



***In Vitro* Effects of Different Combinations of Phytohormones on Callus Induction from Different Explants of Cabbage *Brassica Oleracea* Var. *Capitata* L. Seedlings**

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Abstract

The leading purpose of this work is the development of efficient culture conditions to induce calli from cabbage (*Brassica oleracea* var. *capitata* L.) under *in vitro* conditions. The mature seeds were surface sterilized with combinations of different concentrations of ethanol and NaOCl in different time durations and were germinated on MS basal medium. The results revealed that the best sterilization method of cabbage seeds was by using 70% ethanol for one minute, followed by 15 min in 2% (NaOCl). Seedlings were used as donor sources for hypocotyls, cotyledon leaves, true leaves, and shoot tip explants. These explants were cultured on different combinations of cytokinins (TDZ, BAP, Ad) and auxins (IAA, NAA, 2, 4-D) then implanted in Murashige and Skoog (MS) media. 4 weeks after culturing, a significant difference was found among the explants in response to plant hormones. The maximum percentage of callus induction (100%) was using the combinations of 1 BAP + 1 2, 4-D, 1 BAP + 1 NAA, and 1 BAP + 2 2,4-D mg. l⁻¹. In addition, explants responses varied and the hypocotyls showed a superior result (85.71 %) as compared to other explants. For callus fresh weight, the combination of 0.22 TDZ + 79.9 Ad mg. l⁻¹ had a significant effect, causing the highest fresh weight (0.2745g), while control treatment gave the lowest mean of 0.0066 g. Data showed that cotyledon explants were significantly superior in giving highest callus fresh weight with the mean of 0.1723 g. On the other hand, hypocotyl explants gave the lowest mean, reaching 0.1542 g.

Keywords: *Brassica oleracea* var. *capitata* L., Fresh weight, IAA, NAA, 2, 4-D, hypocotyls, cotyledon leaves, shoot tips

تأثير توليفات مختلفة من الهرمونات النباتية على استحثاث الكالس خارج الجسم الحي من أجزاء نباتية مختلفة من بادرات نبات اللهانة

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الخلاصة

الهدف من البحث هو استخدام الزراعة خارج الجسم الحي لتكوين الكالس من نبات اللهانة

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(*Brassica oleracea L. capitata*) باستخدام توليفات هرمونية مختلفة. عقت البذور الناضجة باستخدام تراكيز مختلفة من الايثانول وبفترات زمنية مختلفة ثم نبتت على وسط MS، اظهرت النتائج كفاءة التركيز 70% من الايثانول لدقيقة واحدة متبوعا ب 15 دقيقة من 2% NaOCl. كما زرعت الاجزاء النباتية المختلفة (السويقه الجينية السفلى، الاوراق الفلجية، الاوراق الحقيقية والقمة النامية) والمستصلحة من بادرات اللهانة على وسط MS المجهز بتراكيز مختلفة من السايبتوكاينينات (TDZ, BAP, Ad) والاكسينات (IAA, NAA, 2, 4-D)، وبعد مرور 4 اسابيع من الزراعة لوحظت اختلافات معنوية بخصوص استجابة الاجزاء النباتية المزروعة حيث اعطت التوليفات (1BAP + 1 2, 4-D, 1 BAP + 1 NAA and 1 BAP + 2 2,4-D) اعلى معدل بلغ 100% لاستحثاث الكالس بالإضافة الى تنوع استجابة الاجزاء النباتية المختلفة وتوق السويقه الجينية السفلى بإعطاء اعلى معدل بلغ 85.71 % بالمقارنة مع الاجزاء النباتية الاخرى. اما بخصوص الوزن الطري للكالس فقد اثرت التوليفة $0.22 \text{ TDZ} + 79.9 \text{ Ad mg. l}^{-1}$ في اعطاء اعلى معدل بلغ (0.2745g) في حين اعطت معاملته المقارنة اقل معدل 0.0066 g، ولم تسجل أي فروق معنويه بين كل من (الاوراق الفلجية، الاوراق الحقيقية والقمة النامية) حيث اعطوا (0.1723, 0.1612 and 0.1643 mg) على التوالي. أوضحت البيانات بان الاجزاء النباتية المأخوذة من الأوراق الفلجية تفوقت معنويا في اعطاء اعلى وزن طري للكالس واعلى متوسط بلغ 0.1723 g. من ناحية أخرى فان الاجزاء النباتية المأخوذة من السويقه الجينية السفلى قد أعطت ادنى متوسط بلغ 0.1542 g.

Introduction

Cabbage (*Brassica oleracea* var. *capitata* L.) belongs to the family of Brassicaceae. It is one of the most common vegetables grown worldwide and often referred to as green cabbage to distinguish it from red cabbage, which has the same scientific name [1]. Cruciferae family is one of the largest families in the plant kingdom, which is famous as a source of medicinal plants as well as its nutritional properties. It includes 338 genera and 3350 species that are distributed worldwide [2].

Methanolic and aqueous crud extracts of *B. oleracea* seeds showed an inhibitory effect on both Gram-positive and Gram-negative bacteria [3]. It was also concluded that *Brassica oleracea* var. *capitata* (red cabbage) is considered as a novel inhibitor for uricase isolated from *Pseudomonas aeruginosa*, possibly used to treat gout disease in the future [4].

Cabbage, *B. oleracea* var. *capitata* L., is one of the most common vegetables in the world. Because it contains high levels of flavonoids and anthocyanins, cabbage has been long used as a herbal medicine. The antioxidant and anti-inflammatory properties of cabbage were also recently reported [5].

The parts of Brassica plants that are used as food and medicine include root, shoot, stem, leaves, leaf buds, flower buds, florets, inflorescence, seeds, seed oil, and callus. Furthermore, these plants are very rich in a variety of nutrients, such as carbohydrates, lipids, protein, vitamins, and minerals, along with phytochemical components of medicinal value [6].

In recent years, major efforts in Brassicaceae research have focused on using tissue culture technology for crop improvement. Direct *in vitro* plant regeneration is a very important step for mass production of genetically identical or uniform plants, due to somatic propagation, which may be used later as vegetative clones. *In vitro* techniques have also been applied to Brassicas from different points of view, resulting in organogenesis, somatic embryogenesis, and regeneration [7- 9]. Considerable research has been carried out in the regeneration of the Brassicaceae crop plants.

The conditions of all experiments are specific for the variety under investigation. Therefore, the main objective of the current study is to test the ability of cabbage explants for callus induction under the influence of different growth regulators using a native cultivar of *Brassica oleracea* var. *capitata* L.

Materials and Methods

This study was performance at the laboratory of tissue culture of the Department of Genetic Engineering, Food and Biotechnology Center, Ministry of Sciences and Technology, Baghdad, Iraq, during the years 2019-2020. Mature seeds of green cabbage local cultivar were purchased from a local market and used in this study.

Seed sterilization and germination

Seeds of green cabbage were surface sterilized with ethanol and sodium hypochlorite (NaOCl), successively at different durations at different periods and concentrations, as shown in Table 1. Ten seeds were cultured in each glass jar, while the treatment was replicated five times. All steps of sterilization were carried out under aseptic conditions in a Laminar Air Flow Hood. Seeds were cultured on Murashige and Skoog, 1962, (MS) salts medium solidified with 7g.l^{-1} of agar (Figure1) and incubated at room temperature ($25 \pm 2^\circ\text{C}$), 16 h light of 1000 Lux intensity, and 8 h darkness [10]. 10 days later, the results of contamination and seed germination were recorded. The seedlings were used as a donor source for explants.

Treatment	Time (min)	Concentrations	References
D.D Water	----	----	-----
Ethanol +NaOCl	5 each	50% (v/v) each	[11]
Ethanol + NaOCl	1+ 15	70%+ 2%	[12]
Ethanol + NaOCl	1+20	70% + 20% (8%)	[13]
Ethanol + NaOCl	1+10	70% + 20% (1.05%)	[14]



Figure 1-Germination of cabbage seeds in culture media

Callus induction medium

For callus induction, cotyledon leaves, true leaves, hypocotyls, and shoot tips were separated and cultured on six types of media, according to previous literature reviewed in T Table 2, (Figure 2). The culture was incubated at the same condition of the germinated seeds mentioned previously, while the results were recorded after 4 weeks.

Table 2- Media combinations used in the present study for the culturing and initiation of callus along with their citing references. TDZ = (Thidiazuron), BAP = (Benzyl Amino Purine), Ad = (Adenine), IAA = (Indole Acetic Acid), NAA = (Naphthalene Acetic Acid), 2,4-D = (Dichlorophenoxyacetic acid).

Media combinations	References	Type of plant	Type of explant
0.5 mg. L ⁻¹ NAA + 0.25 mg. L ⁻¹ TDZ + 3.0 mg. L ⁻¹ BA	[15]	Cauliflower	Hypocotyls, cotyledon
1 mg. L ⁻¹ BAP + 1 mg. L ⁻¹ NAA	[16]	Cabbage	stems, leaves, roots and petioles
MS+ 0.22 mg. L ⁻¹ TDZ + 79.9 mg. L ⁻¹ Adenine	[17]	Cabbage	Hypocotyls, cotyledon
1 mg. L ⁻¹ BAP + 2 mg. L ⁻¹ 2, 4-D	Researcher combination	Cabbage	stems, leaves, cotyledons, Hypocotyls,
1 mg. L ⁻¹ BAP + 1 mg. L ⁻¹ 2, 4-D	Researcher combination	Cabbage	Hypocotyls, cotyledon
0.5 mg. L ⁻¹ BAP + 2.0 mg. L ⁻¹ 2,4-D	[18]	Cabbage and Cauliflower	hypocotyls



Figure 2-Leaves and hypocotyl explants separated from cabbage seedlings and cultured on callus induction media

Statistical analysis

All the tests were performed by using the Completely Randomized Design (C.R.D) with 5 replicates for each treatment. Collected data was analyzed via Gen Stat program [19] and the means were compared via DUNCAN multiple range test.

Results

Seeds sterilization and germination

According to the outcomes of surface sterilization and seed germination in Table 3, the highest contamination percentage (100%) was found at control (D.D water) treatment, while lower (0%) contamination percentage was found at 70% ethanol 1min+2% NaOCl 15 min. For the germination, the highest germination (100 %) was observed when seeds were treated with 70% ethanol 1min+2% NaOCl 15min, with no contamination observed (Figure 1), while a germination ration of 10% was recorded when the seeds were treated with D.D water.

Table 3- Effects of different ethanol and sodium hypochlorite concentrations on seeds contamination (%) and germination (%) of cabbage.

Treatments	Contamination (%)	Germination (%)
D.D Water	100 a	10 c
50% Ethanol 5min+50% NaOCl 5min	32 b	64 b
70% Ethanol 1min+2% NaOCl 15min	0 d	100 a
70% Ethanol 1min+20% NaOCl 20 min	8 cd	100 a
70% Ethanol 1min+20% NaOCl 10min	20 bc	100a

*Each number represents average of five replicates.

** The same letter represents no significant differences among the numbers within the same column.

Effects of explant and growth regulators on callus induction (%)

The results in Table 4 reveal various responses for callus percentage, where most of hormonal combinations exhibited positive effects. However, no significant differences were found among 1 BAP + 1 2, 4-D, 1 BAP + 1 NAA and 1 BAP + 2 2, 4-D mg. l⁻¹ treatments, which gave 100% for each, and control treatment (MS free hormones) which presented the weakest response (4%), as shown in Table 4. For explants response, data in the same table show that hypocotyl explants were significantly superior, giving the highest callus percentage (85.71%), while leaves gave the weakest response of 62.86 % (Figure 3).



Figure 3-Callus induction from leaves explants were cultured in media supported with 1 mg. L⁻¹ BAP + 2 mg. L⁻¹ 2, 4-D

Data revealed that the combinations of 1 BAP + 1 2, 4-D, 1 BAP + 1 NAA, and 1 BAP + 2 2, 4-D mg. l⁻¹ gave 100% callus induction from cotyledons, hypocotyls, leaves, and shoot tips. There were no significant differences among other combinations (0.22 TDZ + 79.9 Ad, 0.5 NAA+ 0.25 TDZ+3 BA mg. l⁻¹ for cotyledons and hypocotyls and 0.5 BAP + 2 2, 4-D mg. l⁻¹ for hypocotyls) which gave induction values of 100 and 92%, respectively (Figure 4).



Figure 4-Shoot, roots, and callus initiated from hypocotyls explants.

Table 4- Effects of different hormonal combinations on % callus induction from different Type of explants

Treatments mg. L ⁻¹	Cotyledons	Hypocotyls	Leaves	Shoot tips	Mean
Control	0 f	8 f	0 f	8 f	4.00 d
0.22 TDZ + 79.9 Ad	100 a	100 a	44 e	64 d	77.00 bc
0.5 BAP + 2 2, 4-D	64 d	92 ab	52 e	80 c	72.00 c
0.5 NAA+ 0.25 TDZ+3 BA	100 a	100 a	44 e	84 bc	82.00 b
1 BAP + 1 2, 4-D	100 a	100 a	100 a	100 a	100 a
1 BAP + 1 NAA	100 a	100 a	100 a	100 a	100 a
1 BAP + 2 2, 4-D	100 a	100 a	100 a	100 a	100 a
Mean	80.57 b	85.71 a	62.86 d	76.57 c	

* Means followed by the same letters are not significantly different from each other within the single variant or the two interactions, at a significance of 5% level according to Duncan's multiple range test.

Effects of Growth Regulators on calli fresh weight

The results in Table 5 reveal various responses for callus fresh weight from different explants in different hormonal combinations, which ranged from 0.3074 g using 0.22 TDZ + 79.9 Ad to 0.0528 g using 1 BAP + 1 NAA. No significant differences were found between 1 BAP + 1 2, 4-D and 1 BAP + 1 NAA, which gave similar results of 0.1366 g and 0.1391 g, respectively. The best combination was 0.22 TDZ + 79.9 Ad with a mean weight reaching 0.2745 g. Data showed that cotyledon explants were significantly superior to give highest callus fresh weight, which reached 0.2786 g, with the highest mean reached 0.1723 g. On the other hand, hypocotyl explants gave the lowest mean, reaching 0.1542 g.

Table 5- Effects of different hormonal combinations on callus fresh weight (g) of cabbage explant's

Treatment mg. L ⁻¹	Type of Explant				Mean
	Cotyledons	Hypocotyls	Leaves	Shoot tips	
Control	0.0000 m	0.0082 m	0.0000 m	0.0184 m	0.0066 f
0.22 TDZ + 79.9 Ad	0.2970 a	0.1998 ef	0.2938 a	0.3074 a	0.2745 a
0.5 BAP + 2 2, 4-D	0.2380 cd	0.1370 iik	0.1756 fgh	0.2140 de	0.1912 c
0.5 NAA+ 0.25 TDZ+3 BA	0.1540 hii	0.1616 fgii	0.1304 ik	0.1672 fghi	0.1533 d
1 BAP + 1 2, 4-D	0.1854 efgh	0.1112 k	0.1128 k	0.1372 iik	0.1366 e
1 BAP + 1 NAA	0.0528 l	0.2126 de	0.1766 fgh	0.114 k	0.1391 de
1 BAP + 2 2, 4-D	0.2786 ab	0.2490 bc	0.2392 cd	0.1916 efg	0.2396 b
Mean	0.1723 a	0.1542 b	0.1612 ab	0.1643 ab	

* Means followed by the same letters are not significantly different from each other within the single variant or the two interactions, at a significance of 5% level according to Duncan's multiple range test.

Discussion

Our results showed that the best treatment for both contamination (%) and germination (%) of white cabbage seeds was 70% ethanol 1min + 2 % NaOCl 15min, as illustrated in (Table 3). The successful establishment of a contamination-free culture is a critical stage in obtaining an aseptic explant [20-21]. Sodium hypochlorite is a strong sterilizing agent that is used widely in *in vitro* applications. However, it was found that the sterilization of sunflower seeds with 5% marketable bleach and dry heating gave the best results to overcome the difficulties of internal bacterial infection [22]. Other researchers used antibiotics [23] or fungicides [24-25] when the repetitive surface sterilization technique become insufficient. In this study, NaOCl showed a positive role as an eliminator of microorganisms, due to the hypochlorous acid activity, which is formed when the NaOCl reacts with H₂O and releases Cl [26]. This result agrees with those reported by other groups [27- 29].

Many researches indicated the important role of synergistic effects of auxins and cytokinins in callus induction media [30- 33], also in addition to the usage of definite concentrations to stimulate either callogenesis or organogenesis of different tissue explants for different plants. Callus initiation depends greatly on the balance of auxins and cytokinins in the cells, which plays a vital role in cell division. Cytokinins are necessary for the growth of plants in tissue culture, as well as the cell divisions that yield undifferentiated callus [34].

Goodwin [35] and Taiz and Zeiger [36] stated that the ideal auxin concentration leads to an increase of rRNA transcription, multiplication, and enzyme activity which will increase the growth of callus. Furthermore, TDZ has shown both auxin and cytokinin-like effects, although, chemically, it is totally different from commonly used auxins and cytokinins [37].

In most Brassica species, regeneration depends on the age of explants [38 – 39], with younger explants resulting in better responses [40]. In addition, *de novo* organogenesis is depending on somatic cell totipotency, which enables plants to regenerate organs from wounded parts or excised explants *in vitro* [41]. This process is commonly divided into several distinct events: callus induction, induced partition of cell identity within the callus, radial patterning within shoot progenitors, and shoot apical meristem morphogenesis [42]. Explants from 3- or 4- days old seedlings gave optimum regeneration rates in different *Brassica* spp.; nevertheless, these explants are too small and, therefore, they are difficult to manipulate [43- 44, 38]. This is the reason why most researchers used explants excised from 5- to 7- or even 10- days old seedlings [45- 47].

In this study, when leaf and hypocotyl explants derived from young seedlings (age 7- 10- d-old) were cultured on callus induction media, they showed better and more varied response to callus (Figure 3), along with indirect organogenesis (Figure 4). The best media combinations for callus induction were 1 BAP + 1 2, 4-D, 1 BAP + 1 NAA, and 1 BAP + 2 2, 4-D mg.l⁻¹, which gave 100% callus induction from cotyledons, hypocotyls, leaves and shoots (Table 4). However, a previous study [18] stated that higher concentrations of cytokinins were important for plantlet regeneration, since higher concentrations of auxin caused only an increase in callus size with no or very little regeneration observed. Our findings agreed with this statement. Some scientists gained rapid shoot multiplication of the transgenic *Brassica oleracea* at MS enhanced with different concentrations of BAP and NAA. Thus, it can be concluded that the age of explants is important for their capability to regenerate.

Conclusions

Based on the results obtained from this study, it is concluded that all explants of cabbage showed a variable response to several hormonal combinations, despite the superiority of hypocotyl and cotyledon leaves explants for callus formation in media containing TDZ, Ad, or BA.

On the other side, a good response was observed indirectly in shoot proliferation in the same media (although all media combinations were for callus initiation) or directly in organogenesis (development of plant organs from callus tissue) in media free hormones. These observations need further studies for soma clonal variations of those plants that were regenerated from callus in this study, along with the need for further screening of abiotic stresses, such as drought or salt tolerance.

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