



## **The Multi-drops and Cross-sections, Efficient Methods for Establishing Cell Suspension Culture of *Cuminum cyminum* L. and Plant Regeneration**

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**Abstract:** The current study aims of the current study is to establish cell suspension cultures of the medicinal plant, cumin *Cuminum cyminum* L. by the application of multiple drops and cross-section techniques. These methods were used in cultivating cell suspensions and *in vitro* plant regeneration. Leaf, stem, hypocotyl and root explants were cultured in Murashige and Skoog (MS) medium containing different concentrations (0.05, 0.5 and 1.0 mg.l<sup>-1</sup>) of naphthalene acetic acid (NAA) and (0.05, 0.1, 1.0 and 2.0 mg.l<sup>-1</sup>) benzyl adenine BA for callus production. The results indicated the high response of cumin, as the percentage of callus initiation was 100%. The plant regeneration percentage reached 91.6%. Moreover, a friable callus of hypocotyls was appropriate for initiation of cell suspension culture in MS medium with 0.5mg.l<sup>-1</sup> NAA and 1.0 mg.l<sup>-1</sup> benzyl adenine (BA). The best density for primordial callus formation was 51.0 ×10<sup>5</sup> cells ml<sup>-1</sup> in both multiple-drops and cross-sections embedding methods. Callus that had been produced from multi drops and sectors had the ability for shoot regeneration. This study clarified the efficiency of these techniques in establishing cell suspension culture and shoot regeneration, which could be promising sources for high production of active compounds in cumin plant.

**Keywords:** Callus, Cumin, Hypocotyls, Medicinal plant, Tissue culture.

### **Introduction**

Cumin (*Cuminum cyminum* L.) is a member of the Apiaceae family and is one of aromatic medicinal herbs (Purohit, 2018). Egypt is the natural home of this plant, which is also grown in India, Iran, China and Pakistan (Lodha & Mawar, 2014). Cumin seeds are used as spices and in other cuisines due to their potent scent (Korinek *et al.*, 2021). The plant's physiological and nutritional qualities are widely established in the scientific literature (Alasadi *et al.*, 2020; Miah *et al.*, 2021). Despite the seeds of cumin is

commonly used as a spice for their characteristic scent (Srinivasan, 2018), they are also used in traditional medicine to treat different diseases. Chemical studies had disclosed that cumin contained a variety of compounds like alkaloids, flavonoids and glycosides (Singh *et al.*, 2017). Moreover, minerals like iron, calcium, copper, manganese, potassium, selenium, and zinc are abundant in this spice. Cumin seeds are also rich in flavonoid and phenolic antioxidants

such as carotenoids, zeaxanthin, and lutein (Allaq *et al.*, 2020).

Plant tissue culture is considered an important technique in both basic science and practical application (Efferth, 2019), this technique was used in callus induction of *Dianthus caryophyllus* (Mahood, 2021) and conservation of *Fragaria ananassa* (Kadhim & Abdulhussein, 2021). Furthermore, it is a good used technique in physiological and genetic studies (Annon & Abdulrasool, 2020). All main families of terrestrial plants heal injured tissue using callus cells that have not undergone differentiation. *In vitro* culture of these callus cells is possible for biotechnological uses (Ferid *et al.*, 2020). Additionally, when friable callus cells have been added to a suitable sterile liquid media and agitated, they have been interspersed through the liquid medium to produce the suspension culture (Neumann *et al.*, 2020). Callus and cell suspension cultures are well-known techniques for producing a range of phytochemicals from different plant tissues such as organosulfur bioactive compounds from garlic (Setiowati *et al.*, 2022).

The stimulation of callus might be extremely important when employed to manufacture and accumulate of medications, such as medicinal and antioxidant substances (Singh *et al.*, 2020). Typically, callus cultures require tiny places and a short time to multiply; they are inexpensive, do not need particular circumstances equipment, and they might be used in various scales (Mamdouh & Smetanska, 2022).

A few studies have documented plant regeneration from callus of *C. cyminum* (Valizadeh *et al.*, 2007; Deepak *et al.*, 2014) and the initiation of cell suspension culture (Ramchandra *et al.*, 2020; Woo *et al.*, 2021). Therefore, the present study aimed to find an

efficient method for plant regeneration from callus by using different explants and establish cell suspension cultures of cumin by applying multi-drops and cross-section techniques.

## Materials & Methods

### Production of axenic seedlings

*C. cyminum* L. seeds were washed with tap water for three minutes, followed by surface sterilization with 70% ethanolic alcohol for one minute, and subsequent 15 min immersion in a commercial bleach solution (2.5%) sodium hypochlorite. Finally, they were washed 3-5 times with sterile distilled water. Sterilized seeds were cultured on solid MS medium (Murashige & Skoog, 1962) free from growth regulators, placed in a culture room at 25°C in the dark for three days and then translocated to fluorescent light (16hrs day<sup>-1</sup>).

### Callus initiation

Approximately 1.0 cm of different explants (Leaf, stem, hypocotyl and root segments) from 30 days old seedlings, were used for callus initiation. They were cultured in MS medium containing combinations of different concentrations (0.05, 0.5 and 1.0 mg.l<sup>-1</sup>) NAA and (0.05, 0.1, 1.0 and 2.0 mg.l<sup>-1</sup>) BA. Moreover, 2, 4-D and Kin with the same previous concentrations were used. All the samples were preserved in a culture room at 25 °C in 16 hours in light followed by 8 hours in the dark in a consequent manner.

### Rooting of regenerated shoots

All shoots that regenerated during callus induction were transferred to agar- solidified MS medium free from growth regulators for rooting.

### Establishment of cell suspension cultures

One g of two months friable hypocotyls callus was placed in 25 ml of liquid MS medium with 0.5 mg.l<sup>-1</sup> NAA and 1.0 mg.l<sup>-1</sup> BA in 50 mL conical flasks; samples were kept in the shaking incubator in the dark at 28°C and velocity of 250 rpm. Then culture media were filtered using a sterile, fine plastic sieve with 46 µm pores to remove the cell aggregates and harvest single cells; after that, the required amount of the same medium was added to fulfill the original size. Then, they were returned to the shaker incubator. The viability of the cells was determined using Evan's blue stain (Birkenhead & Willmer, 1986).

The percent of viable cells was calculated according to the following equation:

$$\text{Viability(\%)} = \frac{\text{Total number of viable cells}}{\text{Total number of cells}}$$

For determining the increase in ratio of cells, 1.5 µL was taken from the cell culture on the first, second, third fourth, fifth and sixth day, and placed on a hemocytometer; the numbers of cells were recorded and their densities were calculated according to Wai-Leng & Lai-Keng (2004).

### Culturing of cell suspensions and callus initiation

The cell suspensions of cumin's hypocotyls callus at different densities were cultured by embedding them in agar using the multiple-drops and cross-section method of Khanpour-Ardestani (2015) with modifications. One ml of cell suspension was mixed rapidly with 1.0 ml of 3% pre-autoclaved liquid agar at 40 °C to prevent hardening. The obtained mixture was placed as approximately identical drops on the bottom of 9.0 cm diameter plastic Petri dishes (5-6 drops dish<sup>-1</sup>), or as sectors. Numbers of cultured drops are 20 for each density. After solidification, 5.0 ml of new

liquid MS medium which was applied in the initiation of cell suspension was added. The samples were put in a culture room under the similar conditions of callus induction and examined microscopically every day to detect their divisions. The maintenance of the cultures was every four days that achieved by getting rid of the liquid medium and adding the same volume of a fresh medium. After the appearance of primordial callus from agar droplets, or sectors, they were transferred via sterile spatula to MS medium supplied with 0.5mg.l<sup>-1</sup> NAA and 1.0 mg.l<sup>-1</sup> BA in 100 ml glass container, at a rate of 2 drops flask<sup>-1</sup>.

## Results & Discussion

### Callus induction and shoots regeneration

Sterilized seedlings of *C. cyminum* were achieved after 28 days of germination on MS medium (Fig. 1, a). The results pointed out that MS medium containing 0.5, 1.0 mg.l<sup>-1</sup> NAA and 1.0, 2.0 mg.l<sup>-1</sup> BA respectively was the best for callus initiation from all explants (Fig. 1b-e).

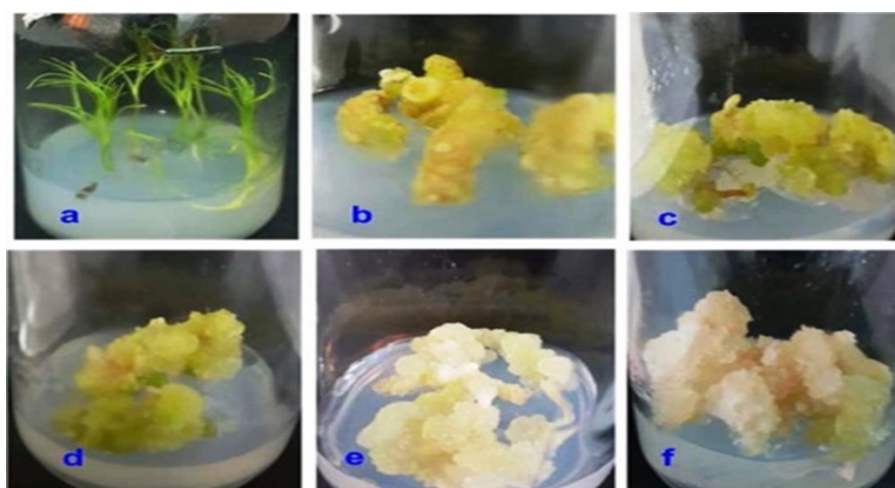
In contrast, combinations of 2, 4 -D and kin did not promote callus induction (Table 1). Kazemi *et al.* (2016) mentioned that MS medium enriched with 1.0 mg.l<sup>-1</sup> NAA and 0.5 mg.l<sup>-1</sup> BAP was the best for callus induction from leaf and hypocotyl explants of cumin. Numerous aspects of development and differentiation are controlled by auxin and cytokinin interactions in a complex way. Now it is better to understand how these hormones work to regulate different physiological and developmental responses according to recent developments in research of metabolism and cell cycle. In addition, the interactions of these hormones at several levels, such as post-translational modification, hormone availability and activity, their involvement in signaling networks and the control of gene expression are revealed by molecular and

genetic investigations (Ikeuchi *et al.*, 2013). The initiated callus was maintained in  $1.0 \text{ mg.l}^{-1}$  NAA with  $2.0 \text{ mg.l}^{-1}$  BA (Fig. 1f). One of the most interesting results of this study was the spontaneous regeneration of shoots from the callus of leaves and stems (Fig. 2a, b) with percentages of 91.6 % and 66.6% respectively in the combination with  $1.0 \text{ mg.l}^{-1}$

$1 \text{ NAA}$  with  $2.0 \text{ mg.l}^{-1}$  BA (Table 2). While the study of Valizadeh *et al.* (2007) clarified that regeneration of cumin occurred in B5 medium fortified with  $0.1 \text{ mg.l}^{-1}$  NAA and  $4 \text{ mg.l}^{-1}$  Kin. Additionally, it was reported, that plant growth regulators affect callus formation and differentiation (Dar *et al.*, 2021; Guo & Ryong, 2021).

**Table (1): Callus initiation from different explants of *C. cyminum* in MS medium supplemented with various concentrations of growth regulators.**

| Growth regulators<br>( $\text{mg.L}^{-1}$ ) | Callus initiation frequency* (%) |                       |                        |                       | Period for callus initiation (days) |       |            |       |
|---------------------------------------------|----------------------------------|-----------------------|------------------------|-----------------------|-------------------------------------|-------|------------|-------|
|                                             | Leaves                           | Stems                 | Hypocotyls             | Roots                 | Leaves                              | Stems | Hypocotyls | Roots |
| <b>NAA + BA</b>                             |                                  |                       |                        |                       |                                     |       |            |       |
| 0.05 + 0.1                                  | 18.7<br>$\pm 0.1606$             | 25.0<br>$\pm 0.1735$  | 43.7<br>$\pm 0.0606$   | 12.5<br>$\pm 1.1649$  | 21                                  | 20    | 20         | 25    |
| 0.5 + 1.0                                   | 100.0<br>$\pm 0.1036$            | 100.0<br>$\pm 0.1426$ | 100.0<br>$\pm 1.06491$ | 100.0<br>$\pm 0.0688$ | 17                                  | 17    | 17         | 19    |
| 1.0 + 0.05                                  | 75.0<br>$\pm 0.0863$             | 68.7<br>$\pm 0.0365$  | 81.2<br>$\pm 1.0706$   | 68.7<br>$\pm 0.1746$  | 19                                  | 19    | 17         | 21    |
| 1.0 + 2.0                                   | 100.0<br>$\pm 0.0179$            | 100.0<br>$\pm 0.1425$ | 100.0<br>$\pm 0.0975$  | 100.0<br>$\pm 0.0316$ | 14                                  | 14    | 14         | 17    |
| 0.0 + 0.0                                   | 0.0                              | 0.0                   | 0.0                    | 0.0                   | 0                                   | 0     | 0          | 0     |
| <b>2,4-D + Kin</b>                          |                                  |                       |                        |                       |                                     |       |            |       |
| 0.05 + 0.1                                  | 0.0                              | 0.0                   | $12.5 \pm 0.1379$      | 0.0                   | 0                                   | 0     | 28         | 0     |
| 0.5 + 1.0                                   | 12.5<br>$\pm 0.1379$             | 0.0                   | $25.0 \pm 0.1735$      | 0.0                   | 21                                  | 0     | 23         | 0     |
| 1.0 + 0.05                                  | 37.5<br>$\pm 0.0279$             | $25.0 \pm 0.1735$     | $25.0 \pm 0.1036$      | 0.0                   | 21                                  | 23    | 21         | 0     |
| 1.0 + 2.0                                   | 50.0<br>$\pm 0.0479$             | 37.5<br>$\pm 0.0279$  | $50.0 \pm 1.0648$      | 25.0                  | 19                                  | 19    | 19         | 23    |
| 0.0 + 0.0                                   | 0.0                              | 0.0                   | 0.0                    | 0.0                   | 0                                   | 0     | 0          | 0     |



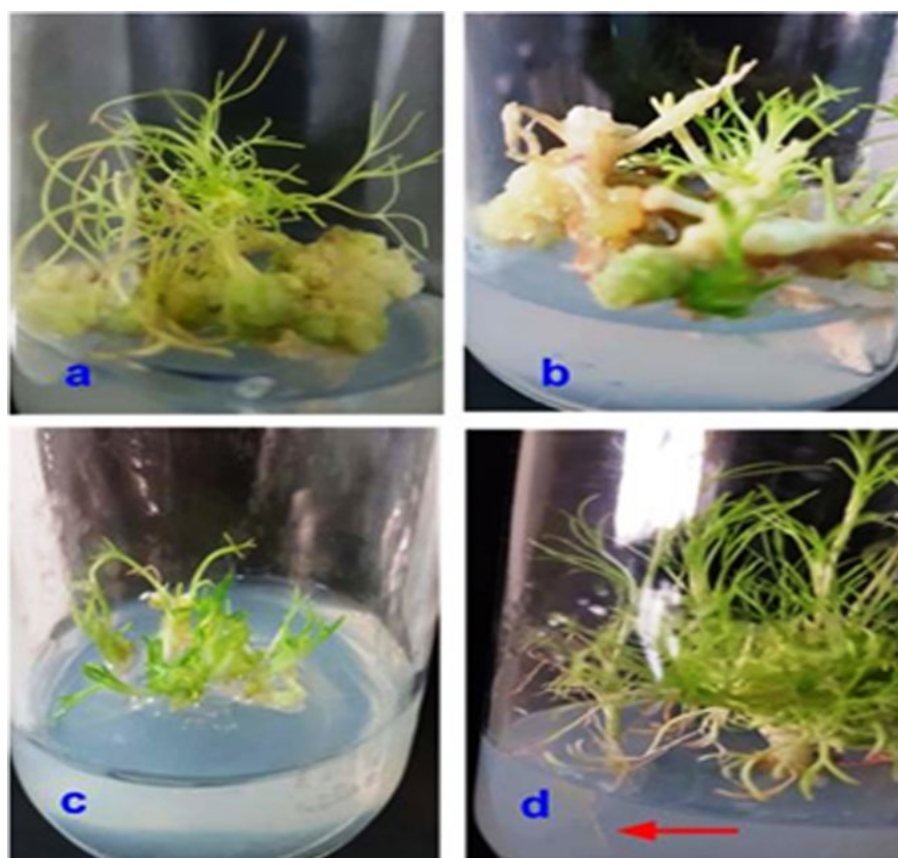
**Fig. (1): Callus induction from different explants of *C. cyminum* L. in MS medium containing  $0.5 \text{ mg.L}^{-1}$  NAA and  $1.0 \text{ mg.L}^{-1}$  BA. (a) 30 days old sterilized seedlings (b) hypocotyls after 20 days (c) stem explants after 20 days, (d) leaves derived callus (e) roots explant after 30 days (f) callus of hypocotyls after 40 days in maintenance medium.**

Furthermore, when these shoots were transferred to MS medium free from growth regulators (Fig. 2c), they were rooted after 40 days (Fig. 2d).

#### Formation of cell suspension cultures

According to our results, the friable callus of hypocotyls was appropriate for the initiation of cell suspension and production of numerous single cells that were enough to create homogenized fine cell suspensions in MS medium with 0.5 mg.l<sup>-1</sup> NAA and 1.0 mg.l<sup>-1</sup> BA. Conversely, It was reported that liquid B<sub>5</sub> medium was suitable for initiating embryonic cell suspension cultures from hypocotyl segments-derived embryonic calli of cumin (Woo *et al.*, 2021). Besides, it was

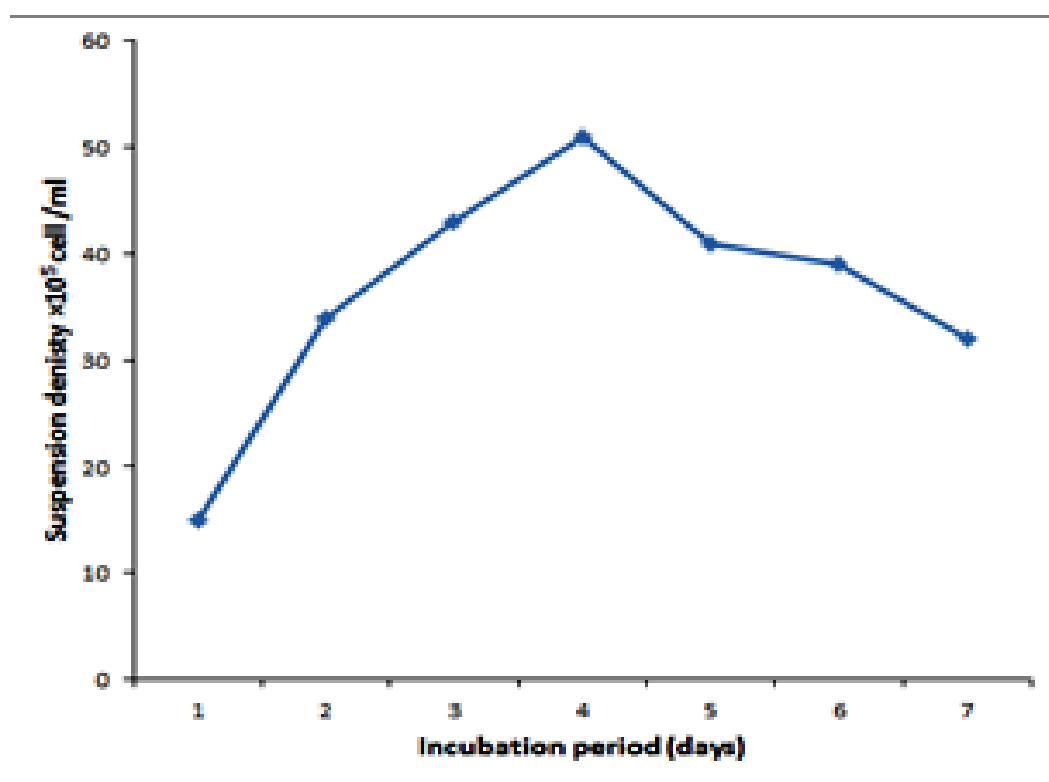
indicated that plant growth regulators play a major role in establishing cell suspension culture (Haida *et al.*, 2019). Data indicated that cell densities increased with time until the fourth day of culture age as the density was 51.0×10<sup>5</sup> cells. ml<sup>-1</sup> (Fig. 3). Furthermore, the viability of these cells was 90%. It seems that cell suspension cultures of cumin displayed a sigmoid growth pattern that began with the lag phase, progressed through the exponential phase, and finally reached the stationary phase. According to Ramchandra *et al.* (2020) cell growth rates of cumin started out slowly, but as the culture went on, they rapidly speeded up.



**Fig. (2):** Plant regeneration from leaf and stem's callus explants of *C. cyminum* L. in MS medium containing 1.0 mg.L<sup>-1</sup> NAA and 2.0 mg L<sup>-1</sup> BA. (a) Shoots regeneration from leaf callus after 40 days (b) shoot regeneration from stem explant after 50 days (c) regenerated shoots after seven days in MS free medium (d) rooting of shoots (arrow).

**Table (2): Shoot regeneration from leaves and stems callus of *Cuminum cyminum* .**

| NAA + BA<br>(mg.l <sup>-1</sup> ) | Shoot regeneration frequency * (%) |                 |
|-----------------------------------|------------------------------------|-----------------|
|                                   | Callus of Leaves                   | Callus of Stems |
| 0.05 + 0.1                        | 0.0±0.0000                         | 0.0±0.0000      |
| 0.5 + 1.0                         | 83.3±0.0739                        | 41.6±0.1689     |
| 1.0 + 0.05                        | 0.0±0.0000                         | 0.0±0.0000      |
| 1.0 + 2.0                         | 91.6±0.9731                        | 66.6±0.6791     |

**Fig. (3): Initiation of cell suspensions of *C. cyminum* L. in MS medium containing 0.5mg.L<sup>-1</sup> NAA and 1.0 mg. l<sup>-1</sup> BA.****Cultivation of cumin's suspension cell**

The results of embedding cell suspension using both multiple-drops and cross-section techniques clarified the efficiency of these methods. On the other hand, follow-up different densities ( $15 \times 10^5$ ,  $39 \times 10^5$ ,  $41 \times 10^5$  and  $51.0 \times 10^5$  cells. ml<sup>-1</sup>) in multiple drops

showed that,  $51.0 \times 10^5$  cells. ml<sup>-1</sup>, was the best in the abundance of its single cells. As the cells began their first division after 28 h and then continued their successive divisions to produce cell colonies (Fig. 4a), consisting of large numbers of cells, that vary in their numbers according to the density (Table 3).

**Table (3): Formation of primordial callus via embedding cell suspensions of *C. cyminum* L. in multiple drops.**

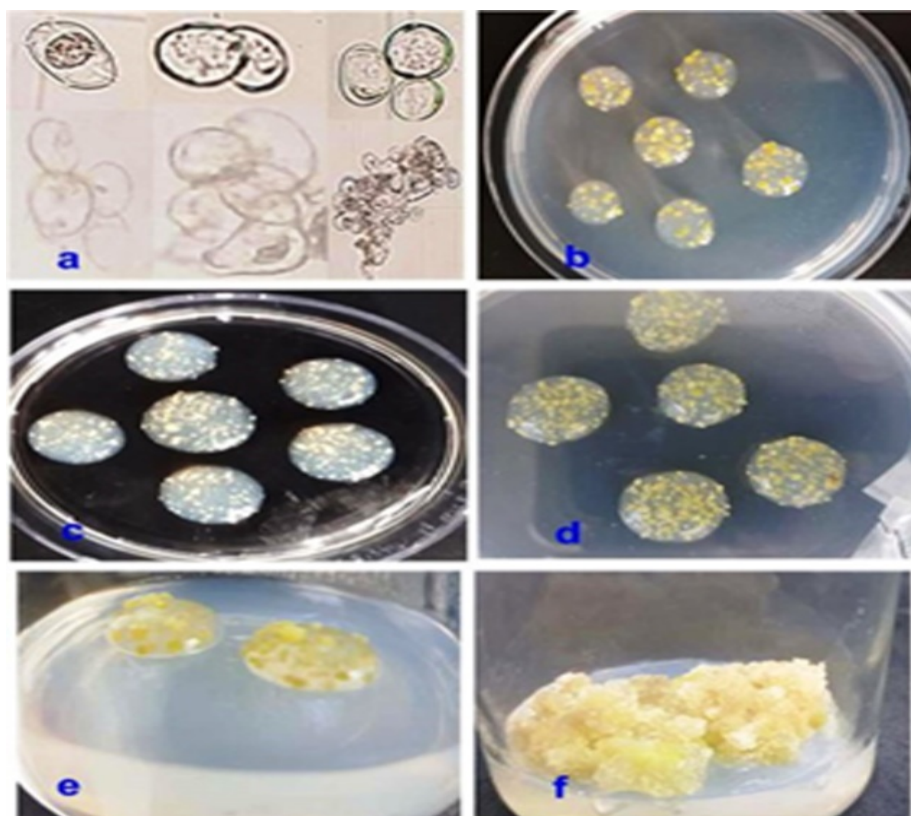
| Cell Densities<br>( $\times 10^5$ cells ml <sup>-1</sup> ) | Total Number          |                        |                            |
|------------------------------------------------------------|-----------------------|------------------------|----------------------------|
|                                                            | Callus-inducing Drops | Cell colonies $\pm$ SD | Primordial Callus $\pm$ SD |
| 15                                                         | 0                     | 0 $\pm$ 0.00           | 0 $\pm$ 0.00               |
| 39                                                         | 16                    | 240 $\pm$ 1.333        | 229 $\pm$ 0.0588           |
| 41                                                         | 16                    | 395 $\pm$ 0.1052       | 389 $\pm$ 0.0526           |
| 51                                                         | 18                    | 655 $\pm$ 0.2105       | 627 $\pm$ 0.1052           |

Furthermore, these colonies developed to numerous white tiny calli primordial within two weeks (Fig. 4b-d). This was related to the viability and biological activities of the cell suspension culture (Tan *et al.*, 2010). In addition, when whole drops were transferred to maintenance medium in glass jars (Fig. 4e), primordial callus increased in size and cracked the agar to produce friable callus after 4-6 weeks (Fig. 4f). Similarly, cell suspension embedded in cross-sections also behaved in the same manner to produce cell colonies, primordial callus and friable calli (Fig. 5a-d; see Fehér, 2019). All cultures

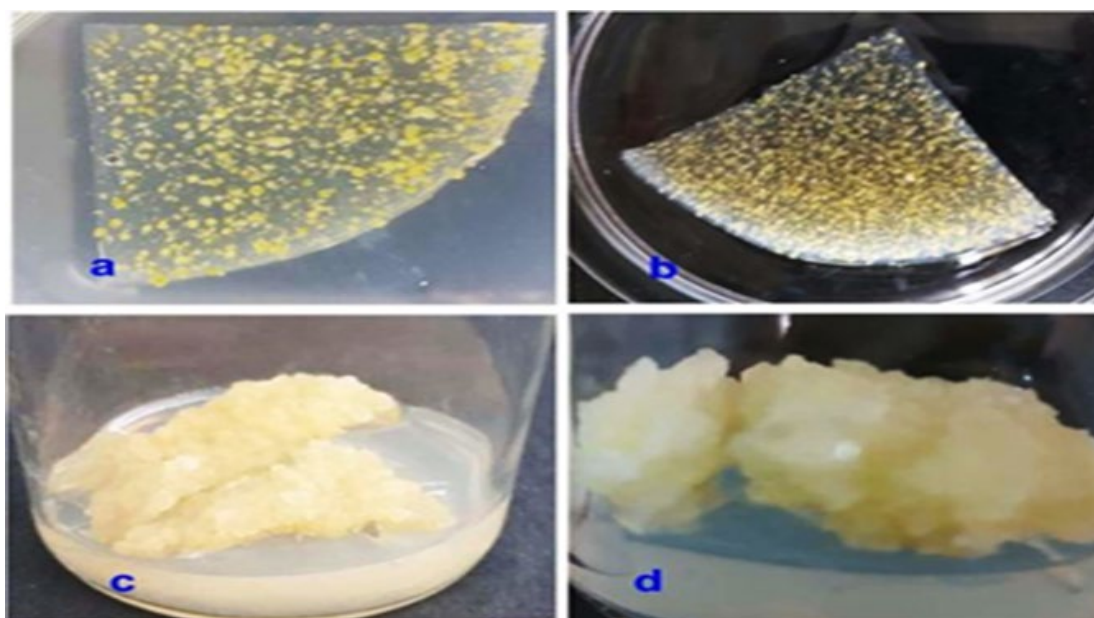
were maintained for four months through a reproducible growth mode; the produced callus was white and friable. It was very obvious, that the sectors technique produced more primordial callus 589, 881 for the densities ( $41 \times 10^5$  and  $51.0 \times 10^5$  cells.ml<sup>-1</sup>) respectively (Table 4) compared to 389, 627 for multiple-drops methods. The use of liquid media and culture suspensions gave good results in producing medium-sized callus cells capable of regeneration (Khanpour-Ardestani *et al.*, 2015).

**Table (4): Formation of primordial callus by embedding different densities of *C. cyminum* L. cell suspensions in cross- sections.**

| Cell Densities<br>( $\times 10^5$ cells ml <sup>-1</sup> ) | Total Number           |                        |                            |
|------------------------------------------------------------|------------------------|------------------------|----------------------------|
|                                                            | Callus-inducing sector | Cell colonies $\pm$ SD | Primordial Callus $\pm$ SD |
| 15                                                         | 0                      | 0 $\pm$ 0.00           | 0 $\pm$ 0.00               |
| 39                                                         | 6                      | 208 $\pm$ 0.263        | 212 $\pm$ 0.1578           |
| 41                                                         | 6                      | 551 $\pm$ 0.421        | 586 $\pm$ 0.052            |
| 51                                                         | 6                      | 889 $\pm$ 0.052        | 881 $\pm$ 0.055            |



**Fig. (4):** Embedding different densities of cell suspension of *C. cyminum* L. in MS medium containing 0.5 mg. L<sup>-1</sup> NAA and 1.0 mg L<sup>-1</sup> BA using the multiple-drops technique.(a) production of cell colonies (b) initiation of primordial callus from density  $39 \times 10^5$  cells.ml<sup>-1</sup>(c)  $41 \times 10^5$  cells.ml<sup>-1</sup> (d)  $51.0 \times 10^5$  cells. ml<sup>-1</sup> (e) the whole droplets in maintenance medium (f) produced callus after six weeks.

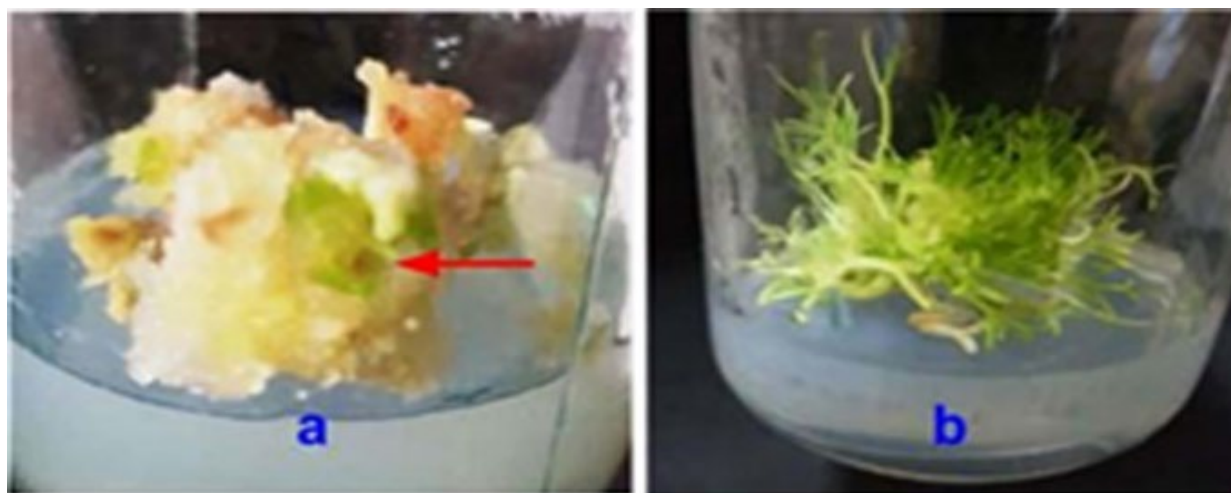


**Fig. (5):** Embedding cell suspension of *C. cyminum* L. in MS medium containing 0.5 mg.l<sup>-1</sup> NAA and 1.0 mg.l<sup>-1</sup>BA using cross sections technique. (a, b) formation of primordial callus, (c) Callus derived from sectors after four weeks (d) callus in (c) after two months.

### Plant regeneration

The results of this study indicated that the callus produced from multi drops and sectors had the ability to shoots regeneration. It was noticed that after four months of sub-culturing, some parts of the callus became green (Fig. 6a). This might refer to the differentiation of chloroplast that had occurred (Bastakis *et al.*, 2018), or it might be the result of the interaction between endogenous hormones and exogenous plant growth regulators. Eventually, these parts developed into a large number of shoots

when transferred to MS medium containing 2 mg.l<sup>-1</sup> BA alone (Fig. 6b). It appears that cytokinin increased the differentiation of shoot buds. According to Fehér, a heterogeneous organization is supported by the fact that only some calli cells can participate in organogenesis or embryogenesis. It is important to note that since the phrase "developmental potency" refers to cells, a callus cannot be either pluri- or totipotent, but it may contain cells with these properties (Fehér, 2019).



**Fig. (6):** Shoot regeneration from callus derived from multi drops and sectors (a) beginning of shoot formation (arrow) (b) regenerated shoots in MS Medium supplemented with 2.0 mg.l<sup>-1</sup> BA.

### Conclusion

In this study, we were able to develop a practical and effective methodology for establishing cell suspension cultures and plant regeneration of *C. cyminum*. Both MS medium fortified with 0.5 mg.l<sup>-1</sup> NAA+1.0 mg.l<sup>-1</sup> BA and MS +1.0 mg.l<sup>-1</sup> NAA + 2.0 mg.l<sup>-1</sup> BA were the best for callus initiation and shoots regeneration. Furthermore, using multiple drops and cross-section methods was very efficient in cultivating cell suspensions, the obtained results revealed the possibility of applying these techniques as the promising

methods to produce active compounds from cumin and other medicinal plants.

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### Contributions of authors

**S.M.S. :** Suggesting the research, designing the experimental approach and writing the manuscript.

**R.F.A.** : Practical experiments and analyzing the data.

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## Conflicts of interest

The authors declare no conflicts of interest.

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## القطرات المتعددة والقطاعات العرضية، طرق كفاءة لإنشاء مزارع المعلقات الخلوية وإنتاج نباتات الكمون

### *Cuminum cyminum* L.

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**المستخلص:** تهدف الدراسة الحالية الى إنشاء مزارع المعلقات الخلوية للنبات الكمون *Cuminum cyminum* L. مع تطبيق تقنيتي القطرات المتعددة والقطاعات العرضية في زراعة هذه المعلقات وإنتاج النباتات خارج الجسم الحي. زرعت قطع الأجزاء المختلفة (الأوراق، السيقان، السيقان تحت الفلقة والجذور) على وسط موراشيغ وسكوك (MS) الصلب الحاوي تراكيز متباينة (0.05، 0.5 و 1.0 ملغم لتر<sup>-1</sup>) نفتالين حامض الخليك NAA و (0.05، 0.1، 1.0، 2.0 ملغم لتر<sup>-1</sup>) بنزائل ادنين BA لغرض استحداث الكالس. اشارت النتائج الى الاستجابة العالية لنبات الكمون، اذ بلغت نسبة الاستحداث 100% ونسبة إنتاج الافرع الخضرية 91.6%. فضلاً عن ذلك كان الكالس الهش لقطع السيقان تحت الفلقة مناسباً لإنشاء مزارع المعلقات الخلوية في وسط MS السائل المجهز بـ 0.5 ملغم لتر<sup>-1</sup> NAA و 1.0 ملغم لتر<sup>-1</sup> BA وكانت الكثافة 51.0 × 105 خلية مل<sup>-1</sup> هي الأفضل في تكوين بادئات الكالس عند زراعة هذه المعلقات بطورها في الاكار باستخدام طريقتي القطرات المتعددة والقطاعات المستعرضة. وأظهر الكالس الناتج من المعلقات الخلوية مقدرة لإنتاج الافرع الخضرية. أوضحت نتائج هذه الدراسة كفاءة كلا التقنيتين في إنشاء مزارع المعلقات الخلوية وإنتاج الافرع الخضرية والتي من الممكن أن تكون مصادر واعدة لإنتاج المركبات الطبية لنبات الكمون.

**الكلمات المفتاحية:** الكالس، الكمون، السيقان تحت الفلقة، النبات الطبي، زراعة الانسجة.