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MULTIPLE SHOOT REGENERATION VIA INDIRECT ORGANOGENESIS FROM SHOOT TIP AND NODAL MERISTEM EXPLANTS OF *CERATOPHYLLUM DEMERSUM* L.

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ABSTRACT

Ceratophyllum demersum L. (Ceratophyllaceae) is a well-known medicinal plant. The objective of this study was to improve an effective protocol for *in vitro* callus formation and plantlet regeneration using shoot tip and different nodal explants of *C. demersum* and to investigate the changes in the photosynthetic pigment contents of shoots emerging from these calli. Murashige and Skoog (MS) media having various concentration of thidiazuron (TDZ, 0.05-0.80 mg/L) were tested for callus induction. With the effect of TDZ, callus induction occurred at high frequency from the explants. Callus regeneration frequencies were determined as 100% in all culture media for 1st nodal explant, as 83-100% for shoot tip and as 66-100% for 2nd nodal explant. The best hormone application in terms of callus concentrations was found as MS medium containing 0.50 and 0.75 mg/L TDZ for all explants. In the second phase, the calli derived from the explants transferred to free-hormone MS (MSO) and MS medium supplemented with 0.10 mg/L 6-Benzylaminopurine (BAP) and 0.10 mg/L Gibberellic Acid (GA_3) for shoot regeneration. Multiple shoot regenerations from subcultured callus in different media were successfully achieved. Maximum number of shoots per callus from shoot tip (108), 1st (96) and 2nd (87) nodal meristem explants were recorded on MS medium fortify with 0.10 mg/L GA_3. The longest shoots were obtained in the MS medium containing 0.10 mg/L GA_3 for the 1st (1.39 cm) and 2nd (1.43 cm) nodal explants and in the MS medium containing 0.10 mg/L BAP for the shoot tip explants (1.25 cm). The highest chlorophyll *a*, *b* and total chlorophyll contents of regenerated shoots were obtained in shoot tip callus cultured in MS medium fortify with 0.10 mg/L BAP, while the maximal carotenoid content was determined in 2nd nodal callus cultured in MS medium supplemented with 0.10 mg/L GA_3. It has been found that the regenerated shoots from the shoot tip callus had higher chlorophyll content. This study is a new protocol for the efficient and rapid production of *C. demersum* via callus induction and showed that high efficiency production system can be established for *C. demersum*.

Keywords: Callus induction, *C. demersum*, nodal explant, photosynthetic pigment.

INTRODUCTION

Medical and aromatic plants produce a wide variety of hydrocarbons and their analogues such as aldehydes, ketones and esters (Qiming *et al*, 2006). Industrial and commercial pharmaceuticals and chemicals are usually products of secondary metabolism in plants. Out of the 350,000 plant species known to date, about 35,000 are used worldwide for medicinal purposes (Shasany *et al*., 2007). Approximately 25% of all medicines prescribed in developed countries have about 100 plant species (Comer and Debus, 1996). *Ceratophyllum demersum* L., a perennial plant of family Ceratophyllaceae (Arber, 2010), is an aquatic medicinal plant and it has been traditionally used in the human treatment such as ulcer, dysentery, wounds, fever (Taranhalli *et al*., 2011). Karale *et al.* (2013) reported that the methenolic extracts of *C. demersum* in albino rats show analgesic, antipyretic and anti-inflammatory effects. The essential oil of *C. demersum* has been found to have anti-inflammatory, antifungal, antibacterial, and antineoplastic activity (Kurashov *et al*., 2016). Moreover, it has been reported that the phytochemical components such as flavonoids, sterols, glycosides, tannins, saponins, alkaloids, terpenes, chlorides of *C. demersum* have allelopathic effects on algae (El-Sheekh *et al*., 2017).

The demand for medicinal and aromatic plants is rapidly increasing, due to their valuable chemistry. One of the most important production methods to be applied to meet this demand is plant tissue culture techniques. Medical plants have a number of advantages in producing by optimizing culture conditions through cell and tissue cultures. These can be listed as (*i*) the environmental factors (climate, geographical difficulties, seasonal constraints) encountered during cultivation of the plant are removed, (*ii*) less land use is provided, (*iii*) prevent the plant from being collected from the nature and protect its generation, (*iv*) it is ensured that economically valuable metabolites, which are present in plants in low quantities, can be produced in sufficient quantity, (*v*) homogeneity, standard quality and productivity are provided in production, (*vi*) helps in the understanding of the biosynthesis mechanisms of the metabolites (Erkoyuncu and Yorgancılar, 2015). Thanks to all these advantages, the production of medical and aromatic plants by tissue culture techniques is an important

research topic. The technique is used in the production of many medical plants such as *Scorzonera ahmet-duranii* (Surucu *et al*., 2018), *Ephedra gerardiana* (Rautela *et al*., 2018), *Stahlianthus campanulatus* Kuntze (Mongkolsawat *et al*., 2018) and *Artemisia abrotanum* (Bolyard, 2018).

The production of callus form from various explants (leaf, shoot tip, internode etc.) is carried out primarily to determine the culture conditions required for the survival and growth of the explants, examine cell growth, obtain products from primary and secondary metabolism, and obtain cell suspension. Large amounts of secondary and bioactive compounds can be obtained from the plants via the callus and cell culture (Ogita*et al*., 2009; Sen *et al*., 2014). The present study was designed for *in vitro* callus formation and plantlet regeneration of *C. demersum*, a high value medicinal aquatic plant, using shoot tip and different nodal meristem explants. In addition, photosynthetic pigment contents of shoots formed on calli were examined. *In vitro* production studies on *C. demersum* have been carried out previously by Karatas *et al*. (2014), Dogan *et al*. (2015) and Emsen and Dogan **(**2018). However, studies on plant regeneration following callus formation have never been conducted before. In this respect, our current work is potentially a significant contribution to the literature.

MATERIALS AND METHODS

The plant materials were obtained from Karamanoglu Mehmetbey University, Karaman, Turkey. Surface sterilization of the explants was accomplished by the method of Karatas *et al*. (2014). After sterilization, shoot meristem, 1st and 2nd nodal meristem explants were isolated under sterile conditions and incubated on MS (Murashige and Skoog, 1962) medium fortify with 30 g sucrose (Duchefa) per liter, 0.65% agar and 0.05, 0.10, 0.20, 0.40 and 0.80 mg/L Thidiazuron (TDZ) in Magenta GA^7 vessels for *in vitro* callus formation. Seven weeks after inoculation, the densities and frequencies of callus were recorded (Table 1).

After seven weeks, the identical compact callus obtained from shoot tip, 1st and 2nd nodal meristem were cultured on MS nutrient media supplemented with 3% sucrose (Duchefa), 0.65% agar (Duchefa) and 0.10 mg/L 6-Benzylaminopurine (BAP), 0.10 mg/L Gibberellic Acid (GA_3) and on MSO (hormone-free) medium to evaluate shoot regeneration (Table 2). Since *C. demersum* is rootless in its natural environment, *in vitro* rooting trials have not been conducted. The elongated shoots were transferred to aquariums containing water at pH 7.0 and 23±1°C 16 h light photoperiod for 30 days for acclimatization. The trials were repeated three times. The pH of all culture media was adjusted to 5.7±1 before autoclaving (1.2 atmospheric pressure, 120°C for 20 min). All explants were incubated under 16 h light - 8 h darkness photoperiod using white LED (Light Emitting Diodes) lights (1500 lux). The data for both callus and shoot regeneration in the MS mediums were recorded after 8 weeks of culture.

For photosynthetic pigment content analysis, the washed fresh plant samples (100 mg) were extracted with 80% acetone. Photosynthetic pigments (chlorophylls and carotenoids) were measured in a spectrophotometer (Thermo Fisher Scientific, Multiskan Go) at 663 nm, 646 nm and 470 nm. All content values were calculated according to Lichtenthaler and Wellburn (1983). Total chlorophyll content was given as chlorophyll-*a* + chlorophyll-*b*.

Data from tissue culture studies and photosynthetic pigment contents were analyzed statistically with IBM SPSS 21 for Windows and post hoc tests were performed using Duncan. Data given in percentages were subjected to arcsine transformation before statistical analysis (Snedecor and Cochran, 1997).

RESULTS AND DISCUSSION

The explants of *C. demersum* for callus induction were cultured in MS nutrient medium with various doses of TDZ (0.05-1.00 mg/L) (Table 1) and a yellowish and light green color were formed on the callus at the end of eight weeks (Fig. 1 a,b,c). Browning at a low rate has been observed on some callus. The first callus formation was observed in the MS nutrient medium containing 0.50 mg/L TDZ for the shoot (on the 14th day) and 1stnodal explant (on the 12th day), while it was obtained in the MS nutrient medium with 0.75 mg/L TDZ for the 2nd nodal explant (on the 12th day). The results showed that callus formation varied according to the explant type and hormone concentration. Irvani *et al*. (2010) cultured root, hypocotyl and cotyledon explants of *Dorema ammoniacum* D. on MS medium containing different concentrations of 2,4-D and NAA alone and in combination with BAP or KIN for callus induction and obtained the 100% effect from root explants on MS nutrient medium with 2 mg/L BAP + 1 mg/L NAA. Further, they observed that callus development began on the 8th day in root segments and on the 20th day in hypoctyl explants. And also the first callus formation in cotyledon explants was observed at 4 weeks. Khalafalla *et al* (2010) reported that the earliest calli formation from *Solanum tuberosum* L. was obtained in MS medium containing 4.0 and 5.0 mg/L 2,4-D on day 7.

Callus regeneration frequencies were determined as 100% in all culture media for 1st nodal explant, as 83.33-100% for shoot tip and as 66.66-100% for 2nd nodal explant. Generally, the calluses are obtained at high frequencies. Similarly, callus formation was reported in other plants cultured on MS medium with TDZ such as *Oriental hybrid* 'Siberia' (Wu *et al*., 2017),

Brassica oleracea L. var. botrytis (Srivastava *et al*., 2017), *Atropa acuminata* (Khan *et al*., 2017) and *Dimorphorchis lowii* (Jainol and Gansau, 2017). Whereas, Dogan *et al*. (2015) cultured the shoot tip and nodal explants of *C. demersum* in liquid MS medium supplemented with TDZ at different doses, and reported that callus did not form in the culture medium. This difference in results may be due to the fact the used liquid culture medium. Because the liquid culture medium may have prevented callus formation. In line with these, Karatas *et al*. (2014) who reported the absence of callus formation of shoot tip and nodal explants of *C. demersum* cultured in the liquid MS medium.

When compared to callus densities for all three explant types, the best hormone application was recorded as MS medium containing 0.50 and 0.75 mg/L TDZ (Fig. 1 a,b,c). The explant that gave the best results in callus experiments was determined as 1st nodal explant compared to other explants. Jainol and Gansau (2017) cultured leaf tip explants *D. lowii* on MS medium containing 0.22-3.0 mg/L TDZ and reported that a maximum percentage of callus formation (28.0±17%) and maximum callus density were determined in MS medium supplemented with 3.0 mg/L TDZ.

In vitro shoot regeneration from plant cells and explants is influenced by plant growth regulators that are added exogenously and by hormone present in the plant hormones (Trigiano and Gray, 2000). Different plant tissues can contain endogenous hormones at different levels and therefore the explant varieties have a critical effect on a successful shoot regeneration (Yucesan *et al*. 2007). In the present study, the calluses obtained from shoot tip, 1st and 2nd nodal explants in the MS medium fortify with TDZ (0.05-1.00 mg/L) were selected and transferred to the MS nutrient medium without plant growth regulator (MSO) and to MS medium containing 0.10 mg/L BAP and 0.10 mg/L GA_3 (Table 2), which is to achieve multiple shoot regeneration by sub-culturing callus. At the end of the first week, shoot tips began to spread on the callus masses, and multiple shoots were clearly observed after three weeks. At the end of eight weeks, shoots were obtained which prolonged and developed on calluses (Fig. 1 d,e,f). Data regarding shoot regeneration frequency of (%), mean number of shoots per callus and mean shoot length were recorded for three explants after eight weeks of culturing (Table 2).

Shoot regeneration frequency was found statistically significant (p<0.01), and was obtained to be 75.00-100.00% from shoot tip explants, 66,67-100.00% from 1st nodal explants and 58.33-100.00% from 2nd nodal explants. The lowest shoot regeneration rates in three explant applications were determined by transferring calli in MS medium containing 1.00 mg/L TDZ to MSO medium. When explants were compared within themselves, the highest percentage of shoot regeneration was determined in the shoot tip explant. 100% shoot regeneration in all subculture applications was obtained from calli transferred from 0.25 mg/L TDZ in three explants. Zale *et al*. (2004) detected 8 to 90% of shoot regeneration from callus in 29 different wheat types.

In BAP, GA_3 and MSO applications, regenerated shoots were obtained from calli through organogenesis (Table 2). Mean number of shoots per calluses of shoot meristem, 1st and 2nd nodal meristem explants ranged between 39.61-108.83, 42.08-96.25 and 33.38-87.08, respectively in the subculture MS medium. Maximum number of shoot per callus from shoot tip (108.83), 1st (96.25) and 2nd (87.08) nodal meristem explants were achieved in culture medium with 0.10 mg/L GA_3. Whereas, minimum number of shoot per calluses from all explants were determined from hormone-free MS medium (MSO). When the BAP, GA_3 and MSO applications were compared, the highest number of shoots per callus was obtained in MS medium containing 0.10 mg/L GA_3. On the other hand, the lowest numbers of shoots on callus were detected in the MSO medium. The results revealed that GA_3 was the best practice in terms of shoot numbers per callus. Similarly, the promoting effect of GA_3 and BAP on *in vitro* shoot proliferation and elongation have been lately reported over a wide range species such as *Jatropha curcas* (Purkayastha *et al*., 2010), *Magnolia* X 'Spectrum' (Wojtania *et al*., 2016), *Solanum tuberosum* L. (Ali *et al*., 2018) and *Cicer arietinum* L. (Kumari *et al*., 2018).

The lengths of shoots from sub-cultured calluses in MSO nutrient medium and MS nutrient medium fortified with 0.10 mg/L BAP and 0.10 mg/L GA_3 were examined (Table 2). Shoot length was ranged between 0.49 and 1.25 cm, between 0.47 and 1.39 cm, and between 0.48 and 1.43 cm on the callus from shoot tip, 1st, and 2nd nodal meristem explants, respectively. The longest shoots were recorded in the culture with 0.10 mg/L GA_3 for the 1st (1.39 cm) and 2nd (1.43 cm) nodal explants and in the culture with 0.10 mg/L BAP for the shoot tip explants (1.25 cm). The shortest shoots were detected from the calluses in the MSO medium. In addition, the shortest shoots were determined from calli containing the highest TDZ concentration (1.00 mg/L). Similar to these findings, the suppression and adverse effects of TDZ on the length of shoots have already been reported (Murthy *et al*., 1998; Chand *et al*., 1999; Fratini and Ruiz, 2002).

Since *C. demersum* is a rootless plant in its natural environment, rooting studies have not been carried out. The *in vitro* regenerated plants were transferred to the aquarium to acclimatize to external conditions. The MS nutrient medium on the plants was carefully removed with tap water. After five weeks, it was recorded that all plants survived (100%).

Photosynthetic pigmentation of plants is affected by many internal and external factors

(Muradoglu *et al.,* 2015). Dogan *et al.* (2018) reported the change in photosynthetic contents due to the heavy metal stress of *C. demersum*. Thomas *et al.* (2016) investigated the effect of nanomolar copper in low radiation on photosynthetic pigment contents of *C. dermersum*. Furthermore studies on the effects of salt (Dogan and Saygideger, 2018), herbicide (Huiyun *et al*., 2009) and ultraviolet (Germ *et al*., 2006) on photosynthetic pigment contents in *C. demersum* have been carried out previously. However, the chlorophyll contents of shoots from the calli produced in tissue culture conditions have not been studied previously. In the present study, the photosynthetic pigment contents (chlorophyll *a*, *b*, total chlorophyll, carotenoid) of regenerated shoots formed by the effects of free-hormone MS (MSO), 0.10 mg/L BAP and 0.10 mg/L GA_3 from callus composed of shoot tip, and 1st nodal and 2nd nodal explants under different TDZ concentrations were evaluated (Fig. 2).

The chlorophyll *a* contents of the shoots from shoot tip, 1st nodal and 2nd nodal explant were determined as 1.34-0.95, 1.18-0.86 and 1.26-0.92 mg/g fresh weight, respectively. The highest content of chlorophyll *a* in shoot tip explant was measured as 1.34 mg/g f.w. from callus with 0.25 mg/L TDZ in MS nutrient medium containing 0.10 mg/L BAP. For 1st nodal (1.18 mg/g f.w.) and 2nd nodal (1.26 mg/g f.w.) explants, the maximum chlorophyll *a* contents were detected in shoots developed from callus with TDZ of 0.05 mg/L in MS medium containing 0.10 mg/L BAP. The lowest chlorophyll-*a* contents in all three explant types were determined in the MSO culture medium, while the excess amounts of chlorophyll *a* were obtained in culture medium containing 0.10 mg/L BAP. The results show that high TDZ concentration affects chlorophyll-*a* content negatively. The results also showed that shoots from shoot tip calli had a higher chlorophyll-*a* than others.

The chlorophyll-*b* contents of the regenerated shoots from callus were recorded as 0.56-0.27 mg/g f.w for shoot tip explants, 0.43-0.23 mg/g f.w for 1st nodal explants and 0.51-0.28 mg/g f.w for 2nd nodal explants. When all experiments were compared, the highest content of chlorophyll-*b* (0.56 mg/g f.w) was obtained in shoots exiting the shoot tip callus medium from MS containing and 0.10 mg/L BAP, whereas the lowest chlorophyll-*b* content (0.23 mg/g f.w.) was determined in MS medium with 0.10 mg/g GA_3. In general, the increase in TDZ doses has been shown to reduce chlorophyll-*b* content. The highest chlorophyll-*b* content was determined in 0.10 mg/L BAP application.

Total shoot chlorophyll contents of regenerated shoots from shoot tip, 1st nodal and 2nd nodal calli were 1.90-1.22, 1.61-1.09 and 1.77-1.17 mg/g f.w., respectively (Fig. 2a,b,c). Chlorophyll content decreased with increasing TDZ concentration. In the spectroscopic measurements, the highest chlorophyll-*b* content was obtained in the shoots from the shoot tip callus cultured in MS medium supplemented with 0.10 mg/L BAP. The lowest chlorophyll-*b* values were generally found in calli produced in high TDZ and in MSO nutrient medium.

Plant carotenoids represent a large class of terpenoids characterized by excellent light-absorbing properties in the blue-green range of the visible spectrum (450-550 nm) and they are conjugated polyene chains which are C_{40} carbohydrates with a double bond chain. The leaves of plants, flowers and fruits in different colors ranging from yellow to red depends on the accumulation of carotenoids in the chromoplasts (Galpaz et al., 2006; Moise et al., 2014).

In the established experiments, the carotenoid contents of shoots emerging from calli were very variable. Carotenoid contents in shoot tip, 1st nodal and 2nd nodal calli were found to be 0.35-0.22 mg/g f.w., 0.31-0.19 mg/g f.w. and 0.36-0.21 mg/g f.w., respectively (Fig. 2d,e,f). The maximum amount of carotenoid content (0.36 mg/g f.w.) was obtained in shoots from the 2nd nodal callus in MS medium fortify with 0.10 mg/L GA_3. On the other hand, the lowest carotenoid content was determined as 0.19 mg/g f.w. in 1st nodal callus. In general, carotenoid quantities at the lowest levels were detected in shoots in the MSO medium.

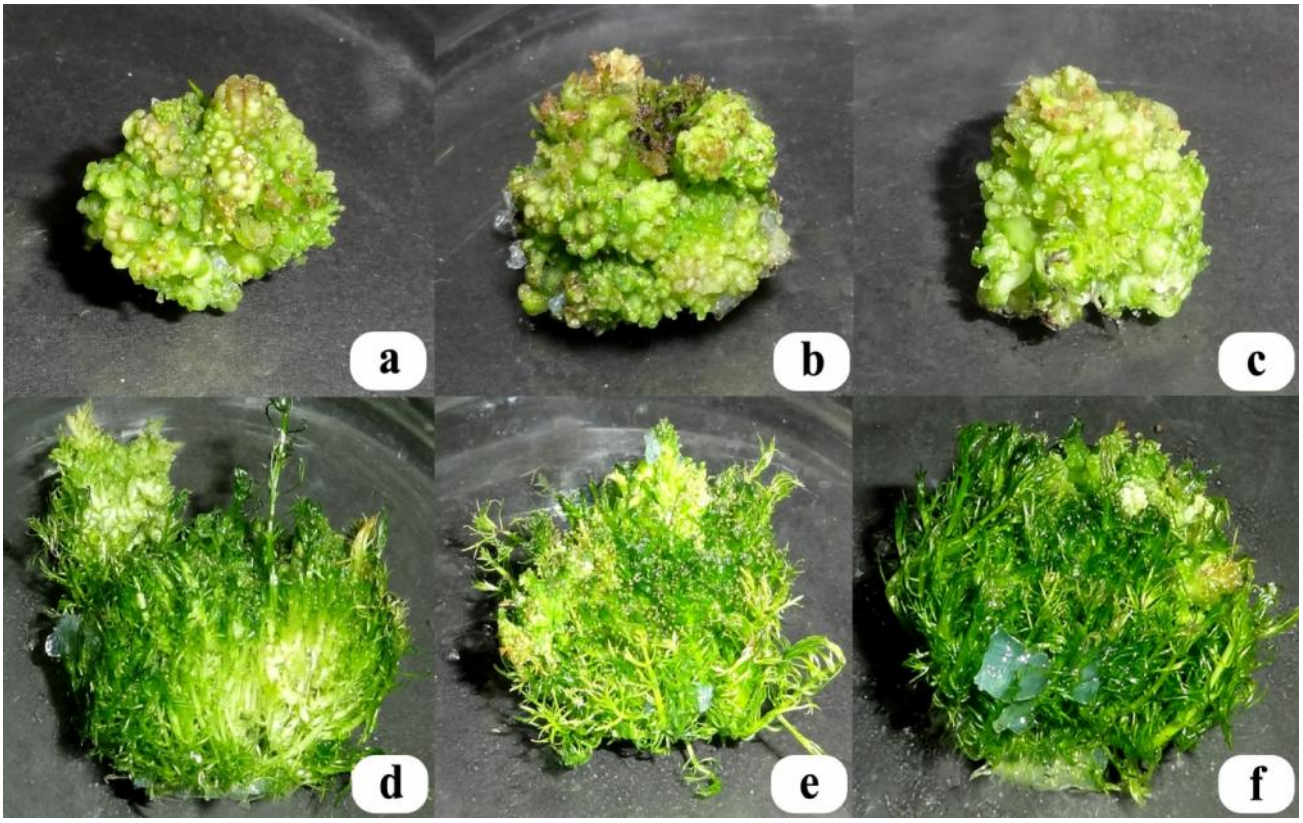
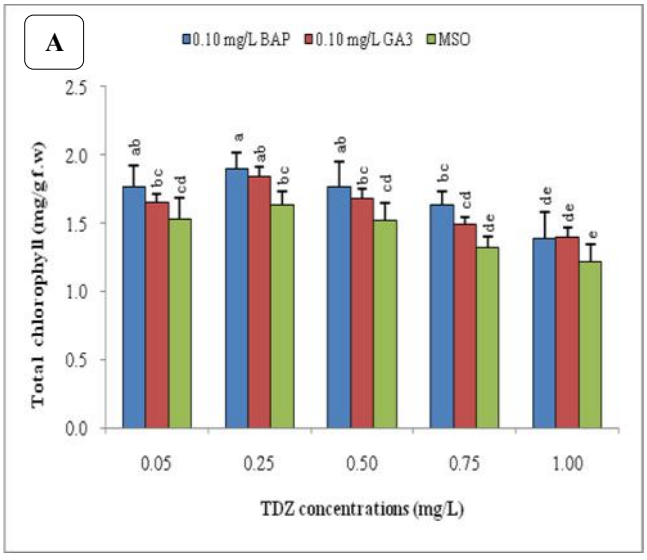
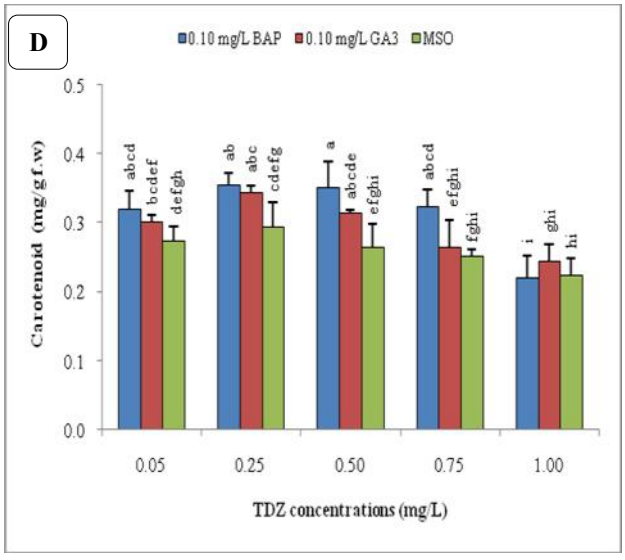


Figure 1. Callus induction and plant regeneration from shoot tip and nodal explants of *C. demersum*. Callus formations from shoot tip (a), 1st (b) and 2nd (c) nodal explants, after eight weeks of culture; Plant regenerations from callus formed shoot tip (d), 1st (e) and 2nd (f) nodal explants, after eight weeks of culture.





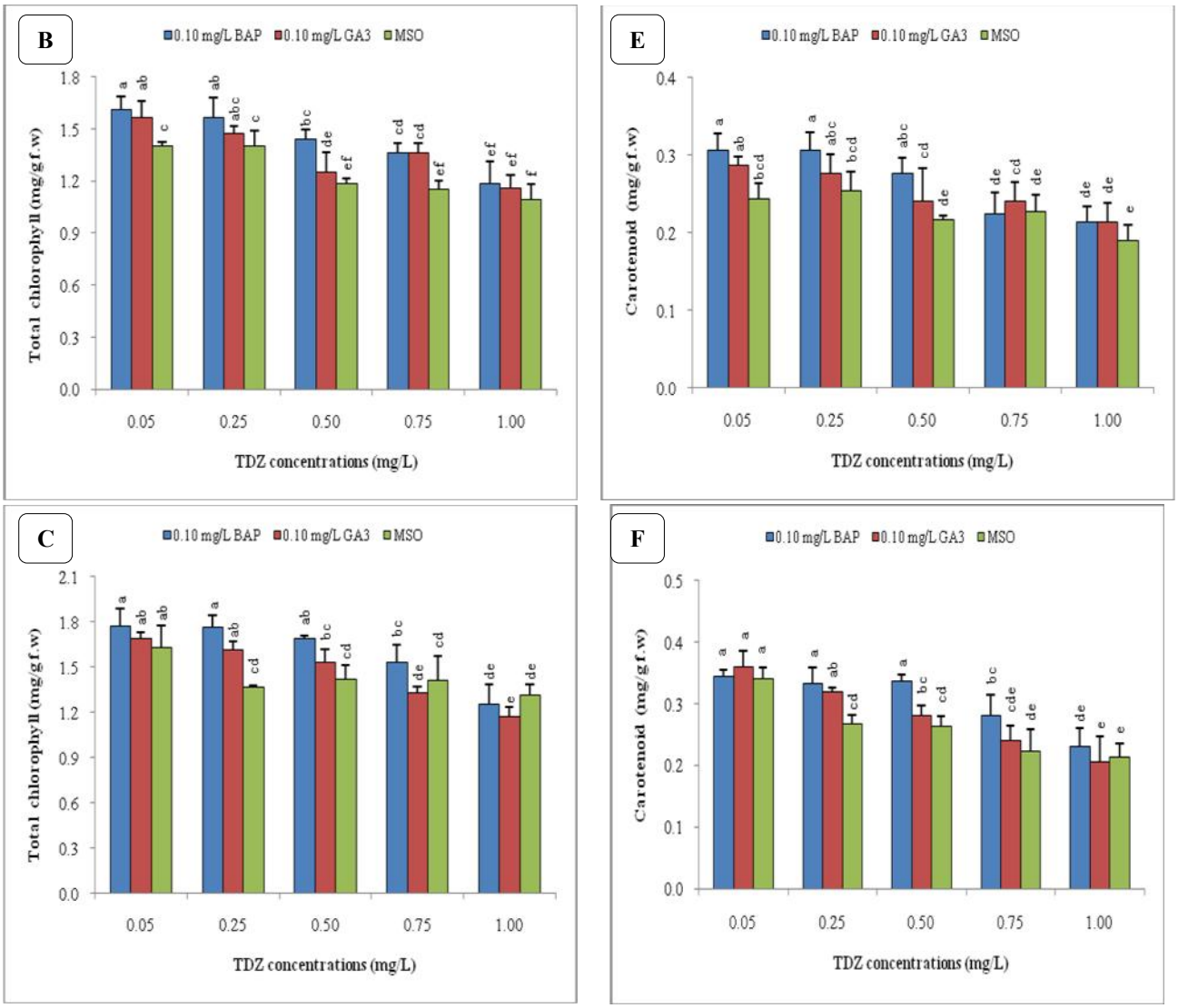


Figure 2. The total chlorophyll and carotenoid contents of regenerated shoots formed by the effects of free-hormone MS (MSO), 0.10 mg/L BAP and 0.10 mg/L GA_3 from callus composed of shoot tip (A,D), and 1st nodal (B,E) and 2nd nodal (C,F) explants under different TDZ concentrations.

Table 1. Effects of different concentrations of TDZ on callus induction from shoot tip and nodal meristem explants of *C. demersum* on MS medium.

TDZ (mg/L)	Shoot tip			1st nodal meristem			2nd nodal meristem		
	Callus frequency (%)	Callus density	First callus formation (days)	Callus frequency (%)	Callus density	First callus formation (days)	Callus frequency (%)	Callus density	First callus formation (days)
0.05	83.33	++	16	100.00	++	14	94.44	+	17
0.25	100.00	++	16	100.00	+++	15	100.00	++	17
0.50	100.00	+++	14	100.00	+++	12	100.00	+++	16
0.75	100.00	+++	15	100.00	+++	15	100.00	+++	15
1.00	94.44	+	18	100.00	++	17	66.66	+	19

Intensive callus: +++, Normal callus: ++, Less callus: +

Table 2. Effects of free-hormone MS (MSO), 0.10 mg/L BAP and 0.10 mg/L GA_3 on regenerative response of callus of *C. demersum*.

First Culture Medium	Subculture	Shoot regeneration frequency (%)			Mean number of shoots per callus			Mean shoot length (cm)		
Growth regulators (mg/L)		Shoot tip	1st nodal meristem	2nd nodal meristem	Shoot tip	1st nodal meristem	2nd nodal meristem	Shoot tip	1st nodal meristem	2nd nodal meristem
	0.10 BAP	100.00^{a}	91.67^{a}	66.67^{bc}	90.75^{def}	86.28^{bc}	82.22^{b}	1.22^{b}	1.35^{b}	1.12^{c}
0.05 TDZ	**0.10 GA_3**	100.00^{a}	100.00^{a}	100.00^{a}	101.25^{b}	91.17^{ab}	85.75^{a}	0.97^{e}	1.10^{e}	0.91^{e}
	MSO	100.00^{a}	91.67^{a}	83.33^{ab}	61.83^{h}	66.44^{fg}	53.00^{g}	0.71^{h}	0.69^{i}	0.74^{g}
	0.10 BAP	100.00^{a}	100.00^{a}	100.00^{a}	96.42^{bcde}	92.92^{ab}	78.75^{c}	1.25^{a}	1.15^{d}	0.95^{d}
0.25 TDZ	**0.10 GA_3**	100.00^{a}	100.00^{a}	100.00^{a}	108.83^{a}	96.25^{a}	87.08^{a}	1.17^{c}	1.39^{a}	1.43^{a}
	MSO	100.00^{a}	100.00^{a}	100.00^{a}	55.50^{hi}	64.33^{g}	61.08^{f}	0.65^{i}	0.62^{j}	0.68^{h}
	0.10 BAP	100.00^{a}	100.00^{a}	100.00^{a}	92.92^{cde}	83.08^{cd}	79.58^{bc}	1.12^{d}	0.97^{g}	0.92^{e}
0.50 TDZ	**0.10 GA_3**	100.00^{a}	100.00^{a}	100.00^{a}	98.50^{bc}	85.92^{bc}	81.75^{bc}	1.13^{d}	1.18^{c}	1.21^{b}
	MSO	100.00^{a}	100.00^{a}	91.67^{a}	51.92^{i}	55.28^{h}	52.05^{g}	0.59^{j}	0.54^{k}	0.57^{i}
	0.10 BAP	100.00^{a}	100.00^{a}	100.00^{a}	89.16^{efg}	74.08^{ef}	72.00^{d}	0.83^{f}	0.96^{g}	0.90^{e}
0.75 TDZ	**0.10 GA_3**	100.00^{a}	100.00^{a}	100.00^{a}	97.17^{bcd}	77.58^{de}	73.16^{d}	0.97^{e}	1.04^{f}	1.14^{c}
	MSO	83.33^{b}	83.33^{ab}	75.00^{abc}	42.22^{j}	45.55^{i}	36.11^{h}	0.51^{k}	0.48^{l}	0.53^{j}
	0.10 BAP	100.00^{a}	100.00^{a}	100.00^{a}	83.41^{g}	66.17^{fg}	62.91^{f}	0.77^{g}	0.91^{h}	0.86^{f}
1.00 TDZ	**0.10 GA_3**	100.00^{a}	100.00^{a}	100.00^{a}	84.92^{fg}	70.75^{efg}	67.83^{e}	0.77^{g}	1.03^{f}	0.91^{e}
	MSO	75.00^{b}	66.67^{b}	58.33^{c}	39.61^{j}	42.08^{i}	33.38^{h}	0.49^{l}	0.47^{l}	0.48^{k}

Means followed by different small letters within columns are significantly different ($p<0.01$)

Conclusion: This work reported *in vitro* callus induction from shoot tip, 1st and 2nd nodal explants of *C. demersum* and plant regenerations from these calluses. So far, this is the first report of using TDZ, BAP and GA_3 for callus formation and plantlet regeneration of *C. demersum*. The most effective hormones for callus formation and density were MS medium containing 0.50 and 0.70 mg/L TDZ for all explant types. The most effective application of shoot numbers per callus was recorded as GA_3>BAP>MSO. The longest shoots were determined in the MS medium including 0.10 mg/L GA_3 for the 1st and 2nd nodal explants and in the MS medium fortify with 0.10 mg/L BAP for the shoot tip explants. Additionally, explant sources and culture conditions were found to be effective on the photosynthetic pigment contents of the regenerated shoots. This work is a new protocol for efficient and rapid production of *C. demersum* via callus induction. It may contribute significantly to the literature in this regard.

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Conflict of interest disclosure: The author declare no conflict of interest.

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