Keywords: adventitious shoot, *Elaeocarpus robustus*, micropropagation, pH, sucrose

要　旨

不定芽を誘導するために*Elaeocarpus robustus*の節間由来のカルスを、様々なスクロース濃度とpH値のシュート再生培地（4.4 μM BA及び0.5 μM NAAを含む改変MS培地）で培養した。実験の結果、スクロースを3%添加した場合に最も良いシュート増殖が認められ、また、pHは5.8に調整した場合が効果的であった。この条件において、最も良いシュート再生率（90%）、培地あたりのシュート数（6.2本）及び培地あたりの平均シュート長（5.3 cm）が得られた。本研究で用いた樹種においては、より高いもしくはより低いスクロース濃度とpHがシュート増殖に適さないことが明らかになった。
キーワード: 不定芽, *Elaeocarpus robustus*, マイクロプロパゲーション, pH, スクロース

1. Introduction

Indian olive (*Elaeocarpus robustus* Roxb.) belonging to the family Elaeocurpaceae is a well-known fruit tree of Bangladesh. Its green fruits are rich in vitamin C, edible in the raw, and can be used for preserves [3]. Although this fruit tree is mainly propagated by seeds, the satisfactory results for efficient plant production have not been obtained so far. Propagation by seeds does not also ensure the genetic characteristics of the mother plants. On the other hand, the micropropagation method for this plant has to severely maintain the clonal fidelity. A better understanding of culture conditions influencing the shoot regeneration is needed for establishing the efficient plantlet production.

Carbohydrates are necessary as sources of energy and carbon substrates for biosynthesis of metabolites in living plant cells. Continuous supply of carbohydrates to plants cultured *in vitro* is essential, since photosynthetic activity of *in vitro* grown tissues is usually reduced. Sucrose is used as most the common carbohydrate source for plants and utilized for tissue culture purpose. During initiation stage, high-sucrose level is required in the medium, whereas its concentration should be decreased in the multiplication stage [6]. This compound is also necessary as an osmotic reagent in media [15]. For all these reasons, carbohydrates

have great potential effects on the physiology, growth, and differentiation of plant cells [5].

The pH in medium is another important culture condition for *in vitro* shoot regeneration. The enzymatic and hormonal activities in plants and nutrient uptake are largely affected by pH level in the tissue cultures [1,6]. The effects of medium pH are more significant for early differentiation of pro-embryogenic cell aggregates. Low pH (*ca.* 3-4) of media seems to prevent differentiation of pro-embryogenic cell aggregates, whereas higher pH levels (5-5.5) favor the formation of globular structures [11]. The solidification with gelling agent in the media is influenced by the pH level. The pH levels more than 6 produce hard-solidified media and pH levels lower than 5 give unsatisfactory solidification [1]. *In vitro* propagation has possibility to offer highly efficient techniques for propagating elite fruit plants. It is well known that among many factors affecting *in vitro* plant regeneration, sucrose concentration and pH level are major important conditions. The objectives of the present study are to determine the optimal sucrose concentration and pH level for efficient induction of adventitious shoots from internode-derived callus of *E. robustus*.

2. Materials and Methods

Six-week-old callus derived from internode of *E. robustus* was used for the experiments. The callus was induced on modified MS (MMS, half strength of major salts, and full strength of minor salts and vitamins) medium supplemented with 2.2 μM BA and 2.3 μM 2,4-D from the juvenile internode segments according to the method of a previous report [16]. For efficient shoot regeneration, three sets of experiment were performed at seven different concentrations (0, 1, 2, 3, 4, 5, and 6%) of sucrose, and pH level was adjusted to 4.8, 5.8, and 6.8. In the first set of experiment, pH was adjusted to 4.8, and sucrose concentration was 0-6%, while in the second and third sets of experiment, pH level was adjusted to 5.8 and 6.8, respectively, at the same concentrations of sucrose. As shoot-regenerating medium, MMS medium supplemented with 4.4 μM BA and 0.5 μM NAA was used as previously reported[16]. The pH in the media was adjusted to objective values by adding 1.0 or 0.1M NaOH or HCl aqueous solution. All media were added with 0.8% (w/v) agar prior to autoclaving at 121 °C for 20 min. Routinely, a melted medium was dispensed into the culture flasks (100 or 200 mL) and plugged with non-absorbent cotton wrapped in one layer of cheese cloth. The cultures were maintained in the culture room at 26 (1 °C and 60% relative humidity under a 16-h photoperiod provided by cool white fluorescent tubes (60 $\mu E \cdot m^{-2} \cdot s^{-1}$). The shoot regeneration frequency, total number of shoots (> 1cm long), and length of shoots per culture were recorded after 8 weeks of cultivation. Each treatment consisting of 20 explants was triplicated (number of sample, N = 60). The differences of the averaged numbers and lengths of shoots among all treatments were tested using analysis of variance (ANOVA) and evaluated by Tukey's multiple comparison test using JMP Statistical Discovery Software (SAS Institute, SAS Campus Drive, USA).

3. Results

Table 1 shows the results of all parameters for shoot regeneration in shoot regenerating medium (MMS medium containing 4.4 μM BA and 0.5 μM NAA) supplemented with different concentrations of sucrose and at different pH levels after 8 weeks of culture. When medium was sucrose free, no shoot regeneration was recorded at all pH levels. Considering sucrose concentration, the percentage of shoot formation, number of shoots per culture, and length of shoots increased gradually with the increase of sucrose concentration in the medium up to 3% and then decreased at all pH levels. In terms of pH levels, the level of pH 5.8 in medium performed the best shoot regeneration among three levels of pH supplementation. However, the best shoot regeneration was obtained in the medium supplemented with 3% sucrose at pH 5.8 among all treatments (Fig. 1A). Under this culture condition, the best results were recorded for percentage of shoot formation (90%), total number of shoots per culture (6.2 shoots), and length of shoots per culture (5.3 cm) (Table 1). The low sucrose concentration (1%) with high level (6.8) of pH in the medium resulted in the lowest shoot regeneration frequency (25%), as well as small regenerated shoot number (1.0) and shoot length (1.0 cm). Figure 1B shows the regenerated shoots in the medium containing 3% sucrose at pH 6.8. Significant differences ($p < 0.05$) for shoot proliferation were recognized among sucrose concentration and pH level. For number of shoots per culture, remarkable statistical differences among sucrose concentrations were recorded in the medium adjusted to pH 5.8, while not in the media at pH 4.8 and 6.8.

Table 1 The effects of sucrose concentration and pH level on adventitious shoot regeneration from internode-derived callus of *E. robustus* after 8 weeks of culture.

Sucrose concentration (%)	pH level	Culture response (%)	Number of shoot (mean ± SE, N=60)#	Length of shoot (cm) (mean ± SE, N=60)#
0		0	0	0
1		30	1.0 ± 0.15d	1.0 ± 0.22d
2		35	2.5 ± 0.41c	2.5 ± 0.35c
3	4.8	50	3.0 ± 0.40c	3.2 ± 0.20c
4		40	2.5 ± 0.25c	2.8 ± 0.22c
6		35	1.8 ± 0.27d	2.5 ± 0.21c
0		0	0	0
1		40	1.6 ± 0.15d	1.3 ± 0.22d
2		50	3.4 ± 0.31c	3.1 ± 0.38c
3	5.8	90	6.2 ± 0.62a	5.3 ± 0.46a
4		70	4.0 ± 0.38b	4.2 ± 0.21b
6		60	1.7 ± 0.22d	3.1 ± 0.30c
0		0	0	0
1		25	1.0 ± 0.16d	1.0 ± 0.25d
2		30	1.5 ± 0.25d	2.2 ± 0.16c
3	6.8	40	2.5 ± 0.32c	3.0 ± 0.35c
4		35	1.8 ± 0.32d	2.5 ± 0.19c
6		30	1.0 ± 0.22d	2.0 ± 0.19c

'#' The values with different alphabets were statistically significant by Tukey's multiple comparison test ($p<0.05$).

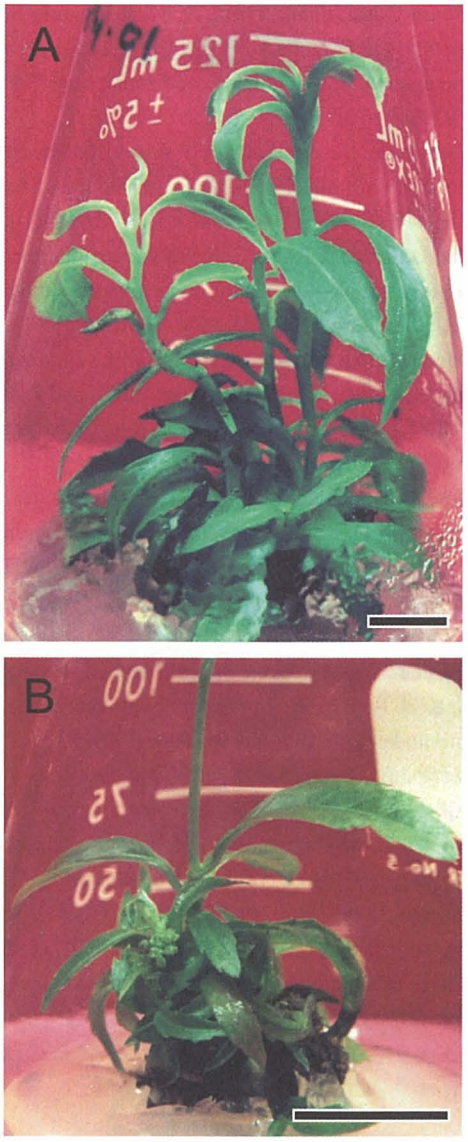

Fig. 1 *In vitro* grown adventitious shoots of *E. robustus* on MMS (4.4 μM BA + 0.5 μM NAA) medium supplemented with 3% sucrose at pH 5.8 (A) and with 3% sucrose at pH 6.8 (B). Bar = 1 cm.

4. Discussion

It is well known that the carbon source in the culture medium is an essential factor as an energy source and for maintaining osmotic pressure [2]. In general, sucrose has been commonly used in the tissue culture media to induce adventitious shoots [17]. In the present study, therefore, sucrose was selected as a carbon source for adventitious shoot regeneration. Three percent sucrose in the medium showed the best performance for shoot regeneration from internode-derived callus of *E. robustus*. The optimal sucrose concentration was 3% for efficient shoot regeneration, which has been reported in some trees, such as *Acacia arabica* [13], *Tectona grandis* [18], and *Calliandra tweedii* [8]. In contrast, lower concentration of sucrose (1.5%) showed the best performance on shoot regeneration in *Araria elata* [7]. Hence, the optimum sucrose concentration varies for shoot regeneration depending on species and genotype as well as culture stage. Between two species of *Acacia*, the maximal shoot yield was obtained in the medium supplemented with 3% sucrose for A. *arabica* [13] and 2% for A. *mangium* [14]. The optimal sucrose concentration was higher in medium at the initial culture stage, while it was lower at the multiplication stage in *Amygdalus communis in vitro* cultured [6]. The lower and higher concentrations of sucrose in the media showed less effective for shoot regeneration from the callus of *E. robustus* in the present study. Similar observations have been reported on shoot regeneration through somatic embryogenesis in *Medicago sativa* [10]. Although the callus cultures can utilize contain lower concentration of sucrose in the medium to support the full potential of biomass growth [15], the reasons for insufficient shoot development are less known. The higher concentration of sucrose in the medium increased the osmotic pressure, which showed inhibitory effects on shoot regeneration due to tissue adjustment to osmotic pressure during culture [15]. Soluble sugars, such as glucose and sucrose, have been reported to function as osmotic stabilizers of tissues grown under osmotic stress [9]. The results of the present study suggest that sucrose is an important factor, for shoot regeneration of *E. robustus*. The shoot multiplication increased with increase in sucrose concentration up to a certain optimum level, 3%.

Another factor pH in the medium was also examined for shoot regeneration. In this study, pH 5.8 was the optimum during the shoot multiplication from callus of *E. robustus*. Although either lower or higher pH level produced a few shoots, the levels caused some abnormalities: thin shoots with very shorter internode, nodes bearing large buds, and thicker basal leaves. There are reports on the optimum pH level for tissue culture of some plants: some showed that pH 5.8 was the optimum [1,8], which are consistent with the present results, while others have reported that a slightly lower level (pH 5.5) produced the highest number of shoots [6]. This might be attributed to genotypic variation of the plants used in the experiments. More slow sucrose uptake from liquid medium compared to solidified medium has been recognized in double-layer medium for micropropagation of *Rosa multiflora* [4]. On the other hand, the hardness of the medium would limit the nutrient uptake [6]. The present study clarified that pH level can influence the shoot development and an optimum pH level gives most efficient shoot regeneration.

In conclusion, this study found that an optimum concentration of sucrose (3%) and pH level (5.8) can gave the best performance on shoot development. The results obtained in this study provide some information on tissue culture, which helps the further advanced researches on this fruit tree.

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