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Callus-mediatede embryogenesis of leaf celery (*Apium graveolens* L. var. *secalinum* Alef.) as a response to malt, peptone and yeast extracts application

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Callus-mediatede embryogenesis of leaf celery (*Apium graveolens* L. var. *secalinum* Alef.) as a response to malt, peptone and yeast extracts application

Abstract: Historically, leaf celery (*Apium graveolens* L. var. *secalinum* Alef. of Apiaceae family is well-known traditional plant that possesses eminent therapeutic characteristics. The current work aimed to develop an efficient protocol for celery embryogenesis via indirect approach of callus cell cultures using biotic elicitors (malt, peptone and yeast extract) at different concentrations (1.5, 2.5, 3.5 g l^{-1}) of each. The optimal explant type to induce calli were the stems and the best medium was MS-medium + 0.5 mg l-1 2,4-D. Fresh mass and growth of celery stem calli were proliferated recording the highest values after 8 weeks of culture, whereas the highest growth rate was achieved after 5 weeks of culture. Malt extract (1.5 g l^{-1}) performed as the best biotic elicitor for the highest value of callus fresh mass. Similarly, growth rate and growth value increased to 1.2 ± 0.009 g/day and 12.1 ± 0.12, respectively. Throughout the histological investigation, the anatomical structure for all elicited treatments of celery stem calli exhibited more potent efficiency of malt than peptone for embryogenesis development then the yeast extract. Meanwhile, elicitor- free MS-medium stopped the callus growth at the stage of globular embryo formation. This work documented the elicitors' efficiency for callus growth and somatic embryo development of *Apium graveolens*. var. *dulce* (Mill.) Pers..

Key words: *Apium graveolens* L. var. *secalinum* Alef., callus, embryogenesis, biotic elicitors, histological investigation

Embriogeneza listne zelene (*Apium graveolens* L. var. *secalinum* Alef.) iz kalusa z dodatki slada, peptona in izvlečkov kvasa

Izvleček: Zgodovinsko gledano je listnata zelena (*Apium graveolens* L. var. *secalinum* Alef.) iz družine kobulnic (Apiaceae) znana tradicionalna rastlina, ki ima izjemne terapevtske lastnosti. Cilj dela je bil razviti učinkovit protokola za embriogenezo listnate zelene posredno iz kultur kalusnih celic z uporabo biotskih elicitatorjev (slada, peptona in ekstrakta kvasa) pri različnih koncentracijah (1,5, 2,5, 3,5 g l^{-1}) vsakega. Optimalna vrsta eksplantata za induciranje kalusa so bila stebla, najboljše gojišče pa MS-medij + 0,5 mg l^{-1} 2,4-D. Sveža masa in rast stebelnega kalusa zelene sta proliferirali z največjimi vrednostmi po 8 tednih gojenja, medtem ko je bila najvišja stopnja rasti dosežena po 5 tednih gojenja. Izvleček slada (1,5 g l^{-1}) se je izkazal kot najboljši biotski elicitor za največjo vrednost sveže mase kalusa. Podobno sta se povečali hitrost rasti in vrednost rasti na 1,2 ± 0,009 g/dan oziroma 12,1 ± 0,12. Skozi histološko preiskavo je anatomska zgradba vseh izzvanih stebelnih kalusov zelene pokazala močnejšo učinkovitost slada kot peptona za razvoj embriogeneze , oba pa večjo kot izvleček kvasa. Medtem je MS-gojišče brez elicitorja ustavilo rast kalusa na stopnji oblikovanja globularnega zarodka. To delo je dokumentiralo učinkovitost elicitorjev za rast kalusa in somatski razvoj zarodkov listanate zelene.

Ključne besede: *Apium graveolens* L. var. *secalinum* Alef., kalus, embriogeneza, biotski elicitorji, histološka preiskava

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1 INTRODUCTION

Apium graveolens L. var. *secalinum* Alef. leaf celery, belongs to family Apiaceae' and it is one of biennial branching herbs that grow in Europe and East Asian countries. This aromatic plant was used for medicinal purposes and then it had been cultivated as a food additive (Hassanen et al., 2015). Also, celery seeds contain an essential oil that can be used in industry or as flavoring or spice (Toth & Lacy, 1992). Celery is a rich source of some bioactive compounds such as phenolic acids, flavonoids in addition to carotene, vitamins and cellulose. So, the different parts of the celery plant were reported to possess different therapeutic effect including anti-inflammatory, antioxidant, antifungal and antibacterial properties as it contains 22 volatile compounds (Zahra et al., 2011). Tissue culture propagation through somatic embryogenesis is an efficient technique for developing micropropagation and for industrial production of celery (Nadel et al., 1995). The addition of plant growth regulators to *in vitro* cultures have different effects on cell division, cell differentiation and growth developing. Auxins are used for callus induction, the most commonly used is 2,4-D. Media fortified with 1 mg l^{-1} 2,4-D gave the best callus induction and shoot regeneration of *Bunium persicum* (Boiss.)F. F.Dtsch , a member of the Umbellifera family (Valizadeh & Kazemi, 2009). Chen (1976) found that 2,4-D and kinetin combinations were the most effective for celery callus initiation. MS medium with 0.5 mg/l 2,4-D and 0.5 mg/l kinetin successfully produced friable calli from petiole and leaf explants as reported by Bruznican et al. (2017). While Hassan (2019) reported that the optimum plant growth regulator for callus initiation and regeneration was BAP (1 mg l^{-}) and 2,4-D (0.5 mg l^{-1}). Also, some reports indicated that transferring callus to media with 1 mg l^{-1} 2,4-D (Wakhlu et al., 1990) or 0.5 mg l^{-1} 2,4-D (Bonianpoor, 1995) resulted in embryogenesis formation. Somatic embryogenesis can be conducted in a direct or indirect method. In the process of indirect somatic embryogenesis, it starts with embryogenic calli formation. These embryonic cells are characterized by large nuclei and cytoplasm with high metabolic activity (Jim'enez & Bangerth, 2001). Induction of somatic embryogenesis is affected by many factors, like plant phenotype, explant type, medium composition, plant growth regulators, organic additives and incubation conditions (Wojcikowska & Gaj, 2017, Rizwan et al., 2020, Gulzar et al., 2020, and Araujo et al., 2021). To induce somatic embryogenesis significantly, high concentrations of auxins such as 2,4-D should be added, while embryo development often needs lower auxin levels (Edwin et al., 2008). Reinert et al. (1966) first reported the induction of adventitious embryos in celery. Al-Abta & Collin (1978) used cell suspension cultures of celery for the somatic embryos production. In addition, embryoid formation in cell suspension cultures of celery was dependent on 2, 4-D addition. Somatic embryogenesis was successfully induced from nodal segments using 1 and 4 mg l^{-1} of 2,4-D alone (Chagas et al., 2023). Embryogenic calli were highly proliferated on culture medium with 1.5 mg l^{-1} 2,4-D (Zhang et al., 2021). The maximum embryogenesis percentage from hypocotyl explants of *B. persicum* was found on 0.5 mg l^{-1} BAP + 1 mg l^{-1} 2,4-D, 0.5 mg l^{-1} BAP + 2 mg l^{-1} 2,4-D, and 1 mg l^{-1} 2,4-D treatments (Ouzhand et al., 2023). Mahadi et al. (2016) showed that the addition of 2 mg l^{-1} 2,4-D and 2 mg l^{-1} BAP gave the best result for embryogenic callus of Calamansi (*Citrus* x *microcarpa* Bunge), while, using 1.0 mg l^{-1} 2,4-D and 2.0 mg l^{-1} BAP gave the best embryo production from cell cultures of *Tecoma stans* L. (Omar et al., 2024). Somatic embryogenesis was also improved by the addition of organic compounds, like casein hydrolysate, yeast extract, malt extract, coconut water, peptone and tryptone, where they provide cultures with sugar, sugar is typically supplied by sucrose, amino acids, phosphate, several microelements and vitamins to enhance growth and development of embryogenic cultures (Al-Khayri, 2011). Malt extract has been applied for callus and somatic embryogenesis induction in many *Citrus* species (Amin & Shekafandeh, 2015). For example, the best result for embryogenic callus formation of sweet orange 'Washington Nave' was obtained on the medium supplemented with 500 mg l^{-1} malt extract (Mazri & Belkoura, 2021). While, addition of 1g l^{-1} of malt extract improved the induction of mulberry secondary somatic embryos (Agarwal, 2004). Also, Borpuzari & Borthakur (2016) reported that maximum callus formation and maturation of globular embryos resulted by addition of 100.0 mg l^{-1} of yeast extract combined with 2,4-D and IAA of *Plumbago rosea* L. inter node explants. Sherif et al. (2018) mentioned that biotic elicitors such as peptone (50 mg l^{-1}), and coconut water (5 %) which were added individually or together are necessary for embryogenesis from intermodal explant of *Anoectochilus elatus* Lindl., *w*hile, 0.5 g l^{-1} or 1 g l^{-1} peptone successfully promoted the embryogenesis from leaf tip segments of *Oncidium* 'Gower Ramsey' (Chen & Chang, 2002). The histological analysis of somatic embryogenesis through various techniques provides detailed knowledge of various stages of somatic embryogenesis like initiation, proliferation, maturation, and conversion and different phases of growth (globular, heart, torpedo, and cotyledonary). Besides, it allows to identify and monitor

the early events of embryogenetic cells such as denser cytoplasm, high nucleus-to-cytoplasm ratio and large nucleolus (Gomes et al., 2017, Campos et al., 2017), this requires more studies to achieve a promising stu-Generally, the objective of our work was to perform a successful protocol of somatic embryogenesis development via screening the efficiency of diverse biotic elicitors (malt, peptone and yeast extracts) at different concentrations on somatic embryos formation and development of *Apium graveolens* L.

2 MATERIAL AND METHODS

2.1 SEEDS STERILIZATION AND GERMINATION OF *APIUM GRAVEOLENS*

Seeds of leaf celery (*Apium graveolens* L. var. *secalinum* Alef.) were obtained from Horticulture Research Institute, Agricultural Research Center, Ministry of Agriculture and Land Reclamation, Egypt. The investigation was carried out in National Research Centre. Seeds of celery were disinfected by 70 % ethyl alcohol for 1 min then washed 3 times with sterile distilled water, then soaked in 45 % sodium hypochlorite (NaOCl) for 20 min before rinsing three times with sterile water. Seeds were placed on MS medium (Murashige & Skoog, 1962) containing 3 % (w/v) sucrose and 0.7 % (w/v) agar. The pH of the culture medium was adjusted to 5.8 before autoclaving. The cultures were maintained in the growth chamber at 25 ± 2 °C with 16 h/8 h photoperiod of fluorescent, 100 µmol photons m^{-2} s^{-1} white light illumination.

2.2 CALLUS INDUCTION OF *APIUM GRAVEOLENS*

Stem and leaf segments were separated from the grown seedlings (4 weeks old) as explants for callus induction. Both stem and leaf segments were cultured on solidified MS medium containing various supplementation: 0.5 mg l^{-1} dichlorophenoxyacetic acid (2, 4-D), 0.5 mg l^{-1} 2, 4-D +1.0 mg l^{-1} benzyl aminopurine (BAP) and 0.5 mg l^{-1} 2, 4-D +1.0 mg l^{-1} kinetin (kin). Cultures were incubated under temperature of 25 ± 1 °C and light conditions of 16 h/8 h photoperiod and 100 µmol m^{-2} s ^{1}. Callus fresh mass were recorded every week through 8 weeks.

2.3 ELICITATION OF *APIUM GRAVEOLENS* CALLUS CULTURES BY DIFFERENT BIOTIC ELICITORS.

Several levels (1.5, 2.5 and 3.5 g l^{-1}) of malt extract, yeast extract and peptone extract as biotic elicitors were supplemented after callus induction to the callus culture medium fortified with 0.5 mg l^{-1} 2, 4-D. Cultures were kept in the growth chamber under temperature of 25 ± 1 °C and light conditions of 16 h photoperiod and 100 µmol m^{-2} s^{-1}. Callus fresh mass were recorded every 10 days through 60 days.

2.4 PARAMETERS OF *APIUM GRAVEOLENS* CALLUS CULTURE GROWTH

The cultures were implemented in triplicates and the growth parameters were calculated using two parameters as follows:

(a) Growth rate: $GR = Pt–P0/10$ (g/day).

(b) Growth value: $GV = Pt–P0/P0$

Pt = mass (g) of calli at the end of every ten days

P0 = starting mass (g) of the callus.

2.5 HISTOLOGICAL INVESTIGATION OF *APIUM GRAVEOLENS* CALLUS CULTURES.

The anatomical structure of *Apium graveolens* stem calli treated with the different elicitors was studied. Microtechnique practices were conducted at the Department of Agricultural Botany, Faculty of Agriculture, Cairo University, within the second season. Samples were fixed for at minimum forty-eight hours in (formaldehyde, acetic acid, alcohol mixture solution (F.A.A), dehydrated, and then embedded in paraffin wax (Sass, 1951). Parts that were cut on a rotary microtome at a thickness of 15-20 microns were stained with crystal violet/erythrosine before mounting in Canada balsam. Slides were investigated microscopically and photomicrographically.

2.6 STATISTICAL ANALYSIS

IBM SPSS statistics subscription software was used. The significance level was investigated by the analysis of variance analysis (ANOVA). Three replicates of 5 explants per replicate were used in each treatment. Data are shown as means ± standard errors (SE) and compared by LSD test at p value less than 0.05.

3 RESULTS

3.1 *IN VITRO* SEEDLINGS GERMINATION OF *APIUM GRAVEOLENS*.

Apium graveolens seeds were cultured on MS medium under sterilization conditions, the application of sterilization procedure in our experiment was efficient for further micropropagation; this examination was similar to those mentioned by Bruznican et al. (2017) who used 70 % ethanol following by sinking in 5 % NaOCl containing 3 drops of Tween 20 for fifteen minutes to decrease the contaminants on the seed surface. Beginning of *Apium graveolens* seed germination was obtained on MS basal medium after 2 weeks culture (Fig. 1), and continued to germinate at 3 and 4 weeks of culture (Figs. 2, 3). Celery *in vitro* seedlings were obtained after 8 weeks of seeds cultivation (Fig. 4) without any auxin or cytokinin in culture medium. This is close to the results of Bruznican et al. (2017) who obtained seedlings on hormone-free medium. Data tabulated in Table 1 showed the germination percentage of celery seeds and their lengths for 8 weeks of culture; maximal percentage of seeds germination was between the seventh and the eighth week of culture (83.10 ± 0.59, 86.57 ± 0.87, respectively), with seedling lengths (4.77 ± 0.15cm and 5.00 ± 0.58cm, respectively). A clear difference was observed in either seeds germination percent or seedlings length (cm) at the eighth week of culture comparable to the other periods, whilst there was no significance in seedlings length from the fifth to the seventh week of culture, and there was slightly significance in seeds germination percent among 5, 6 and 7 weeks of culture. Therefore, it was recommended to let seedlings grow for 8 weeks to obtain normal and healthy considerable amounts of celery *in vitro* seedlings.

Table 1: Percentage of seeds germination and seedlings length during 8 weeks of culture

Incubation period	Seeds germination %	Seedlings length (cm)
Week 1	1.00 ± 0.29g	0.75 ± 0.14e
Week 2	46.97 ± 1.16f	0.75 ± 0.14e
Week 3	65.10 ± 2.89e	2.73 ± 0.15d
Week 4	70.00 ± 5.77 de	3.77 ± 0.15c
Week 5	73.10 ± 1.16cd	4.27 ± 0.15bc
Week 6	78.00 ± 0.58bc	4.27 ± 0.15bc
Week 7	83.10 ± 0.59ab	4.77 ± 0.15bc
Week 8	86.57 ± 0.8a	5.00 ± 0.58a
LSD 0.05	5.91	0.61

Mean values accompanied with different superscript letters were significantly different ($p < 0.05$).

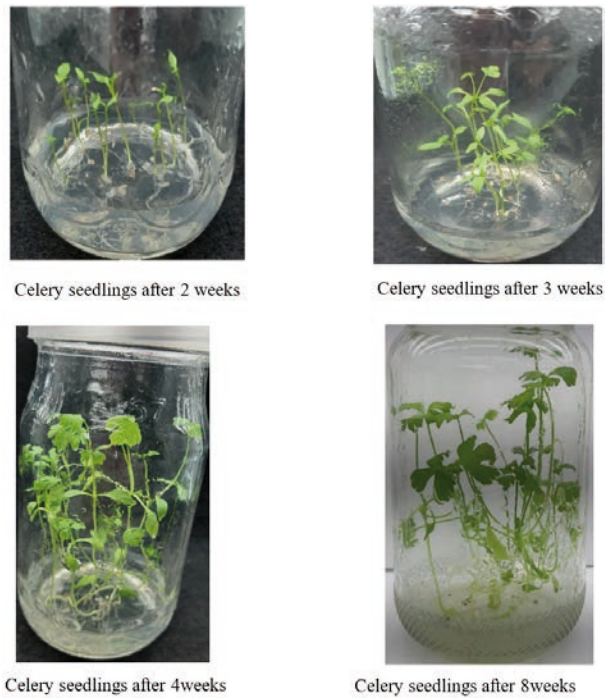

Figure 1: Phases of seedlings formation of celery

3.2 CALLUS INDUCTION OF *APIUM GRAVEOLENS IN VITRO* SEEDLINGS.

About 1 cm of healthy leaf and stem of celery seedlings were aseptically excised and then sub-cultured on MS-medium including 0.5 mg l^{-1} 2, 4-D alone or combined with 1 mg BAP^{-1} or 1 mg l^{-1} kin for callus initiation stage. All treatments exhibited different percentages of callus induction with variable significance of both of stem and leaf explants as shown in Table 2. It was observed that, maximal percent of calli (83.3 ± 0.58 %) in high significant difference clearly appeared using stem explant at treatment of 0.5 mg l^{-1} 2,4-D compared to the leaf explant (55 ± 5.77) on the same distinct medium. Meanwhile, addition of 1 mg l^{-1} BA or kin with 0.5 mg l^{-1} 2, 4-D decreased significantly callus induction in both stem and leaf explants (Fig. 2). So, it was recommended to use stem as the optimal explant rather than the leaf explant (Fig. 3) to be cultured in medium fortified with little concentration of 2,4-D (0.5 mg l^{-1}) alone to induce callus cultures of celery for two months of cultivation.

On the basis of previous results, stem calli of *Apium graveolens* were re-cultured on the same cer-

Table 2: Callus induction of celery stem and leaf explants after two months of cultivation

Treatments	Callus induction%	
	Stem	Leaf
0.5 mg l^{-1} 2,4-D	83.30 ± 0.58^{a}	55.00 ± 5.77^{ab}
0.5 mg l^{-1} 2,4-D + 1 mg l^{-1} BA	53.30 ± 0.58^{ab}	25.00 ± 0.58^{bcd}
0.5 mg l^{-1} 2,4-D + 1 mg l^{-1} Kin	16.70 ± 0.58^{cd}	10 ± 0.58^{d}
$LSD_{0.05}$	36.42	

Mean values accompanied with different superscript letters were significantly different ($p < 0.05$).

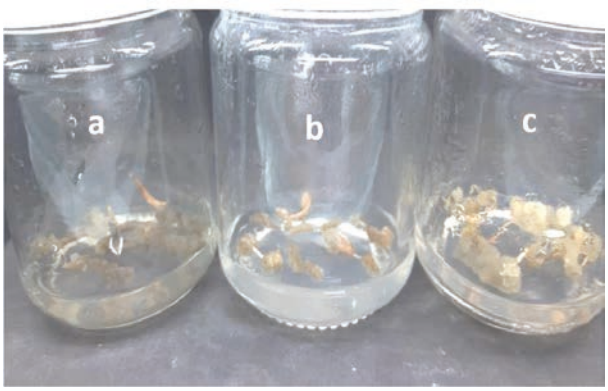

Figure 2: Stem explants of celery after one month of culture on 0.5 mg l^{-1} 2, 4-D + 1 mg l^{-1} BA (a), 0.5 mg l^{-1} 2,4-D + 1 mg l^{-1} Kin (b) and 0.5 mg l^{-1} 2,4-D (c).

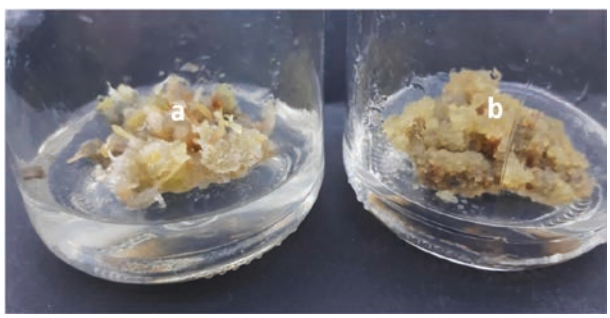

Figure 3: Callus induction from celery leaf (a) and stem (b) explants on MS-medium + 0.5 mg l-1 2, 4-D after two months of culture

tain medium (0.5 mg l^{-1} 2, 4-D) for callus induction, and maintained for 8 weeks to study these parameters; fresh mass, growth rate and growth value (Table 3). The highest values of fresh mass and growth value were recorded with clear significant differences (7.2 ± 0.06 g and 13.4 ± 0.16, respectively) after the eighth week of

Table 3: Fresh mass, growth rate and growth value of celery stem calli grown on medium containing 0.5 mg l^{-1} 2.4-D during 8 weeks of culture

Incubation period of cultures	Fresh mass (g)	Growth rate (g/day)	Growth value
Week 1	1.50 ± 0.29h	0.14 ± 0.04g	2.00 ± 0.58g
Week 2	1.80 ± 0.06g	0.19 ± 0.01g	2.60 ± 0.12g
Week 3	3.00 ± 0.06f	0.36 ± 0.01f	5.00 ± 0.12f
Week 4	3.80 ± 0.06e	0.47 ± 0.02e	6.60 ± 0.12e
Week 5	4.50 ± 0.29d	2.07 ± 1.46a	8.00 ± 0.58d
Week 6	4.90 ± 0.06c	0.63 ± 0.01d	8.80 ± 0.12c
Week 7	5.80 ± 0.06b	0.76 ± 0.01c	10.60 ± 0.12b
Week 8	7.20 ± 0.06a	0.96 ± 0.01b	13.40 ± 0.16a
LSD 0.05	0.38	0.05	0.75

Mean values accompanied with different superscript letters were significantly different ($p < 0.05$).

culture. However, the growth rate increased continuously recording the optimal value (2.07 ± 1.46 g/day) at the fifth week of culture before decreasing significantly from the sixth to the eighth week. Among all tested periods of culture, a considerable significant difference in all determined parameters for callus cultures production through the eight weeks of subculture was noticed. Subsequently, the fifth week of culture was chosen as the preferable period to make subculture of stem calli due to the successive proliferation of callus recovery comparable to other periods. Contrarily, the mass production of callus cultures was achieved after eight weeks of culture (Fig.4).

Figure 4: The produced calli from celery stem explants on MS-medium + 0.5 mg l^{-1} 2, 4-D after 8 weeks of culture

3.3 EFFECT OF BIOTIC ELICITORS ON GROWTH DYNAMIC PARAMETERS OF *APIUM GRAVEOLENS* STEM CALLI

In this part of work, clusters of *Apium graveolens* stem calli were studied for their propagation capacity by re-culture onto the same callusing medium (0.5 mg l^{-1} 2,4-D) supplemented with some biotic elicitors such as malt extract, yeast extract and peptone extract at concentrations; 1.5, 2.5, 3.5 mg l^{-1} of each. Fresh mass, growth rate and growth value are the determinant parameters which had been calculated for callus growth dynamic parameters of *Apium graveolens* during 60 days of culture as illustrated in Tables 4, 5 and 6.

3.3.1 Fresh mass of the enhanced stem calli of *Apium graveolens* by biotic elicitors.

In Table 4, all biotic elicitors (malt extract, yeast extract, peptone extract) in all their various concentrations (1.5, 2.5, 3.5 g l^{-1}) increased significantly the fresh calli growth from 10 days of culture till recording the highest values at the end of experiment (60 days). Likewise, medium devoid of elicitors (control) behaved the same. Furthermore, it was observed apparent significance differences among all augmentation treatments in all determined time periods. Both of malt (1.5 g l^{-1}) and peptone (1.5 and 2.5 g l^{-1}) extracts augmented significantly callus cultures in all different periods of time. Maximum values with high significant difference were achieved in callus fresh mass (13.1 ± 0.15 g, 12.9 ± 0.12 g) at the treatments of 1.5 g l^{-1} malt extract and 1.5 g l^{-1} peptone extract, respectively after 60 days of culture compared to the non-elicited treatment (9.5 ± 0.13 g). Data resulted from Table 4 reveal that all used elicitors increase the callus cultures proliferation of celery compared to the control; except the treatment of yeast extract at 1.5 g l^{-1} concentration which decreased the callus fresh mass values (6.7 ± 0.11 g, 7.4 ± 0.12 g, 8.5 ± 0.15 g) compared to the control (9.5 ± 0.13 g) at the periods of 40, 50 and 60 days of culture, respectively.

3.3.2 Growth rate of the enhanced stem calli of *Apium graveolens* by biotic elicitors.

Data in Table 5 revealed in the growth rate values of *Apium graveolens* stem calli which had been augmented by malt, yeast and peptone extracts in all their different concentrations. Callus growth rate was directly proportional to boost in the periods of time from 10 days till 60 days of culture for either elicitors-containing media or the control, except the treatment of yeast extract at 3.5 g l^{-1} concentration exhibited high value till 50 days of culture. Similar to the achieved results in Table 4, 1.5

Table 4: Fresh mass of celery stem calli grown on MS-Medium containing malt, yeast and peptone extracts

Incubation period of cultures	Control	Malt extract			Yeast extract			Peptone extract		
		1.5 g l^{-1}	2.5 g l^{-1}	3.5 g l^{-1}	1.5 g l^{-1}	2.5 g l^{-1}	3.5 g l^{-1}	1.5 g l^{-1}	2.5 g l^{-1}	3.5 g l^{-1}
10 days	5.80 ± 0.07^{A-a}	7.30 ± 0.17^{A-b}	7.10 ± 0.12^{A-b}	6.50 ± 0.11^{A-a}	6.60 ± 0.12^{A-a}	6.50 ± 0.15^{A-a}	5.90 ± 0.11^{A-a}	7.30 ± 0.11^{A-b}	7.20 ± 0.15^{A-b}	7.10 ± 0.11^{A-b}
20 days	6.10 ± 0.09^{A-a}	8.50 ± 0.1^{B-d}	8.20 ± 0.17^{B-d}	7.50 ± 0.14^{B-c}	7.40 ± 0.12^{B-c}	6.70 ± 0.11^{A-b}	6.10 ± 0.12^{A-a}	7.40 ± 0.14^{A-c}	8.20 ± 0.10^{B-d}	7.40 ± 0.12^{A-c}
30 days	6.60 ± 0.12^{B-a}	10.40 ± 0.09^{C-d}	9.80 ± 0.12^{C-c}	8.60 ± 0.17^{C-b}	8.50 ± 0.15^{C-b}	8.40 ± 0.17^{B-b}	6.60 ± 0.09^{B-a}	8.10 ± 0.15^{B-b}	9.80 ± 0.11^{C-c}	9.70 ± 0.15^{B-c}
40 days	7.40 ± 0.1^{C-b}	11.00 ± 0.12^{D-g}	10.50 ± 0.09^{D-f}	8.90 ± 0.14^{C-d}	9.10 ± 0.12^{D-d}	9.00 ± 0.15^{C-d}	6.70 ± 0.11^{B-a}	8.20 ± 0.13^{B-c}	10.00 ± 0.1^{C-e}	9.90 ± 0.11^{B-e}
50 days	8.50 ± 0.15^{D-b}	11.60 ± 0.12^{E-e}	11.40 ± 0.12^{E-e}	9.50 ± 0.11^{D-c}	9.60 ± 0.09^{E-c}	9.60 ± 0.11^{D-c}	7.40 ± 0.12^{C-a}	10.90 ± 0.11^{C-d}	10.70 ± 0.12^{D-d}	10.80 ± 0.15^{C-d}
60 days	9.50 ± 0.13^{E-b}	13.10 ± 0.15^{F-e}	11.80 ± 0.09^{F-d}	10.40 ± 0.12^{E-c}	10.40 ± 0.15^{F-c}	10.30 ± 0.12^{E-c}	8.50 ± 0.15^{D-a}	12.90 ± 0.12^{D-e}	12.80 ± 0.15^{E-e}	11.90 ± 0.12^{D-d}

Values are introduced as means ± standard errors of three replicates. Values introduced with different capital letters within a column or with different small letters within a row are significantly different at $p < 0.05$.

Table 5: Growth rate of celery stem calli grown on MS-Medium containing malt, yeast and peptone extracts

Incubation period of cultures	Control	Malt extract			Yeast extract			Peptone extract		
		1.5 g l^{-1}	2.5 g l^{-1}	3.5 g l^{-1}	1.5 g l^{-1}	2.5 g l^{-1}	3.5 g l^{-1}	1.5 g l^{-1}	2.5 g l^{-1}	3.5 g l^{-1}
10 days	0.40 ± 0.01^{A-a}	0.60 ± 0.01^{A-c}	0.60 ± 0.02^{A-c}	0.56 ± 0.01^{A-b}	0.56 ± 0.02^{A-b}	0.55 ± 0.01^{A-b}	0.50 ± 0.01^{A-a}	0.63 ± 0.29^{A-c}	0.64 ± 0.01^{A-c}	0.63 ± 0.01^{A-c}
20 days	0.50 ± 0.01^{B-a}	0.70 ± 0.01^{B-d}	0.70 ± 0.02^{B-d}	0.65 ± 0.01^{B-c}	0.58 ± 0.02^{A-b}	0.57 ± 0.01^{A-b}	0.52 ± 0.01^{A-a}	0.72 ± 0.02^{B-d}	0.65 ± 0.11^{A-c}	0.66 ± 0.01^{A-c}
30 days	0.50 ± 0.01^{C-a}	0.90 ± 0.02^{C-e}	0.80 ± 0.02^{C-d}	0.74 ± 0.02^{C-c}	0.62 ± 0.02^{A-b}	0.73 ± 0.02^{B-c}	0.56 ± 0.01^{B-a}	0.87 ± 0.02^{C-d}	0.88 ± 0.01^{B-d}	0.86 ± 0.01^{B-d}
40 days	0.60 ± 0.01^{D-b}	1.00 ± 0.02^{C-e}	0.90 ± 0.02^{D-d}	0.79 ± 0.03^{C-c}	0.80 ± 0.01^{B-c}	0.80 ± 0.01^{C-c}	0.57 ± 0.01^{B-a}	0.94 ± 0.01^{D-d}	0.92 ± 0.02^{B-d}	0.91 ± 0.02^{B-d}
50 days	0.70 ± 0.01^{E-b}	1.00 ± 0.01^{D-e}	0.90 ± 0.01^{E-d}	0.86 ± 0.0^{D-c}	0.87 ± 0.01^{C-c}	0.86 ± 0.01^{D-c}	0.63 ± 0.02^{C-a}	0.97 ± 0.02^{D-d}	0.99 ± 0.01^{C-d}	0.98 ± 0.01^{C-d}
60 days	0.80 ± 0.01^{F-b}	1.20 ± 0.01^{E-f}	1.00 ± 0.02^{F-d}	0.93 ± 0.02^{E-c}	0.94 ± 0.01^{D-c}	0.93 ± 0.01^{E-c}	0.65 ± 0.03^{C-a}	1.18 ± 0.01^{E-e}	1.17 ± 0.02^{D-e}	1.09 ± 0.02^{D-d}

Values are introduced as means ± standard errors of three replicates. Values introduced with different capital letters within a column or with different small letters within a row are significantly different at $p < 0.05$.

Table 6: Growth value of celery stem calli grown on MS-Medium containing malt, yeast and peptone extracts

Incubation period of cultures	Control	Malt extract			Yeast extract			Peptone extract		
		1.5 g l^{-1}	2.5 g l^{-1}	3.5 g l^{-1}	1.5 g l^{-1}	2.5 g l^{-1}	3.5 g l^{-1}	1.5 g l^{-1}	2.5 g l^{-1}	3.5 g l^{-1}
10 days	4.90 ± 0.07^{A-a}	6.30 ± 0.07^{A-c}	6.20 ± 0.12^{A-c}	5.60 ± 0.09^{A-b}	5.50 ± 0.17^{A-b}	5.60 ± 0.15^{A-b}	5.00 ± 0.09^{A-a}	6.40 ± 0.17^{A-c}	6.30 ± 0.15^{A-c}	6.30 ± 0.09 $^{A-c}$
20 days	5.10 ± 0.06^{A-a}	7.50 ± 0.06^{B-c}	7.30 ± 0.09^{B-c}	6.50 ± 0.09^{B-b}	6.30 ± 0.22^{B-b}	6.40 ± 0.12^{B-b}	5.20 ± 0.15^{A-a}	7.40 ± 0.2^{B-c}	7.30 ± 0.18^{B-c}	6.40 ± 0.1^{A-b}
30 days	5.50 ± 0.07^{B-a}	9.40 ± 0.10^{C-e}	8.80 ± 0.13^{C-d}	7.50 ± 0.15^{C-c}	7.40 ± 0.23^{C-c}	7.40 ± 0.17^{C-c}	5.30 ± 0.17^{A-a}	8.70 ± 0.12^{C-d}	8.80 ± 0.12^{C-d}	6.70 ± 0.12^{B-b}
40 days	6.50 ± 0.07^{C-b}	10.10 ± 0.12^{D-e}	9.40 ± 0.15^{D-d}	8.20 ± 0.12^{D-c}	8.30 ± 0.18^{D-c}	8.20 ± 0.12^{D-c}	6.00 ± 0.17^{B-a}	9.30 ± 0.10^{D-d}	9.50 ± 0.12^{D-d}	9.40 ± 0.17^{C-d}
50 days	7.50 ± 0.06^{D-b}	10.60 ± 0.1^{E-e}	9.90 ± 0.0^{E-d}	8.40 ± 0.10^{D-c}	8.50 ± 0.15^{D-c}	8.40 ± 0.10^{D-c}	6.10 ± 0.1^{B-a}	9.90 ± 0.10^{E-d}	9.70 ± 0.20^{D-d}	9.70 ± 0.19^{C-d}
60 days	8.60 ± 0.09^{E-b}	12.10 ± 0.12^{F-e}	10.80 ± 0.12^{F-d}	9.20 ± 0.09^{E-c}	9.30 ± 0.17^{E-c}	9.40 ± 0.12^{E-c}	7.40 ± 0.23^{C-a}	11.00 ± 0.12^{F-d}	10.90 ± 0.23^{E-d}	10.90 ± 0.10^{D-d}

Values are introduced as means ± standard errors of three replicates. Values introduced with different capital letters within a column or with different small letters within a row are significantly different at $p < 0.05$.

g l^{-1} of both of malt and peptone extracts gave higher values of callus growth rate (1.2 ± 0.01 and 1.18 ± 0.01, respectively) than 1.5 g l^{-1} of yeast extract (0.94 ± 0.01), likewise 3.5 g l^{-1} yeast extract decreased significantly the callus growth rate (0.65 ± 0.03) than elicitors free medium (0.8 ± 0.01).

3.3.3 Growth value of the enhanced stem calli of *Apium graveolens* by biotic elicitors.

Data tabulated in Table 6 revealed significantly an ascending growth value for *Apium graveolens* stem calli grown on elicitors containing media, along with the medium devoid of elicitors during 60 days of culture. There were non-significant differences between 40 and 50 days of culture in all concentrations of yeast extract, 3.5 g l^{-1} malt extract, 2.5 and 3.5 g l^{-1} peptone extract. Likewise, there were non-significant differences between 10 and 20 days of culture in treatments of yeast and peptone extracts at 3.5 g l^{-1} concentration of each, beside elicitors free medium. On the other hand, the highest growth value of celery callus cultures was verified significantly using 1.5 g l^{-1} malt extract (12.1 ± 0.12), followed by using 1.5 g l^{-1} peptone extract (11 ± 0.12) after 60 days of culture. Surprisingly, among all these augmentation treatments to increase callus growth value, it was observed that the highest concentration of yeast extract (3.5 g l^{-1}) reduced callus growth value significantly at 40 (6.0 ± 0.17), 50 (6.1 ± 0.12), 60 (7.4 ± 0.23) days of culture compared to the control (6.5 ± 0.07, 7.5 ± 0.06, 8.6 ± 0.09, respectively).

3.4 SCREENING OF SOMATIC EMBRYOS DEVELOPMENT AND DIFFERENTIATION IN *APIUM GRAVEOLENS* STEM CALLI BY HISTOLOGICAL INVESTIGATION

In this part of the study, the augmented biomass of celery stem callus cultures which had been delivered from the previous work were examined for their ability for differentiation and embryogenesis to form somatic embryos. Clusters of stem calli which were grown for 4 weeks on MS-Medium elicited by malt, yeast and peptone extracts (1.5, 2.5, 3.5 g l^{-1} of each) were screened for their possibility for embryogenesis and differentiation by histological assay. Data resulted from the histological examination for all elicited treatments revealed these findings: calli on elicitors free MS-medium (0.5 mg l^{-1} of 2, 4-D, control) led to globular embryo formation (Fig.5). Meanwhile, MS-medium supplemented with 3.5 g l^{-1} of malt extract as biotic elicitor was the optimal structure to form a torpedo shaped embryo (Fig. 6) as an advanced stage of indirect somatic embryogenesis. So, we recommend malt extract at 3.5 g l^{-1} concentration as the best biotic elicitor for embryogenesis development. However, peptone extract containing medium at 1.5 g l^{-1} concentration led to formation of an early heart (Fig. 7, a) and late heart shaped embryo (Fig. 7, b). So, 1.5 g l^{-1} of peptone could be considered as good biotic elicitor for synchronization in the indirect somatic embryogenesis. On the other hand, MS-medium fortified

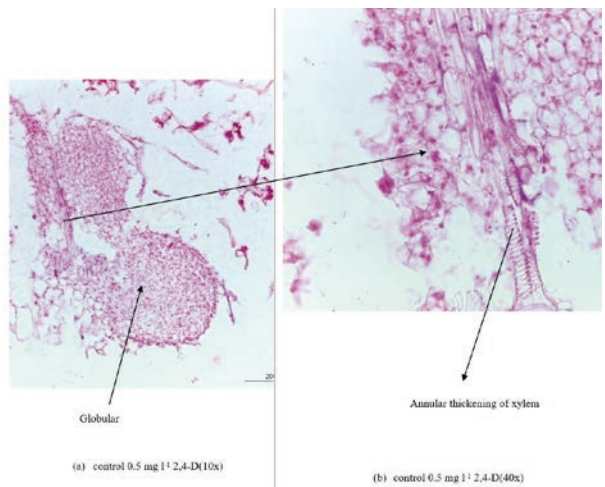

Figure 5: Celery late globular embryo derived from stem calli under the effect of plant growth regulator (0.5 2,4-D) as a control without elicitor (a). Magnified portion showing the formation of xylem vessels with annular thickening (b) after 4 weeks of celery stem calli culture.

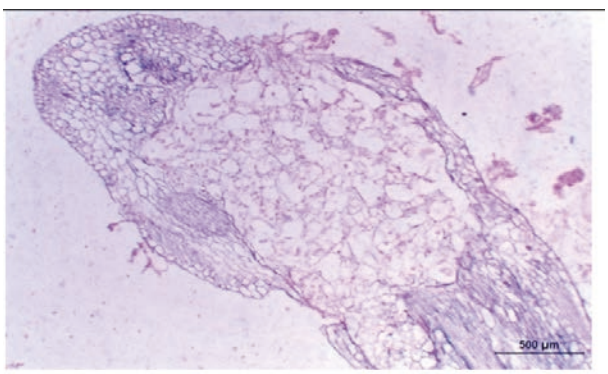

Figure 6: Torpedo shaped embryo formed as an advanced stage of indirect somatic embryogenesis using malt as an elicitor after 4 weeks of celery stem calli culture

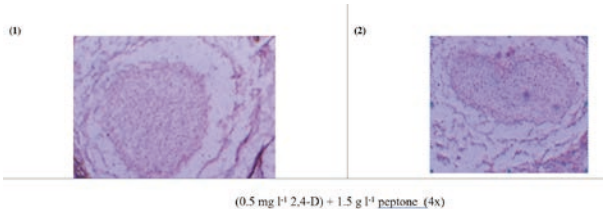

Figure 7: Early heart shaped embryo (1) and late heart shaped embryo (2) showing a synchronization in indirect somatic embryogenesis using peptone as an elicitor after 4 weeks of celery stem calli culture.

with 2.5 g l^{-1} of yeast extract helped in heart shape formation (Fig. 8), therefore yeast extract (2.5 g l^{-1}) acted as valuable biotic elicitor for embryogenesis progression. All other treatments of biotic elicitors exhibited normal parenchyma cells without any differentiation structure.

4 DISCUSSION

The obtained results in Tables 2 and 3 considered 2,4-D addition individually in callus culture medium as the optimal auxin in celery calli propagation. This finding is in accordance with Soorni et al. (2012) who confirmed by statistical analysis the substantial role of 2,4-D at 1 mg l^{-1} concentration on callus induction efficiency of *Cuminum cyminum* L. leaf explants. Also, Babiker et al. (2021) who gained callus cultures of *Chrysanthemum* from leaf or internode explants using 2,4-D at 0.5 mg l^{-1} or 2 mg l^{-1} concentrations. As well Mahood et al. (2022) who obtained the highest percentage of callus induction (90 %) using stem explants following by leaf explants (80 %) using 1 mg l^{-1} 2,4-D in *Gazania rigens* (L.) Gaertn. callus culture medium after four weeks of cultivation. From the previous findings, it could be recommended to use 2,4-D as stimulator for callus growth, 2,4-D had distinctive characteristics; it was hardly decomposed by heating during the sterilization process or by the enzymes resulted from the used explants (George et al., 2008, Al-Khayri, 2011).

On basis the obtained results in Tables 4, 5, and 6, a positive relationship between the propagation increment of celery callus cultures and the time periods increment for these cell cultures incubation could be observed throughout the experiment. Elicitor containing media with their different concentrations raised significantly the calculated growth measurements (fresh mass, growth rate and growth value) for celery callus cultures more than medium devoid of elicitors (control). In this regard, supply of biotic elicitors (malt, yeast, and peptone) in celery calli medium increased the callus proliferation dependent on lower concentrations of those elicitors. This suggestion matches the findings in Tables 4, 5 and 6 which indicated the highest elicitors' effectiveness on mass production of celery callus cultures which had been investigated for fresh mass and growth rate at the lowest concentration of each elicitor. Among the achieved results previously, malt extract followed by peptone extract at 1.5 g l^{-1} concentration of each contributed to attaining the biggest amount of cellular biomass of celery compared to the others. This was attributing to consider that malt and peptone extracts act as efficient elicitors for callus cultures propagation of *Apium graveolens* when used in low concentrations. The significant importance of biotic elicitors (malt, yeast and peptone) in cell cultures propagation could be due to considering malt extract as enriched carbohydrate source; yeast extract as good source of minerals, vitamins and amino acids, and peptone extract as high organic nitrogen source (Badr-Elden, 2017, Vasil & Hildebrandt, 1966, Parc et al., 2007). Based on the obtained results, we recommend adding biotic elicitors in celery callus culture medium, particular malt and peptone extracts which were more preferable than yeast extract for celery biomass production. This recommendation is close to what have been achieved by Sawy et al. (2005), Hussain et al. (2016) and Badr-Elden, (2017) who attained the highest callogenesis in ovules of *Citrus* and kinnow mandarin using high concentration (500 mg l^{-1}) of malt extract in callus induction medium. Likewise, both Parc et al. (2007) and Nhut et al. (2008) referred to the considerable effect of peptone extract in cell proliferation medium as the most sufficient elicitor for strong biomass increment tobacco and avocado, respectively. Otherwise, Eshaghi et al. (2021) recorded lower callus mass (0.152 g) in wheat using yeast extract than when using casein hydrolysate (0.230 g). Besides, Sidhar & Aswath (2014) mentioned the positive effect of moderate yeast extract concentrations on shoot multiplication rate and plant regeneration of *Stevia rebaudiana* (Bertoni) Bartoni. However, Kikowska et al. (2015) added yeast extract at 200 mg l^{-1} to *Eryngium planum* L. shoot cultures medium for biomass accumulation enhancement, and Nadeem et al. (2018) increased by two-fold the fresh and dry weights of *Linum usitatissimum* L.. Cultures over the control using MS-medium supplemented with 200 mg l^{-1} of yeast extract.

In line with the achieved findings as shown in Figures 5, 6, 7 and 8, we selected specific concentration of each biotic elicitor for callus culture medium of *Apium graveolens* for successful somatic embryogenesis and differentiation. Our results were in harmony with the others who supported this idea; plant growth regulators and elicitors played a substantial role in embryogenesis and the development stages of somatic embryos dependent

on concentration and type. Herein some of these reports; Hwankim & Janick (1989) who observed the critical role of 2,4-D in embryogenesis of *Apium graveolens* according to its concentration, where low concentration had poor effect on embryos formation while high concentration led to embryos suppression. Besides, Mazri et al. (2013) used PGRs for olive somatic embryogenesis, Chen & Chang (2002) obtained the best response for direct embryos formation in Oncidium 'Gower Ramsey using 0.5-1 g l^{-1} of peptone and Al-Khayri (2011) gained embryos higher than the control using yeast extract at 1 g l^{-1} concentration. As well Mazri & Belkoura (2021) affirmed the effectiveness of malt extract concentration (500 mg l^{-1}) on somatic embryos formation and differentiation of citrus calli. Likewise, the development of somatic embryogenesis for plant regeneration in various citrus species was achieved by Amin & Shekafandeh (2015), Sawy et al. (2005) and Gholami et al. (2013).

To sum up, authors indicated the significant importance of 3.5 g l^{-1} malt extract as the best biotic elicitor for embryos development, followed by 1.5 g l^{-1} peptone extract to form late stage of heart shaped embryo, then 2.5 g l^{-1} yeast extract for somatic embryogenesis, to be added in MS-medium + 0.5 mg l^{-1} 2, 4-D to efficiently induce somatic embryos with their development for differentiation. This recommendation is due to the importance of amino acids content in yeast extract, carbohydrates content in malt extract and organic nitrogen content in peptone extract which stimulated the embryogenesis and plant growth. These investigations were in agreement with Badr-Elden (2017) and Carimi et al. (1998) who reported the promotive impact of malt extract as carbohydrate source for citrus somatic embryogenesis and stimulation of the early cotyledonary stage of embryos germination in the in vitro rescue of *Citrus* x *aurantium* L.. Additionally, proembryo morphology of calli and proliferation depended on malt extract concentration (Mazri & Belkoura, 2021). Furthermore, the biosynthesis of endogenous amino acid at early stage of protocorm development could not be adequate for faster and healthy growth of orchid protocorm, therefore addition of amino acids from peptone could enhance the growth of orchid protocorm, and the growth of embryo which was related to easily absorption of water (Setiari et al., 2016). Typically, adding more yeast extract to the MS medium at higher concentrations limited growth, but adding less yeast extract had shown to have positive effects (Safwat et al., 2014). Ultimately, in this work we affirmed the extreme necessity to add PGRs (0.5 mg l^{-1} 2.4-D) with biotic elicitors (malt (3.5 g l^{-1}), peptone (1.5 g l^{-1}) and yeast (2.5 g l^{-1}) extracts) in callus culture medium of *Apium graveolens* L. for investigation of somatic embryogenesis development. Therefore, addition of exogenous PGRs and elicitors interacted with the plant hormones and modified their levels, which resulted in cell division, differentiation, growth and morphogenesis.

5 CONCLUSION

An efficient and reliable protocol of somatic embryogenesis was performed as permanent natural application for obtaining progeny homogeneous plants. We employed several biotic elicitors (malt, peptone and yeast extracts) with different concentrations for callus induction, somatic embryogenesis development and embryo differentiation. In the present research, a comprehensible correlation between the elicitors and somatic embryos formation was shown that reflects their importance in the somatic embryogenesis development which was dependent on the elicitor type and concentration. So far to the authors' knowledge, the effectiveness of biotic elicitors on somatic embryos enhancement of celery has not been reported before. Indeed, our research achievements provided new insightsthrough the elicitors for boosting the embryogenesis development and differentiation. All biotic elicitors possessed an impressive performance, in particular malt extract at 3.5 g l^{-1} concentration. Malt extract was a perfect starting point for the study of elicitors-based embryogenesis for differentiation and indirect plant regeneration promotion to be a boon in regeneration mass production of the identical and homogeneous plants of *Apium graveolens.*

6 STATEMENTS & DECLARATIONS

Availability of original data: The authors declare that all original data are included in the manuscript as well they read and approved the final draft of the manuscript

The ethical standards: The authors performed this article without any human studies to compliance the ethics procedures

Conflict of interest: The authors declare that they have no conflicts of interest

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Authors' contributions: All authors designed and performed the experiments, analyzed and wrote the original draft, and edited the manuscript. All authors agree for submission of manuscript to the journal

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