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Research Article
Development of a Highly Efficient Callus Induction and Regeneration Protocol for Sugarcane using Apical Meristem

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Abstract

Background and Objective: Sterilized plants derived from apical meristem culture are proposed to overcome some disease problems in sugarcane. However, the success rate of this technique depends upon cultural media compositions. Thus, this study aimed to determine the optimum concentration of 2,4-D and light conditions on callus development derived from the apical meristem and to stimulate the development of sugarcane shoots and roots. **Materials and Methods:** The apical meristem of the KK3 genotype was used. A 5 × 2 factorial in Completely Randomized Design (CRD) with 20 replications was assigned. Five concentrations of 2,4-D namely 0, 1, 2, 3 and 4 mg L^{-1} were assigned as factor A, while light and dark conditions were assigned as factor B. For a further investigation on the shoot and root regenerations, four concentrations of coconut water namely 0, 10, 20 and 30% (v/v) were applied in the MS media and were assigned in CRD with 20 replications. **Results:** MS media supplemented with 2 mg L^{-1} 2,4-D under dark conditions could generate the highest callus induction rate (100%), big callus sizes (0.72 cm) and a good callus weight (65.75 mg). A 10% of coconut water was the optimum concentration, yielding the most prolific shoots per callus (25 shoots), the longest shoots (4 cm) and the most prolific roots per callus (43 roots). **Conclusion:** Application of 2 mg L^{-1} 2,4-D under dark conditions provided the most effective callus induction of sugarcane and 10% (v/v) coconut water could generate the most prolific shoots and roots per callus.

Key words: Sugarcane seedling, callus induction, plantlet regeneration, coconut water, callus size

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sugarcane plays a major role in the economy of several countries in the tropical and subtropical regions of the world[1]. Thailand is well known as the fourth largest sugarcane producer and the second-largest sugar exporter of sugarcane in the world. Along with Brazil, China and India, Thailand contributes 60% of the total raw-sugar production[2]. Among available sugarcane cultivars released to farmers, Khon Kaen 3 (KK3) is the most popular cultivar widely planted in Thailand, accounting for 63% of the total sugarcane land area[3]. However, sugarcane production faces several problems restricting its yield potential, one of which is disease incidence[1,4]. White leaf disease is one of the major sugarcane diseases that are responsible for over 30 M US dollars losses in the Thai sugarcane industry each year[5]. Unfortunately, there is no sugarcane genotype equipped with resistance genes for this disease to date. Adopting the techniques of sterilized plant tissue culture could relieve this problem. Plant tissue culture technique belonged to *in vitro* culture was a plant propagation on culture media to stimulate the development of shoots and roots[6,7]. Tissue culture is also applied in such research of varietal selection under various conditions, genetic transformation, micropropagation and somaclonal variation[8].

Sugarcane tissue culture technique, the component of all young parts can be used as an explant[9]. Normally, the young leaves are preferable for tissue culture since this tissue could produce a higher number of *in vitro* shoots compared to other plant parts[10]. In contrast, apical meristem tissue is the most disease-free tissue as the apex tissue has no either xylem or phloem[11,12]. Thus, apical meristem culture is more reliable for plant propagation in numerous plant species, for instance, wheat[13], lemon verbena[14] and lotus[15]. In sugarcane, there are two available *in vitro* methods to generate sugarcane plantlets from apical meristem tissue: Direct induction from tissues[16-18] and callus induction and then shoot regeneration[19]. The second *in vitro* method takes an advantage which generates a large number of plants rapidly, callus induction could produce more prolific seedlings by 20-30 times[19]. Moreover, callus is also used in genetic improvement such as chromosome doubling using colchicine[20] and genetic transformation[21].

Also, factors determining the efficiency of this method such as external hormone, media composition and growing condition are another interesting issue to investigate. 2,4-D is preferable for sugarcane callus induction tissue culture due to it provide an effective higher percentage of callus induction compared to others[7,22,23]. Studies dealing with callus induction and then shoot regeneration step by step from apical meristem are scarce. A previous study using the young leaves for tissue culture reported that the use of 2,4-D at 3 mg L^{-1} was able to induce callus of sugarcane[19] while coconut water could stimulate both shoots and roots from the callus[24]. Besides, light and dark conditions altered the callus development[25]. Khamrit *et al.*[19] had initiated *in vitro* study of sugarcane young leaves using 2,4-D together with coconut water to induce callus and plantlet of sugarcane, however, such kind of study evaluated across light and dark conditions combined with 2,4-D has not been reported. It was assumed that those factors may also contribute to the callus induction of sugarcane via apical meristem. Therefore, this study aimed to determine the optimum concentration of 2,4-D and light conditions on callus induction derived from apical meristem tissue and developments of both shoots and roots of the callus. This information would be useful for providing a suitable protocol of sugarcane tissue culture technique for several purposes including disease-free plant production and induction of mutant callus.

MATERIALS AND METHODS

Study area: This study comprised of two experiments: Callus induction from the apical meristem and plant regeneration. Both experiments were conducted at the Department of Biology, Faculty of Science, Khon Kaen University, Thailand and Agronomy Field Crop Station, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand during April, 2019-May, 2020.

Plant materials: The ten-month-old stalks of *S. officinarum* 'KK3' cultivar were collected from the Faculty of Agriculture, Khon Kaen University. Stalk materials 'KK3' were firstly selected for the following criteria such as vigorous and disease-free and the selected stalks were then cut into Single Budded Nodes (SBN). Then, the SBNs were sterilized in Clorox 20% for 3 min and then washed in the water three times to eliminate as many pathogens and insects that may infect the sample. The SBNs were planted in a seedling tray with sterile sandy soil. This soil media was previously incubated at 150°C for 3 hrs. Then, the sterilized water was applied to the media to maintain the optimum soil moisture content. The SBNs were placed and covered with sand and then covered the box with the lid. Trays were incubated in the glasshouse for four weeks. The water regimes were daily maintained. Shoots that emerged from SBNs were used as materials for callus inductions.

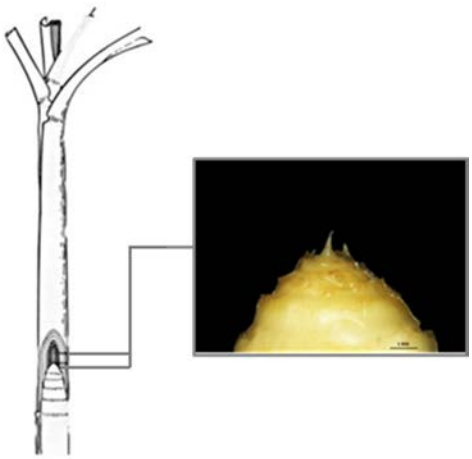

Fig. 1: Sugarcane apical meristem position and the sample cutting into small fragments (5-10 mm) for *in vitro* culture

Sterilization: At 4 weeks after planting, the top of stems of the sugarcane seedling were collected as explant. The explants were thoroughly rinsed under running tap water to unstick surface adhered particles and then washed with 2% (v/v) soap solution. Exterior leaves were removed and an inner leaf possessing apical meristems was sterilized in 70% alcohol for 3 min. Disinfection was done with 38% Clorox plus Tween 20 (2-3 drops in 100 mL sterile) for 5 min. After that, 1-2 leaf sheaths were removed. Then they were surface-sterilized for 3 min in 15% Clorox (commercial bleach) plus Tween 20 (2-3 drops in 100 mL sterile). These small sections of cylinders were washed with distilled water at least four times under sterile conditions. The sterilized tissues were cut into small fragments (5-10 mm) for *in vitro* culture in Fig 1.

Experiment 1

Callus induction from apical meristem: The prepared plant materials derived from SBMs were then used for callus induction in the tissue culture laboratory. A 5×2 factorial in a completely randomized design was performed. Factor A was different concentrations of 2,4-D at 0, 1, 2, 3 and 4 mg L^{-1}, while factor B was in light and dark conditions. Each treatment consisted of 20 replications. The cylindrical apical meristem was cultured on Murashige and Skoog (MS) callus media provided with sucrose 20 g L^{-1}, myoinositol 0.10 g L^{-1} and different concentrations of 2,4-D. One apical tissue was planted on a media plate. The callus samples on the plates were kept under both light conditions with a period of 16/8 hrs (light/dark) at 25±2°C providing 40 µmol $m^{-2}s^{-1}$ and dark condition with a period of 0/24 hrs (light/dark) at 25±2°C. The pH of callus media was maintained at 5.8 with 1 N NaOH solution. Making a solid media, 8 g L^{-1} of agar was used. The media was autoclaved for 20 min at 120°C under 200 psi pressures. All tissue samples were maintained under the same media by subculturing every 2 weeks.

Data collection: The number of callus of each treatment was recorded immediately after planting the tissue on media (number of planted tissue). At 4 weeks after planting callus, the induced callus was counted and callus induction rate was then calculated as described by Mostafiz and Wagiran[26] using the following Eq:

$$\text{Callus induction rate} = \frac{\text{Number of induced callus}}{\text{Total number of planted tissues}} \times 100$$

The size of the formative callus was collected as the average length of all sides for the formative callus, for each treatment. The formative callus of each sample was measured for fresh weight. The samples were oven-dried at 70°C for 72 hrs and the dry weight of those samples was then measured.

Statistical analysis: The statistical analysis was conducted using the Statistic 10 analytical software. The data were subjected to analysis of variance following factorial experiments in a completely randomized design. The mean comparison for all significant parameters was done using the Least Significant Difference (LSD) test at 5%.

Experiment 2

Plant regeneration: Four concentration levels of coconut water (v/v) namely 0, 10, 20 and 30% were assigned in a completely randomized design to investigate the shoot and root developments. Each treatment consisted of 20 replicates with two calli per replicate. The four old week calli were kept in the dark with a 0/24 hrs (light/dark) photoperiod and treated with 2 mg L^{-1} 2,4-D at 25±2°C providing 40 µmol $m^{-2}s^{-1}$. Calli were cut into approximately 1 cm^2 and transferred to the basal MS media composed of 30 g L^{-1} sucrose, 0.10 g L^{-1} myoinositol, 2 mg L^{-1} 2,4-D and different concentrations of coconut water (0, 10, 20 and 30% (v/v)). The callus cultures were incubated at 25±2°C with a 16/8 hrs (light/dark) photoperiod providing 40 µmol.$m^{-2}s^{-1}$. The pH of MS media was adjusted at 5.8 using 1N NaOH solution. Agar was used at 8 g L^{-1}, Activated charcoal 0.5 g L^{-1} media was autoclaved both for 20 min at 121°C under 15 psi pressures.

Data collections: At four months after regeneration, the samples of regenerated calli were counted on root and shoot numbers and 5 random plant samples were then measured for shoot height and root length. The samples were oven-dried at 70 °C for 72 hrs and both shoot and root dry weights were then measured.

Statistical analysis: The statistical analysis was conducted using the Statistic 10 analytical software. Data were subjected to analysis of variance following a completely randomized design. The mean comparison for all significant parameters was done using the 5% Least Significant Difference (LSD) test.

RESULTS

Embryogenic callus induction: The results showed that there was an interaction between 2,4-D of media and light condition and both factors significantly contributed to callus induction of sugarcane. Different concentrations of 2,4-D applied under both light and dark conditions altered percent callus induction, callus size and callus fresh and dry weights. Under these conditions, the explants grown under hormone-free 2,4-D media were not able to form any callus or other morphogenesis and they showed a higher rate of browning. Under the dark condition, callus could be generated in all treatments containing 2,4-D, showing 100% callus induction in Fig. 2a. Meanwhile, under light conditions, the rates of callus induction treated with 1, 2, 3 and 4 mg L^{-1} of 2,4-D were 100, 100, 90 and 0%, respectively. In addition, the highest values of callus length (0.72 cm), fresh weight (65.75 mg) and dry weight (11.94 mg) were found in 2 mg L^{-1} of 2,4-D under the dark condition in Fig. 2(b-d) This was higher than the values under the light condition with the same concentration, for instance, 0.34 cm of callus length, 37.05 mg of fresh weight, 6.81 mg of dry weight. Higher concentrations of 2,4-D reduced the callus size and weight. In addition, at a higher concentration of 4 mg L^{-1} and cultured under the light, callus induced tissue and tissue death were observed in Fig. 3. However, this current study reports a successful and high-frequency regeneration protocol of the apical meristem through embryogenic callus formation. Application of 2 mg L^{-1} of 2,4-D under dark conditions provided the best

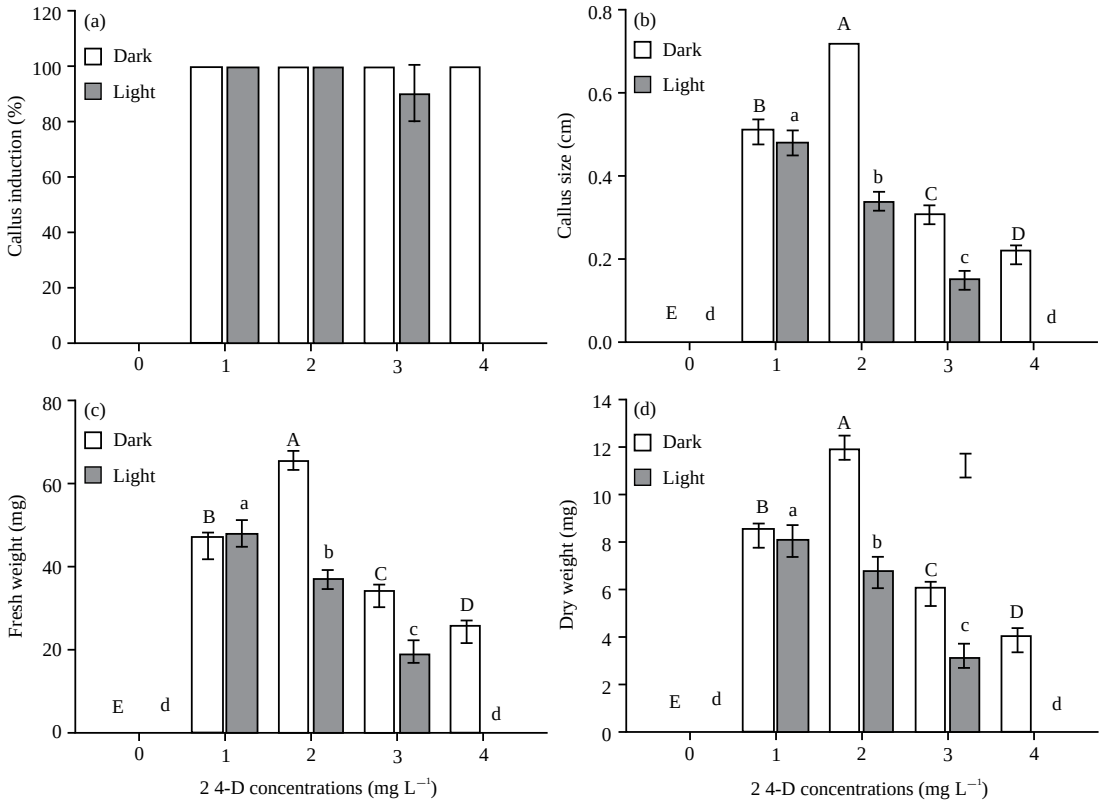

Fig. 2(a-d): *In vitro* responses in different concentrations of 2,4–D under light and dark conditions (a) Callus induction (%), (b) Callus size (cm), (c) Fresh weight (mg) and (d) Dry weight (mg) at four weeks after culture,

*Means in the same column within the same letters (capital letters for dark condition and small letters for light condition) are not significantly different

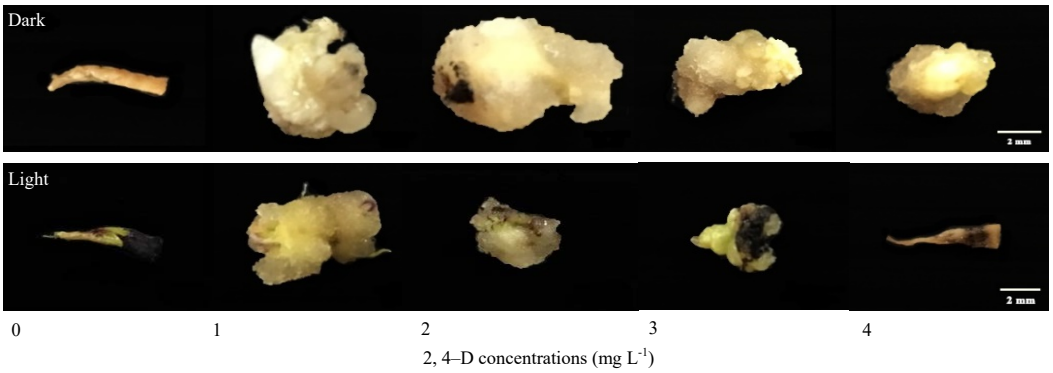

Fig. 3: Induced callus of *Saccharum* 'KK3' on MS medium supplemented with different concentrations of 2,4–D under dark and light conditions at 4 weeks after culture

condition for callus induction of sugarcane. Under this condition, calli appeared cream or yellow and performed impressive callus growth and size. These evidence could not be found when the explants were exposed by the light.

Plantlet regeneration: The calli were obtained from substantial MS media containing 2 mg L^{-1} 2,4-D. This formula was then treated with 0, 10, 20 and 30 % (v/v) of coconut water for four months. Both shoots and roots could be developed from the callus inductions on MS media supplemented with coconut water. An additional 10% (v/v) of coconut water into the MS media was the best option, inducing 25 shoots per callus, 43 roots per callus and 0.07 g root dry weight per callus in Fig. 4(a-c). Although two concentrations of coconut water: 10% (v/v) and 20% (v/v) were not significant on shoot dry weight in Fig. 4d, plant height in Fig. 4e, root length in Fig. 4f, shoot fresh weight in Fig. 4g and root fresh weight in Fig. 4h, these levels were still higher than 30% v/v treatment. An additional 30% (v/v) of coconut water just induced 12 shoots and 6 roots per callus Fig. 4a and b. Plantlet responses to different concentrations of coconut water were illustrated in Fig. 5. As a control treatment, only calli were able to be formed but neither shoot nor root was generated in Fig. 5a. Once 10% (v/v) of coconut water had been applied, both shoots and roots of plantlet could be formed and developed well in Fig. 5b. A similar appearance was noticed on 20% (v/v), yet the overall performance was unstable in Fig. 5c. It could be seen that the roots of the 10% (v/v) treatment were more robust and longer than that of the 20% (v/v) treatment. In contrast to those given treatments, 30% (v/v) generated both shoots and roots in smaller quantities in Fig. 5d. This result illustrated that the application of 10% (v/v) of coconut water could provide not only the best results for callus induction and development but also economically benefit the users.

The different stages of *in vitro* culture in this study were illustrated in Fig. 6. At 14 days after treatment, the surface of an induced callus turned slightly brown as shown in Fig. 6a. Then, the green spots were noticed on proliferating callus surfaces in Fig. 6b. The callus surfaces producing prolific shoots and roots in Fig. 6c were later isolated and transferred onto fresh MS media for further growth and development. After culturing in the laboratory, Seedlings were transplanted into the plastic bag under evaporation greenhouse in Fig. 6d and were then transplanted into the field in Fig. 6e.

DISCUSSION

Plant growth hormones are one of the major factors determining the success rates of callus induction. Besides, the effectivity also depended upon the concentration of auxin[27]. The common type of auxin used for callus induction to date is 2,4-D[28]. By nature, the constant darkness surrounding plant growing media would lead to the increasing level of internal auxin, thus, it resulted in the cell expansion, elongation and callus induction[27]. In addition, 1 mg L^{-1} of 2,4-D under light conditions could induce the callus. Once the explants had been subjected to 2,4-D, the tissue growth was restricted, thus, the callus would be produced. These results confirmed the role of light during callus induction as indicated by the appearance of a green spot that represented alive calli under light conditions. The green spots were the chlorophyll pigmentation, synthesized by the chlorophyll NADPH-protochlorophyllide oxidoreductase[29]. However,

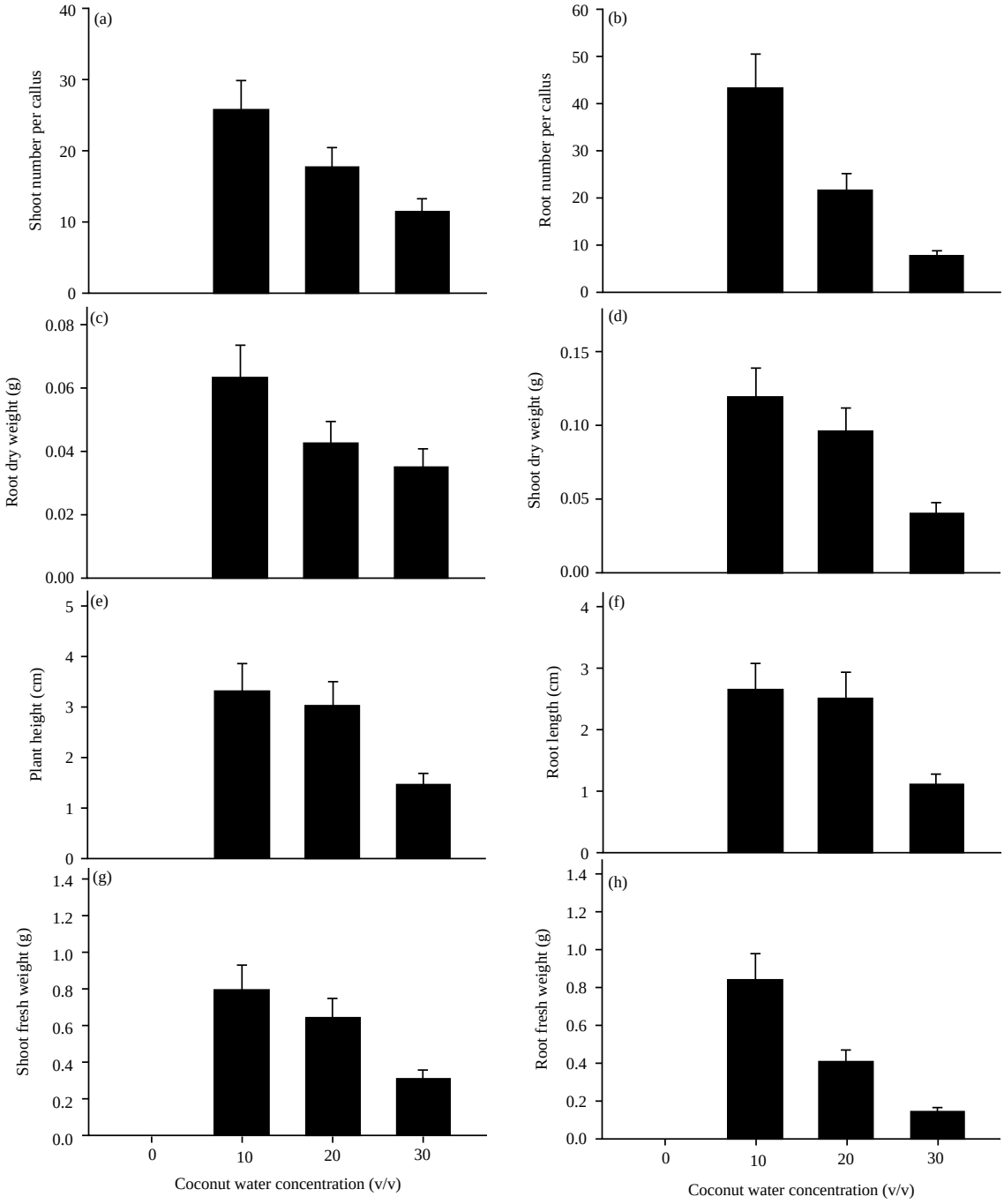

Fig. 4(a-h): Effect of different concentrations of coconut water at 0, 10, 20 and 30% (v/v) on direct shoot regeneration of sugarcane 'KK3' (a) Shoot number per callus, (b) Root number per callus, (c) Root dry weight (g), (d) Shoot dry weight (g), (e) Plant height (cm), (f) Root length (cm), (g) Shoot fresh weight (g) and (h) Root fresh weight (g)

photosensitivity with 2,4-D also showed that plants respond to higher concentrations of toxins. Therefore, 2,4-D was an essential Plant Growth Regulator (PGR) to induce callus from apical meristem, inhibit organogenesis and stimulate cell division to form callus. Previous studies reported that 1-2 mg L^{-1} of 2,4-D was the minimum concentration required for callus induction[30]. However, other studies also reported that MS media containing 3 mg L^{-1} of 2,4-D was an ideal condition for callus induction of sugarcane soft leaf tissue[23]. Therefore, the contradictions might be attributed to the different

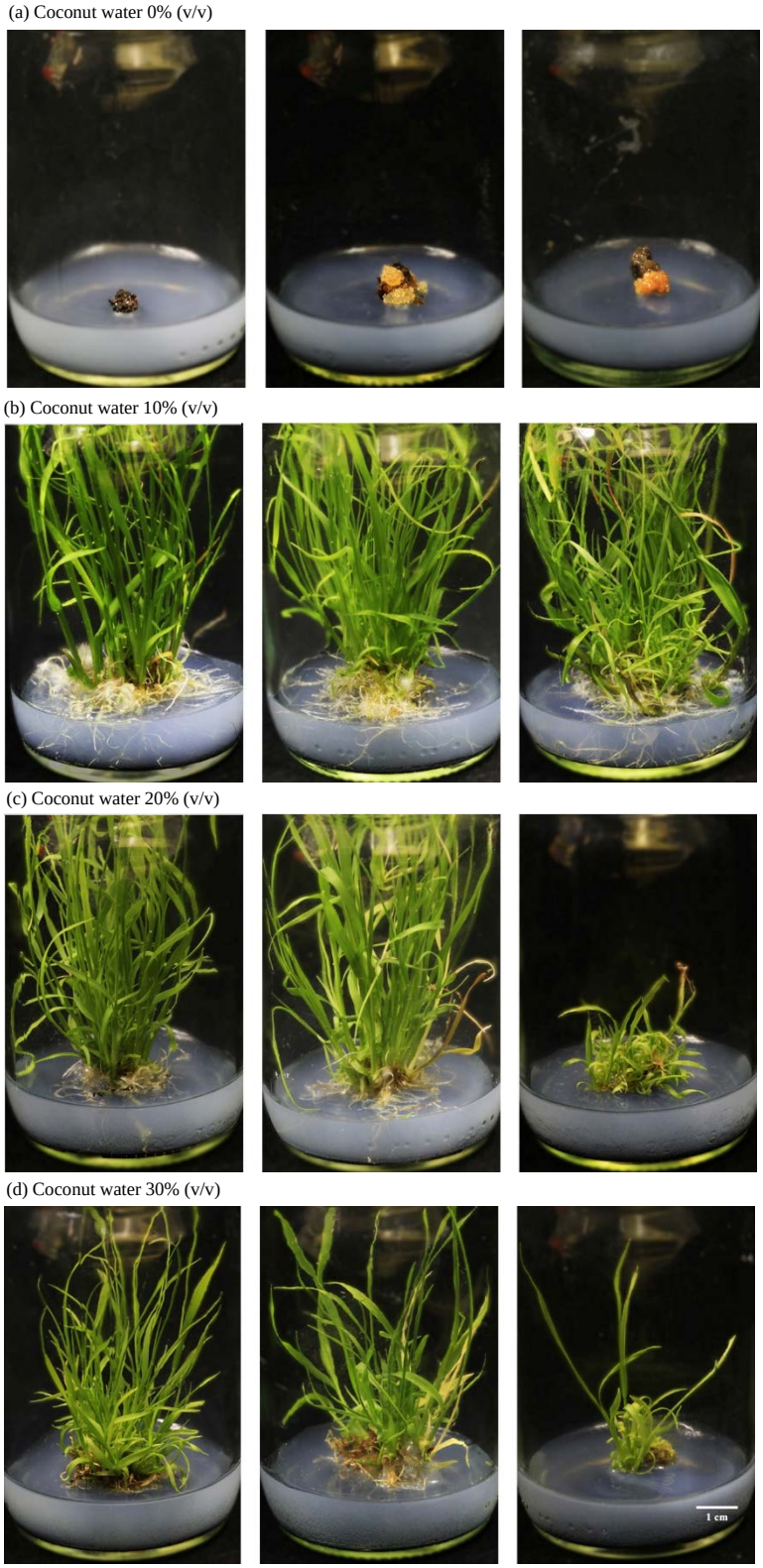

Fig. 5(a-d): Direct shoot and root regenerations of sugarcane 'KK3' after inducing embryogenesis on MS media supplemented with different concentrations of coconut water at eight weeks after culture, (a) Coconut water 0% (v/v), (b) Coconut water 10% (v/v), (c) Coconut water 20% (v/v) and (d) Coconut water 30% (v/v)

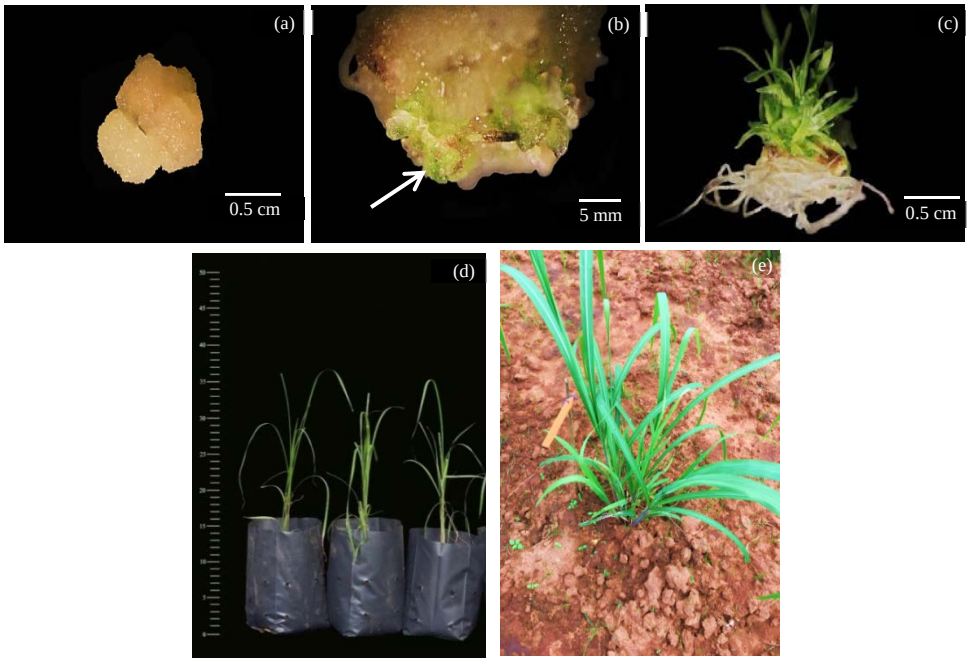

Fig. 6(a-e): Different stages of *in vitro* response in different culture media, (a) Sugarcane callus with 2 mg L^{-1} 2,–4–D under dark conditions, (b) the surface of callus grown under light condition and green spot (arrow), (c) Regeneration shoot and root, (d) The seedlings are aged 1 month after culturing in the laboratory and (e) The seedlings are 2 months old after transplanting into the field

genotypes used in those studies. As known *in vitro* studies, callus induction was highly genotyped dependent including in sugarcane[16]. Both types and concentrations of PGRs required for callus induction depended upon plant species[31].

The efficiency of plantlet regeneration was different when cultured on MS media supplemented with different concentrations of coconut water. Organic supplements were widely used in *in vitro* propagation because of their benefit in supporting plant growth[32]. Several complex organic substances including coconut water are usually added to the plant tissue culture. This study suggested that the optimum concentration for promoting shoot and root numbers was 10% (v/v) coconut water. This result corroborated the previous investigations in orchid plants revealing that the longest shoots and roots and the highest fresh weight were obtained from the growing media with an additional 100 mg L^{-1} (10%) coconut water[33]. Besides, MS media containing 10-15% coconut water showed better shoot and root inductions than MS media supplemented with 4 mg L^{-1} kinetin[34]. Coconut water contained biochemical compounds including potassium, sodium, calcium, iron, vitamins and sugars that promoted the cell divisions, plant growth rate and shoot multiplications[35,36]. Amino acids increased the number of shoots by inducing the cell divisions and more than ten natural N6-substitute adenine compounds, along with zeatin presenting in the coconut water, may be involved in cell division tended towards multiple shoot formation[37]. It acts as a complex multifunctional growth promoter that influences several growth parameters like shoot number and length due to the presence of organic and inorganic elements[38]. However, the somatic embryogenesis was inhibited by the application of 20% coconut water[29]. Higher concentrations of coconut water resulted in lower plant growth. Similarly, Asghar *et al.*[33] revealed that a higher coconut water concentration turned to be an inhibitor for plant shoot development.

Current results indicated that 10 % (v/v) coconut water was the most effective for plantlet regeneration of sugarcane. Since the optimum concentration of coconut water required for shoot and root regenerations was plant species dependent or even genotype-dependent, further investigations of this study covering different sugarcane genotypes are other interesting issues to carry out.

CONCLUSION

This current study reports the successful efforts to generate a high callus induction rate and regeneration of *Saccharum* 'KK3' from the apical meristem. Application of 2 mg L^{-1} 2,4-D under dark conditions provided the most effective callus induction of sugarcane. However, a higher

concentration of 2,4-D resulted in plant toxicity. For plantlet regeneration, 10% (v/v) coconut water was promising as it could generate the most prolific shoots and roots per callus. The information obtained in this study is useful for establishing a reliable protocol for *in vitro* based sugarcane seedling propagations.

SIGNIFICANCE STATEMENT

This study discovers the optimum combination between the concentration of 2,4-D and light conditions on callus development derived from apical meristem tissue and to stimulate the development of sugarcane shoots and roots that can be beneficial for sugarcane researchers are interested *in vitro* culture techniques. This study will help the researcher to uncover the multiply plants in a short time that are disease-free that many researchers were not able to explore. Thus, a new theory of proper protocols for *in vitro* based sterilized sugarcane seedling propagations may be arrived at.

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