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Effect of nutrient media enhanced with plant-growth regulators on indirect somatic embryogenesis induction for the tissue culture of *Digitalis purpurea*

Mohammed Ahmed Al-Oqab[1]*, Salim Zaid[1], Youssef Al-Ammouri[2]

[1]Department of Plant Biology, Faculty of Science, Damascus University, Damascus, Syria.
[2]Department of Pharmacognosy, Faculty of Pharmicy, Syrian Private University, Damascus, Syria.

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ABSTRACT

The aim of this study was to establish effective protocols in indirect somatic embryogenesis induction for the tissue culture of *Digitalis purpurea* in several nutrient mediums enhanced with plant-growth regulators and in the presence of light and dark factors. The results show the superiority of the Vollosovich (5C01) medium in terms of the days required for callus formation compared to the Murashige and Skoog (MS), Linsmaier and Skoog (LS), and Gamborg (B5) media in dark and light conditions. According to the callus induction index, the leaf explants were superior to the stem and root explants. Moreover, leaf explants had the highest number of indirect somatic embryos in the MS medium, with 12.50 and 14.25 in the dark and light, respectively. On the other hand, the 5C01 medium was superior for the callus induction and formation index. The results show a 95% callus induction rate at a concentration of 2 mg/L of NAA+1 mg/L of Kin in the dark and light treatments compared to all the studied treatments in the MS, LS, and B5 mediums. At a concentration of 0.25 mg/L 2.4-D+2 mg/L BA, the MS medium was better for the induction and formation index of indirect somatic embryos of the studied Callus induction from leaf explants by 52.75% in the dark treatments and by 90% in the light treatments.

1. INTRODUCTION

Digitalis purpurea is one of the most important plants of high medicinal value. It is a biennial herbaceous plant belonging to the Plantaginaceae family [1]. The leaves of *D. purpurea* were included in the London Pharmacopoeia in 1650 [2]. The first medical, scientific experiments for its use as a reliable treatment for heart patients date back to 1785, conducted by Doctor William Withering [3,4]. The medical importance of *D. purpurea* is that it contains cardiac glucosides, such as digoxin, used in treating congestive heart failure.

Consequently, in 1990, the FDA approved its use as a treatment for heart failure and atrial fibrillation. The effectiveness of digoxin is due to its effect on the sodium-potassium pump through its effect on the vagus nerve [5]. Several studies on the efficacy of digoxin in the treatment of breast and prostate cancer have recently been published [6,7]. In addition, a number of scientific reports stated that the antiviral effects of digoxin and some other cardiac glycosides, such as Ouabain, block Cov-2 transcription, or replication, which lead to the inhibition of the virus post-viral life cycle. Hence, recent research results indicate that digoxin and Ouabain may be alternative and effective treatments as antidotes to COVID-19, with potential additional therapeutic effects for patients with cardiovascular disease [8]. However, the difficulties in synthetic chemistry regarding the complexities of the skeletal structure of cardiac glucosides and the impossibility of their chemical synthesis make plants the only sources from which to obtain these cardiac glycosides [9]. Such traditional cultivation methods are no longer feasible in light of the challenges faced with traditional methods of propagation as well as the seasonal and environmental conditions. Verma *et al*. [9] reviewed the futility of the natural propagation of digitalis seeds, as this is an ineffective method for producing an adequate stock of seeds and is associated with a low germination rate and other risks. Probert *et al*. [10] reviewed the difficulties that affect the quality of seeds, such as processing methods and storage conditions. Consequently, researchers attempted to produce digitalis containing high glucosides through traditional cultivation. Still, the offspring were not stable, and repairing the traits required long-term programs that were cumbersome and expensive [10].

Therefore, biotechnological research in recent decades has focused on techniques of the tissue culture of digitalis species as an approach that goes beyond seasonal restrictions, overcomes the difficulties associated with traditional agriculture, and achieves sufficiency for the increasing human demand.

*Corresponding Author:
Mohammed Ahmed Al-Oqab, Department of Plant Biology, Faculty of Science, Damascus University, Damascus, Syria.
E-mail: prsmaloqab @ gmail.com

Somatic embryo propagation is one of the most effective techniques for the micropropagation of medicinal plants, a valuable technology for the production of clonal plants, and a promising tool for exploring diversity and highlighting many new and valuable properties [11]. Somatic embryogenesis induction occurs directly from explants or indirectly through the callus stage [12-14].

The callus-differentiation stage is the key to establishing the *in vitro* indirect regeneration system [15]. The indirect somatic embryo induction process occurs through an organized series of distinct embryonic stages, such as spherical, cardiac, and torpedo [5]. The success of indirect somatic embryo induction depends on factors such as the plant type, cultivation conditions, explant types, nutrient medium, plant-growth regulators, materials and other additives, and heat and light factors [16,17].

2. MATERIALS AND METHODS

2.1. Research Location

The research was carried out at the Biotechnology Laboratory of Medicinal Plants, the National Commission for Biotechnology, Damascus, Syria, and at the Tissue Culture Laboratories, Department of Plant Biology, Faculty of Science, Damascus University, Syria, during the period of 2018–2021.

2.2. Plant Material

The studied explants (leaves, stems, and roots) were obtained from *in vitro* plantlets of the studied species, *D. purpurea*, grown in glass (T = 25 ± 1°C; light = 2000 Lux 16/8; E_g = 30 days; H = 70) at the Laboratory of Medicinal Plant Biotechnology at the National Commission for Biotechnology, Syria.

2.3. Preparation of Nutrient Media

The nutrient media were prepared—MS [18], B5 [19], LS [20], and 5C01 [21] —and sterilized in an autoclave (T = 121°C; P = 1.2 Bar). These nutrient media were supplemented with a series of plant-growth regulators, auxins (2.4D, NAA, and IAA), and cytokinin (Kin, BAP, and BA) in graded concentrations (0.25, 0.5, 1, and 2 mg/L) and single and compound cases. Explants (leaves, stems, and roots) were planted on the studied media at a rate of 20 explants for each treatment (concentration) and four replications in each of the studied media (pH = 5.8; agar = 8 g/l; sucrose = 3%). Cultivation was carried out under sterile conditions in a JSCR-1200 SB laminar device. Then, the cultivated explants were incubated according to two groups – the first in dark conditions and at a temperature of (25 ± 1°C) for 30 days, and the second group in light-cycle conditions of 16 h (2000 Lux) at a temperature of (25 ± 1°C) for 30 days. The initial observations included the number of days required for callus formation responses in light and dark conditions, the shape and texture of the callus, and other visual observations. Afterward, the calluses were transferred to a subculture for indirect somatic embryo induction treatments according to the light and dark groups and temperature conditions (25 ± 1°C).

The visual and microscopic observations were recorded (number of Buds), and the induction ratios were restricted to the formation of somatic embryos in all the studied treatments. The best-enhanced hormonal combinations for inducing and forming calluses and indirect somatic embryos were selected. The developing embryos in dark and light conditions were transferred and separated from the embryonic calluses and grown in glass in MS, LS, B5, and 5C01 with the same concentration of the plant-growth regulators (PGR_S).

2.4. Experimental Design and Statistical Analysis

All the experiments were carried out using the split-split-plot design, with an average of 20 samples for each treatment and four replicates. The data were analyzed using the Mstat-C statistical analysis program to calculate the values of the least significant difference at a 0.01% level of significance and the values of the coefficient of variance (CV%).

3. RESULTS AND DISCUSSION

3.1. Days Required for Callus Formation

The days required for the callus formation response varied according to the medium type, growth regulators, explant type, and explant level within the same species. Table 1 indicates the efficiency of the 5C01 and MS mediums in terms of the day's index required for callus formation in the dark conditions compared to the B5 and LS mediums. Furthermore, the superiority of the leaf explants in all the studied nutrient mediums compared to the stem and root explants. These results are agreed with the results of Besher [22] on the superiority of the 5C01 medium in inducing a callus response from *Hyoscyamus aureus*. Furthermore, the results agree with the findings of Zang *et al*. [15] regarding the superiority of the MS medium in inducing a callus response from the leaves of *Digitalis hamiltonii*.

Table 1 shows the superiority of the 5C01 medium, followed by the MS medium, in terms of the callus formation index in the light compared to the B5 and LS media, as well as the superiority of the leaf explants compared to the stem and root explants in the callus formation index of the studied nutrient media. Moreover, it can be noticed that light contributed to inducing the formation of Calli in the MS, B5, and LS media compared to their formation in the dark. In contrast, the light slowed down the induction of the formation of calluses in the 5C01 medium. This result may be due to the internal interactions between the medium components under the influence of light. Therefore, the effect of light on the total concentration of growth regulators (internal and external) was reflected in the induction response and its temporal timing. These findings agree with the results of Chen *et al*. [17] on the

Table 1: Required days for starting callus formation.

Treatment	Dark				Light 16/8			
	Leaves	Stems	Roots	Mean	Leaves	Stems	Roots	Mean
MS (DPM1)	13	16	14	14.33[b]	12	15	14	13.66[a]
B5 (DPM2)	21	24	26	23.66[d]	19	22	24	21.66[c]
LS (DPM3)	14	18	18	16.66[c]	14	17	18	16.33[b]
5C01 (DPM4)	9	9	12	10[a]	12	13	14	13[a]
Mean	14.25[a]	16.75[b]	17.5[c]		14.25[a]	16.75[b]	18[c]	

Means in the table are followed by different letters; the letters represent the order of the mean value which starts from the best value (a) and decline, respectively, according to the letter order

effect of light on the induction and formation of somatic embryos in Howorthia and the effect of light on the activity of growth regulators and the modification of internal hormone levels.

3.2. Indirect Somatic Embryogenesis Induction

Table 2 reveals the superiority of the 5C01 and MS media regarding the number of days needed to form indirect somatic embryos in dark conditions compared to the LS and B5 media, in addition to the superiority of the leaf explants as a source of indirect somatic embryo induction compared to the rest of the other studied explants in the dark conditions. These results are in agreement with the results of Verma *et al*. [23] that the calluses induced from leaf explants of *Crocus species* on the MS medium formed somatic embryos earlier than in the other explants and that the MS medium was the best medium for the development of somatic embryos compared to the B5 and LS media.

The results presented in Table 2 show the superiority of the MS medium, followed by the LS and B5 media, compared to the 5C01 medium regarding the number of days needed for somatic embryo formation in the light cycle 16, as well as the preference for the leaf explants compared to the rest of the studied explants. The results of this study agree with the results of [24], indicating that light is one of the most critical factors controlling plant growth and development, as well as the results of Farhadi *et al*. [25], indicating that light is one of the most significant factors affecting the regeneration and micropropagation systems in a number of crops, where the interactions among light, endogenous auxins, and cytokinin directly or indirectly affect the regeneration systems in plants [17].

3.3. Somatic Embryo Formation Percentages

The results presented in Table 3 show the superiority of the MS medium regarding the indirect somatic embryo formation percentages in the dark compared to the rest of the studied media, with higher percentages of calluses induced from the leaf explants compared to the other studied explants in the dark.

On the other hand, the MS and LS media were superior regarding the rate of somatic embryo formation compared to the studied B5 and 5C01 media in the presence of the light factor. The leaf explants were superior to the other studied explants in the light condition. This may be due to the leaves being the newest part of the plant, which is consistent with the results of Verma *et al*. [23] regarding *Crocus species*, as the newer parts were the most responsive to somatic embryogenesis and plant renewal. The results also agree with the results of Krishnan *et al*. [26], indicating that the callus induction average varied according to the type of explant, while the differences in the response between the nutrient media were due to the difference in nitrogen source and quantity, which is consistent with the results of Mandal and Laxminarayane [11].

In comparing the light and dark factors, the results presented in Table 3 show significant differences that confirm the preference for light conditions in inducing somatic embryos. This may be due to the effect of light on the medium components, specifically on the growth regulators, and stimulating internal hormones. In addition, light works to form the embryonic callus, which is preferred for inducing indirect somatic embryos. These results agree with the results of [27,28] on the importance of light in inducing the development of indirect somatic embryos from Oncidium callus cultures. Moreover, our results agree with those of Verma *et al*. [23] about the superiority of the response rate of leaf explants on the MS medium for *Crocus oliveri* compared to other studied media.

The results of this study are in consonance with the earlier report of Gural *et al*. [29] on *Digitalis davisiana* Heywood about the presence of significant differences in bud renewal depending on the explant type, plant type, hormonal combination, and components of the basal medium. The results also agree with those of other studies showing that calluses derived from different explants with different (variable) potentials lead to different proportions and forms of regeneration and morphology [11,30-32].

3.4. The Number of Somatic Embryos

The results presented in Table 4 show the superiority of the MS medium regarding the number of embryos in both the dark and

Table 2: Required days for forming indirect somatic embryos.

Treatment	Dark				Light 16/8			
	Leaves	Stems	Roots	Mean	Leaves	Stems	Roots	Mean
MS (DPM1)	15	17	19	17[b]	15	16	17	16[a]
B5 (DPM2)	16	18	20	18[d]	15	17	18	16.66[c]
LS (DPM3)	15	18	19	17.33[c]	15	16	18	16.33[b]
5C01 (DPM4)	15	17	18	16.66[a]	16	18	19	17.66[d]
Mean	15.25[a]	17.5[b]	19[c]		15.25[a]	16.75[b]	18[b]	

Means in the table are followed by different letters; the letters represent the order of the mean value which starts from the best value (a) and decline, respectively, according to the letter order

Table 3: The percentages of indirect somatic embryo formation of the *Digitalis purpurea* explants in the dark and light conditions.

Treatment	Dark				Light 16/8			
	Leaves	Stems	Roots	Mean	Leaves	Stems	Roots	Mean
MS (DPM1)	52.75	37.5	23.75	38[a]	90	67.5	27.5	61.66[a]
B5 (DPM2)	45.25	31.25	21.25	31.58[c]	73.5	51.25	21.25	48.66[c]
LS (DPM3)	49.5	32.5	21.25	34[b]	82.25	60	26.25	56.166[b]
5C01 (DPM4)	45.75	26.25	18.75	30.25[d]	80	41.25	20	47.083[d]
Mean	48.312[a]	31.875[b]	21.25[c]		81.43[a]	55[b]	23.75[c]	

Means in the table are followed by different letters; the letters represent the order of the mean value which starts from the best value (a) and decline, respectively, according to the letter order

Table 4: The number of indirect somatic embryos of the *Digitalis purpurea* explants in the dark and light conditions.

Treatment	Dark				Light 16/8			
	Leaves	Stems	Roots	Mean	Leaves	Stems	Roots	Mean
MS (DPM1)	12.5	10.25	8.5	10.41[a]	14.25	11.25	9.75	11.75[a]
B5 (DPM2)	7.25	8.25	5.5	7.00[d]	8.5	8.75	6.75	8.00[c]
LS (DPM3)	9.75	9	6.25	8.33[b]	11.25	9.75	7.5	9.50[b]
5C01 (DPM4)	8.25	8.5	5.25	7.33[c]	9	8.75	6.25	8.00[c]
Mean	9.43[a]	9[b]	6.37[c]		10.75[a]	9.62[b]	7.56[c]	

Means in the table are followed by different letters; the letters represent the order of the mean value which starts from the best value (a) and decline, respectively, according to the letter order

light conditions compared to the rest of the other studied media, the superiority of the leaf explants compared to the stem and root explants, and the preference of light for inducing the number of indirect somatic embryos from the *D. purpurea* explants. These results agree with the findings of Mongomake *et al*. [33], showing that the number of buds formed per plant was significantly higher when incubated with an 8–16-h light cycle.

It was observed that the induction of some of the explants within the same medium was superior, as was the presence of some replicates without induction or within minimum limits. The lack of an appropriate number of induced somatic embryos in some of the explants or media was due to competition for mineral elements, the release of an inhibitory molecule, or the lack of a molecular signal [34].

In view of the superiority of the leaf explants regarding inducing calluses and indirect somatic embryos, the best hormonal combinations were selected for inducing indirect somatic embryos resulting from the tissue culture of *D. purpurea* leaves and the cell types isolated from them.

3.5. Selection of the Best Hormonal Combinations for the Rate of Callus Formation from *D. purpurea* Leaves

The callus stage is key in any system of the regeneration or micropropagation of indirect somatic embryos [15]. The quality of the callus plays an essential role in indirect plant regeneration, and compressed embryonic calluses were selected for successful *in vitro* regeneration [35]. The formation and development of indirect somatic embryos are also greatly influenced by the density of the culture (callus texture) [34], and the patterns of calluses differ depending on the type of nutrient media according to Gural *et al*. [29] on *Digitalis davisiana* Heywood.

The results presented in Table 5 show the selection of the best hormonal combinations for the rate of callus formation and maintenance in light and dark within the studied nutrient media. The concentrations of treatments for the selected growth regulators, where the combinations or treatments had higher rates of callus formation, were chosen compared to all the studied treatments in the presence of medium variables and concentrations of plant-growth regulators, taking into account the physical and morphological properties of the chosen callus. The results show that a concentration of (2 mg NAA + 1 mg Kin)L^{-1} in the 5C01 medium was effective at a rate of 95%, and a concentration of (2 mg 2.4D + 1 mg BA)L^{-1} in the MS medium induced callus formation rates of 85% and 90% in the dark and light, respectively, compared to the rest of the studied and selected combinations in the LS and B5 media and the presence of dark and light factors [Figure 1a-d]. Moreover, Table 5 also shows an improvement in the rate of callus induction in the MS, LS, and B5 media when comparing callus induction in dark and light conditions.

Table 5: The best-selected combinations for the rate of callus generation from leaves in the dark and light conditions for *Digitalis purpurea*

Treatment	Medium name	PGR_S concentration	Callus induction %	
			Dark	Light 16/8
DPM1	MS	(2 mg 2.4D+1 mgBA) L^{-1}	85[b]	90[b]
DPM2	B5	(2 mg 2.4D+1 mgBA) L^{-1}	70[d]	75[d]
DPM3	LS	(2 mg NAA+2 mgBA) L^{-1}	75[c]	85[c]
DPM4	5C01	(2 mg NAA+1 mgKin) L^{-1}	95[a]	95[a]

The results of this study agree with the results of Besher [22] on the preference for the 5C01 medium for the induction and development of calluses of *H. aureus* and with Zang *et al*. [15] on the advantage of the MS medium in inducing calluses of *D. hamiltonii* leaves.

3.6. Selection of the Best Hormonal Combinations for the Proportion of Indirect Somatic Embryo Formation

The results presented in Table 6 show that a concentration of (0.25 mg 2.4D + 2 mg BA)L^{-1} in the MS medium resulted in the highest induction rate for indirect somatic embryos in the dark compared to the other studied treatments in the MS medium and all the treatments in the other nutrient media, followed by a concentration of (0.5 mg NAA + 2 mg BA)L^{-1} in the LS medium. The studied treatments in the 5C01 and B5 media resulted in the lowest percentages of induction of indirect somatic embryos. Moreover, the results show that a concentration of (0.25 mg 2.4D + 2 mg BA)L^{-11} in the MS medium achieved the highest induction rate for indirect somatic embryos (90%) in the light compared to the rest of the studied media and treatments.

The table also shows the discrepancy in the critical role of growth regulators according to the studied nutrient medium and the quality of the growth regulator. The effective superiority of one auxin over another within the nutrient media was attributed to the effective absorption and mobilization of the growth regulator or the rapid mobilization at the targeted sites [36]. The results of this study agree with those of Lijalem and Feyissa [37], which showed that different groups of growth regulators showed different responses in the formation of somatic embryos in *Securidaca longipedunculota*. They are also in agreement with the results of Gural *et al*. [29] on *Digitalis davisiana* Heywood, showing that the presence of significant differences in the renewal of shoots depending on the explant type, plant type, hormonal combination and components of the basal medium, and the timing and requirements of growth regulators for the growth of somatic embryos differs according to the studied species [38].

According to Tables 5 and 6, the explants in the light conditions exhibited embryonic calluses, distinguished by the asynchronous appearance of numerous structures, such as spherical, core, and

torpedoes. These structures were more pronounced in the stage of the third sub-culture while appearing in lower proportions in the explant calluses induced in the dark conditions in the fourth sub-culture [Figure 2a-d].

These results agree with those of Mongomake *et al*. [33] on the asynchronous formation of somatic embryos accompanied by several structures at different stages of development on the same structures of the callus. The subculture also allowed the calluses to gain mass in addition to somatic embryos [26]. There is a need to take into account the temporal estimation processes for the selection of the studied treatments to avoid obstacles to efficient somatic embryo induction resulting from the death of cells in contact with the agar surface or the formation of a brown callus (browning phenomenon), which limits the formation of somatic embryos, as high percentages of brown color reflect a decrease in physical embryonic development [38]. Furthermore, a callus or explant in contact with the agar surface shows a low response rate regarding the formation of somatic embryos. It comprises semi-dead cells due to poor breathing; the lack of oxygen leads to a poor supply of free energy [39,40]. These results agree with Chen *et al*. [17], showing that light is an important factor in improving regeneration efficiency in tissue cultures.

Table 6: The best-selected combinations for the generation of indirect somatic embryos in the dark and light conditions for *Digitalis purpurea.*

Treatment	Medium name	PGR_S concentration	Indirect somatic embryos %	
			Dark	Light 16/8
DPM1	MS	(0.25 mg 2.4D+2 mgBA) L^{-1}	52.75[a]	90[a]
DPM2	B5	(0.5 mgNAA+2 mgBA) L^{-1}	45.25[d]	73.50[d]
DPM3	LS	(0.5 mgNAA+2 mgBA) L^{-1}	49.50[b]	82.25[b]
DPM4	5C01	(1 mgNAA+1 mgKin) L^{-1}	45.75[c]	80[c]

Indirect somatic embryos were developed in a plant in the dark after embryonic sprouts were transferred and grown in nutrient media according to the selected combinations presented in Table 6. Clear differences between the effect of the nutrient medium quality and the plant-growth regulators can be observed [Figure 3a-d].

On the other hand, Figure 3a-d shows the development of indirect somatic embryos in the light in a plant after they were transferred

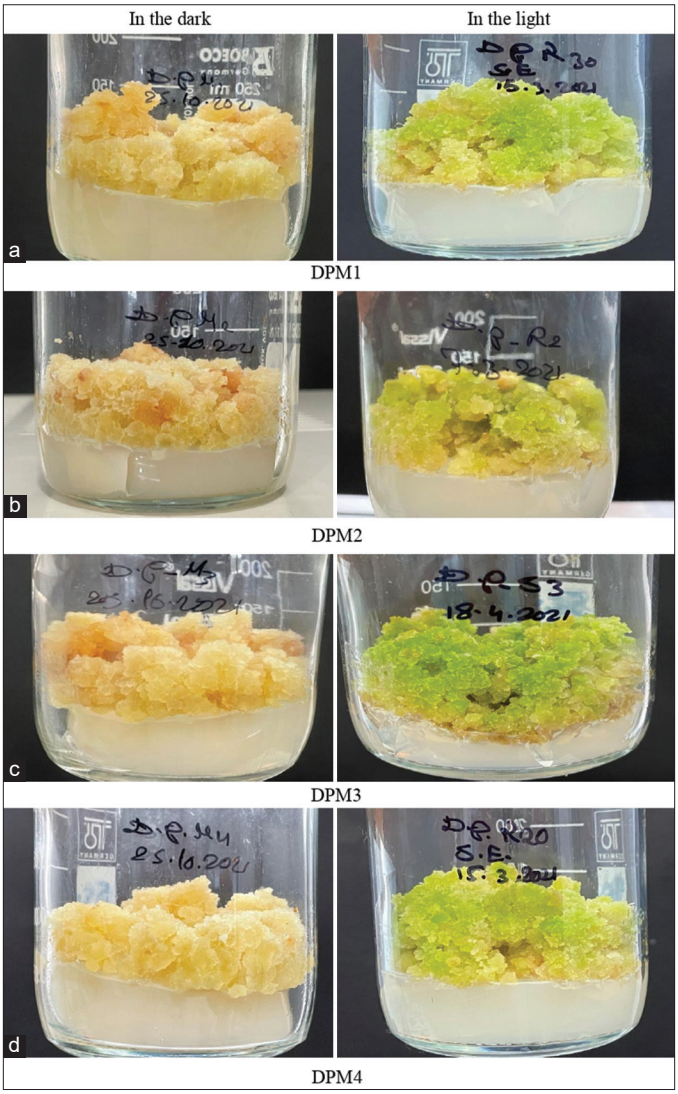

Figure 1: The best cell lines selected from calli induced in the dark and light conditions.

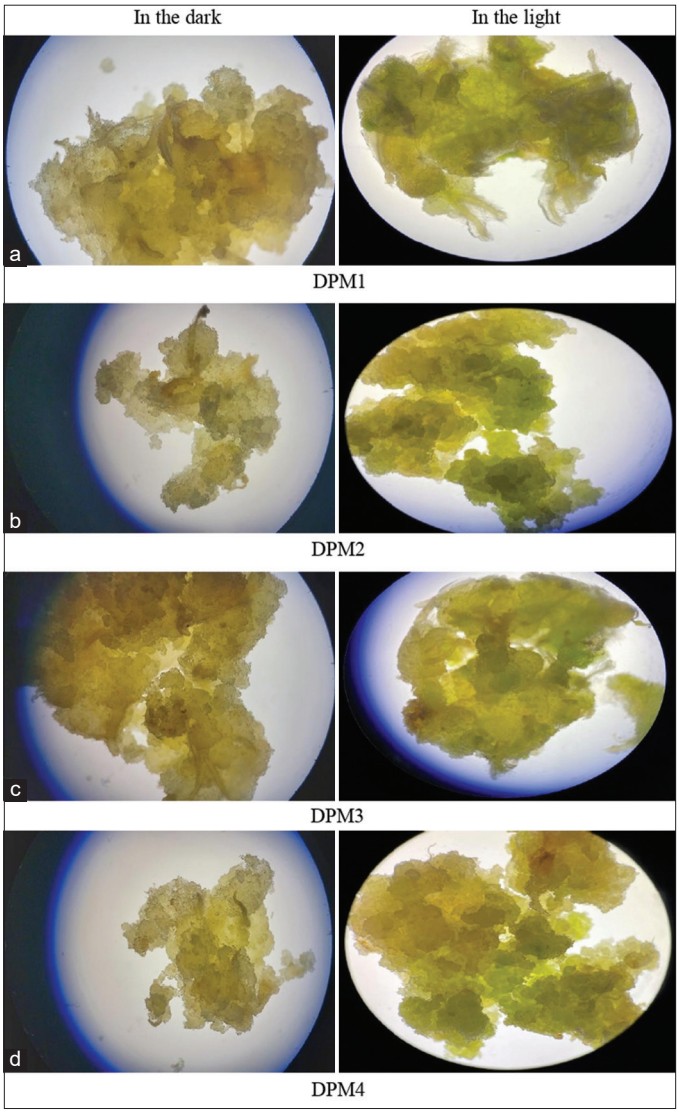

Figure 2: Growth buds from indirect somatic embryos induced in the dark and light conditions.

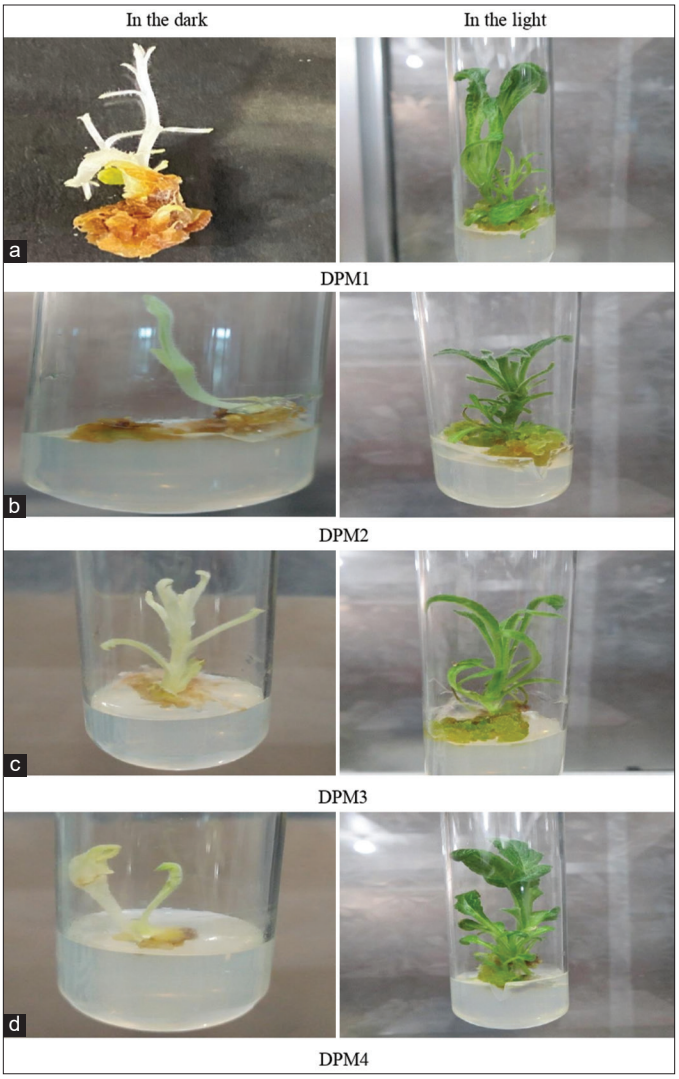

Figure 3: Development of the selected indirect somatic embryos in the dark and light conditions.

to nutrient media containing hormonal combinations, according to Table 6. The effect of growth regulators that enhanced the nutrient medium was noted. In the presence of the light factor that fully promoted growth and development, compared to the embryos in Figure 3, the differences caused by the light factor in the development of indirect somatic embryos and their accurate reproduction can be observed.

4. CONCLUSIONS

Based on the present experimental investigation, the following findings have been drawn; the 5C01 and MS media were the optimum media for callus induction in *D. purpurea*, while the MS and LS media were preferred for indirect somatic embryogenesis formation and induction. It has been also observed that the basal nutrient medium and the type and concentration of growth regulators were critical and essential factors for callus and indirect somatic embryogenesis induction. Moreover, the type of vegetative explant and light were essential factors in the induction of indirect somatic embryos from calluses.

5. AUTHORS' CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Mohammed Ahmed Al-Oqab. The first draft of the manuscript was written by Mohammed Ahmed Al-Oqab, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

6. FUNDING

The authors did not receive support from any organization for the submitted work.

7. DATA AVAILABILITY STATEMENT

The data presented in this study are available upon request from the corresponding author.

8. CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

9. ETHICAL APPROVAL

This research did not involve experiments with human or animal participants.

10. PUBLISHER'S NOTE

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