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Callus Induction and artificial Seed production in Blackberry (Rubus fruticosus L.) Using Tissue Culture Techniques.

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ABSTRACT

The experiment was conducted in the laboratory of cell and tissue culture, Department of Horticulture and Landscape Design, College of Agriculture, University of Kirkuk, Iraq. The explants were collected from plants species (Mora Senza, Spain), from August 1, 2024, to March 1, 2025.

Callus and artificial seed production of blackberry plants were stimulated using tissue culture technology effort by cultivating a single plant cell or a group of plant cells in a regulated environment, plant tissue culture technology makes use of the capacity of these cells to develop into entire plants. The explants were grown on (WPM) supplemented with plant growth regulators: 2,4-D at concentrations of (0.0, 0.25, 0.50, 0.75 and 1.0) mg L^{-1} to determine the best medium for the form callus from the leaf explant and the apical explant. The data indicate that the highest response rate (100%) was in the leaf explant at all concentrations of 2,4-D except the control treatment, and in the apical explant the response rate was 100% at 0.75 mg L^{-1} 2,4-D. In addition, the largest callus size was observed in the leaf explant and the apical explant (47, 27) at 1 mg L^{-1}, respectively. Statistical analysis showed that the greatest callus weight was in the leaf explant (2.0734) g at 1 mg L^{-1} 2,4-D, but in the apical explant the largest callus weight (0.8766) g was at 0.75 mg L^{-1} 2,4-D. We used sodium alginate with calcium chloride to produce artificial seeds at a ratio of 1 g/50 ml of water, it was stored in the refrigerator for 40 days.

***Keywords*:** 2,4-D, callus, WPM, *in vitro*, Blackberry.

INTRODUCTION

Blackberry are a diverse group of species and hybrids in the genus Rubus including *Rubus fruticosus* L. (*Rubus fruticosus* L.) belong to family Rosaceae[1]. Described [1] its fruit include a high percentage phenolic flavonoid phytochemical. These substances have various biological activities, so there is an increasing demand for blackberries. Blackberries flourish in temperate climates and show high adaptability to various ecological conditions. It grows quickly and often establishes in woodlands, scrublands, hillsides and hedgerows and can colonize large areas quickly.

However, blackberries seeds need deep dormancy after drying and must be stored in cool room for months or years which may lead to a significant decrease in the seeds viability and pointed out that in blackberry propagation vegetative way are easy, but it needs large areas for cultivation and ant weeds. Moreover, propagated layering also easy but rooting takes time and effort [2]

By cultivating a single plant cell or a group of plant cells in a regulated environment, plant tissue culture technology makes use of the capacity of these cells to develop into entire plants. In 1902, Gottlieb Haberland proposed the intriguing concept of in vitro plant cell culture, known as " Totipotentiality " This theory went on to provide important biological discoveries[3].

Callus tissue refers to an undifferentiated mass of plant cells produced by in vitro-grown explants on a modified solid medium. This tissue can be used in biological and biotechnological studies in addition to generating active metabolites [4]; [1], [2]). Widely employed in both academic research and biotechnological industries, callus tissue has proven essential for diverse applications ([5][2]).

The application of in vitro propagation in seed industries has been steadily gaining momentum due to the increasing demand for high-quality, disease-free, and uniform planting material. In vitro propagation allows the rapid multiplication of elite or genetically superior plant lines, ensuring uniformity and quality across large-scale seed production [6]. Explants can be sourced from virus-free meristematic tissues, significantly reducing the risk of pathogen transmission in seed industries [7].

The aim of this study to establish callus cultures from shoot apices and leaf cuttings by evaluating the effects of different auxin concentrations. and to develop artificial seeds from shoot apices and assess their viability and using a gelatinous encapsulation material.

Materials and Methods:

The experiment was conducted in the laboratory of cell and tissue culture, Department of Horticulture and Landscape Design, College of Agriculture, University of Kirkuk, Iraq, from August 1, 2024, to March 1, 2025.

Leave parts and apical tip from blackberry plants were utilized as explants for the study. These segments were trimmed to lengths of 1–2 cm using a sterile surgical blade and forceps. Initial surface cleaning involved placing the explants in a container with tap water mixed with standard detergent, followed by continuous agitation for 10 minutes. Subsequently, the explants were thoroughly rinsed with distilled, sterilized water and transferred into a laminar airflow cabinet. Under sterile conditions, the explants were disinfected by immersion in commercial sodium hypochlorite (NaOCl) solution with an initial concentration of 6%. To optimize disinfection parameters, varying concentrations of NaOCl (2%, 4%, and 6%) were tested at exposure durations of 2, 4, and 6 minutes. Following the sterilization treatment, the explants were rinsed three successive times, each lasting five minutes, using sterile distilled water.

Next, the explants were prepared for cultivation in (WPM) medium supplied supplemented with 6 mg L^{-1} agar + 30 mg L^{-1} sucrose + 1gm of activated charcoal and with 2,4-D at concentrations of (0.0, 0.25, 0.50, 0.75, 1.0) mg L^{-1} are added to a glass of water containing one liter of distilled water. The pH of the medium was adjusted to between 5.76 and 5.8. A total of 25 mL of nutrient medium was dispensed into 240 mL glass containers which were sterilized in an autoclave at 121°C for 20 minutes. The glass containers were then transferred to the growth chamber, where the cultures were incubated under an illumination intensity of 3000 lux. The light cycle consisted of 16 hours of light followed by 8 hours of darkness, and the equipped with white fluorescent tubes temperature set to (25 ± 2°C). Once the culture medium solidified, explants were transferred and planted in the callus medium. Data were collected after 4 and 8 weeks from transplantation.

For the production of industrial seeds, it was prepared the solutions:

1. Sodium alginate solution dissolved in sterile distilled water at a ratio of 1:50 g/ml and used to coat the sections.
2. Calcium chloride solution dissolved in sterile distilled water at a ratio of 1:50 g/ml.
3. Sterile distilled water in a Baker as needed.

The apical tips (somatic embryos) produced from the multiplication stage of blackberry plants were taken and immersed for 10-20 seconds in the sodium alginate solution. After coating the sections, the sections were placed in a separate container and immersed in the calcium chloride solution for 10-20 seconds. Following this, they were immersed in sterile distilled water, where they solidified and formed transparent gelatinous spheres. These spheres were stored in Petri dishes, covered, and refrigerated for 40 days.

A completely randomized design (RCD) was used for the experiment, with averages compared using the polynomial Duncan's multiple range test at the 5% probability level. Ten replications were used for each treatment, with each replicate consisting of one plant part [8]. All analyses were performed using SAS statistical software.

Results and Discussion:

Sterilization experiments

The results in Chart 1 show the effects of sodium hypochlorite (NaOCl) concentration, sterilization Period, and their interaction on contamination levels. Complete decontamination (100% contamination-free) was achieved after four weeks when explants were treated with 4 ml of (NaOCl) for 2, 4, or 6 minutes. Similar results were also observed with (NaOCl) concentrations of 2 ml and 6 ml when the sterilization period was maintained at 4 minutes. Table 1 indicates that sodium hypochlorite effectively sterilized the explants, resulting in 100% contamination free cultures, perhaps due to the decomposition of sodium hypochlorite to give chlorine and sodium hydroxide, which can be removed with distilled water [9]. This result agrees with [10], [11].

* Values with similar characters for each factor as accordingly to the Dunkin Multipliers test are not substantially different below the 5% prospect level.

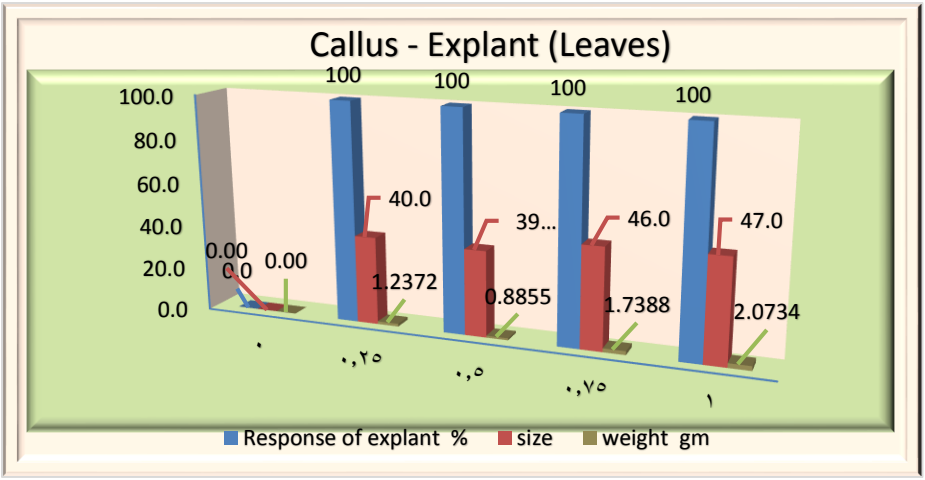
Table 1: Response of blackberry explants to sodium hypochlorite and sterilization Periods.

Period(min) sodium hypochlorite concentration(ml)	2	4	6	sodium hypochlorite concentration impact (ml)
2	90 b	100 a	100 a	97a
4	100 a	100 a	100 a	100 a
6	100 a	100 a	80 b	93 a
Period impact (minute)	97 a	100 a	93 a	

Impact of 2,4-D on the Callus of leaf explant

The data in Chart 1 show that adding 1.0 mg L^{-1} in 2,4-D to the culture medium (WPM) resulted in a 100% response in the callus induction (after 8 weeks), when contrasted with the control condition. Statistical analysis showed that adding 1.0 mg L^{-1} of 2,4-D also resulted in the greatest wet weight of callus (2.0734 gm) when contrasted with the other concentrations. Additionally, the largest size of callus (47.0) was achieved with the same treatment. These results agree with those of [12][13].

Chart 1: Impact of 2,4-D on leaf callus induction after 8 weeks of cultivation**.**

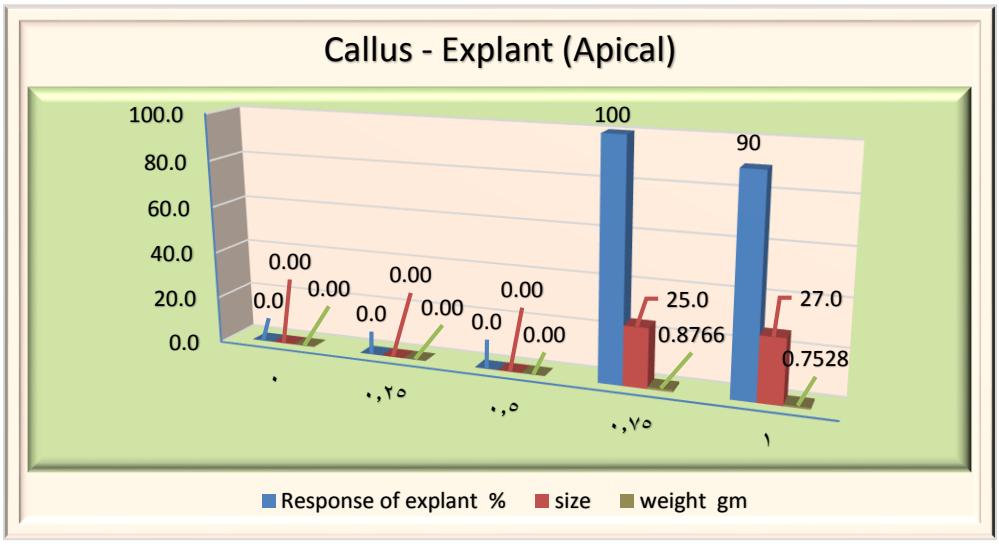


* Values with similar characters for each factor as accordingly to the Dunkin Multipliers test are not substantially different below the 5% prospect level.

Impact of 2,4-D on the Callus of apical explant

The data in Chart 2 show that adding 0.75 mg L^{-1} in 2,4-D to the culture medium (WPM) resulted in a 100% response in the callus induction (after 8 weeks), when contrasted with the control condition. Additionally, greatest wet weight of callus (0.8766 gm) was achieved with the same treatment. Statistical analysis showed that adding 1.0 mg L^{-1} of 2,4-D also resulted in the largest size of callus (27.0) when contrasted with the other concentrations.

Chart 2: Impact of 2,4-D on apical callus induction after 8 weeks of cultivation.



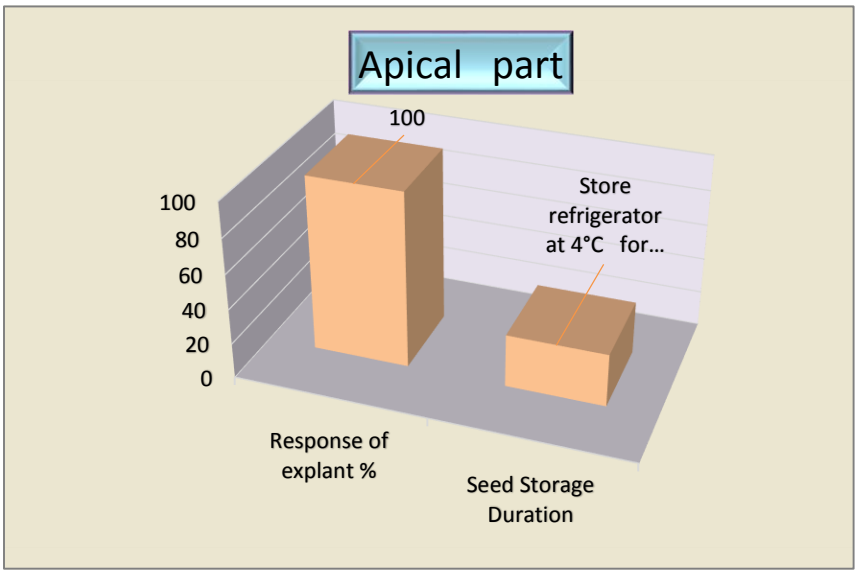
* Values with similar characters for each factor as accordingly to the Dunkin Multipliers test are not substantially different below the 5% prospect level.

Callus formation is the result of the proliferation of undifferentiated and unorganized cells originating from a wounded or excised plant tissue [14] [15]. As indicated in Tables 19 and 20, the process of callus induction is influenced by the potential energy of interconnected cells, alongside intrinsic factors related to the genetic composition of the plant cells [16]. A critical factor regulating callus development is the concentration of plant growth regulators, particularly cytokinins and auxins, in the culture medium [17] Moreover, the success of callus induction is influenced by several factors, including the genotype of the plant [18], the composition of the culture medium, the origin of the explant, as well as its age and nutritional status [19] This result agree with [20] [21] [22]&[23].

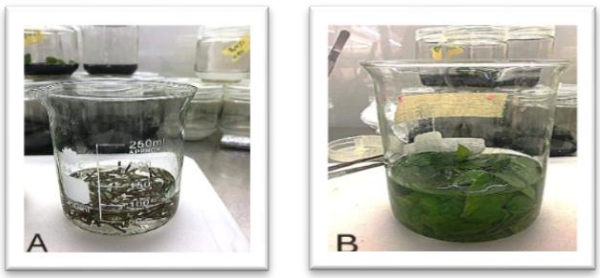
Seed Industrial

The results in Chart 3 showed that the explant (Apical part) succeeded 100% for 40 days. This is due to the effect of sodium alginate and calcium chloride, which led to the formation of a transparent gelatinous mass around the explant. The results indicate that the survival of the artificial seeds for 40 days and their success is due to the storage method. This result agrees with [24] [25].

Chart 3: Impact of storage & wrapping seed industrial on apical part.



* Values with similar characters for each factor as accordingly to the Dunkin Multipliers test are not substantially different below the 5% prospect level.



A. Apical part B. Leaf parts

Figure 3-3: Explant Sterilization

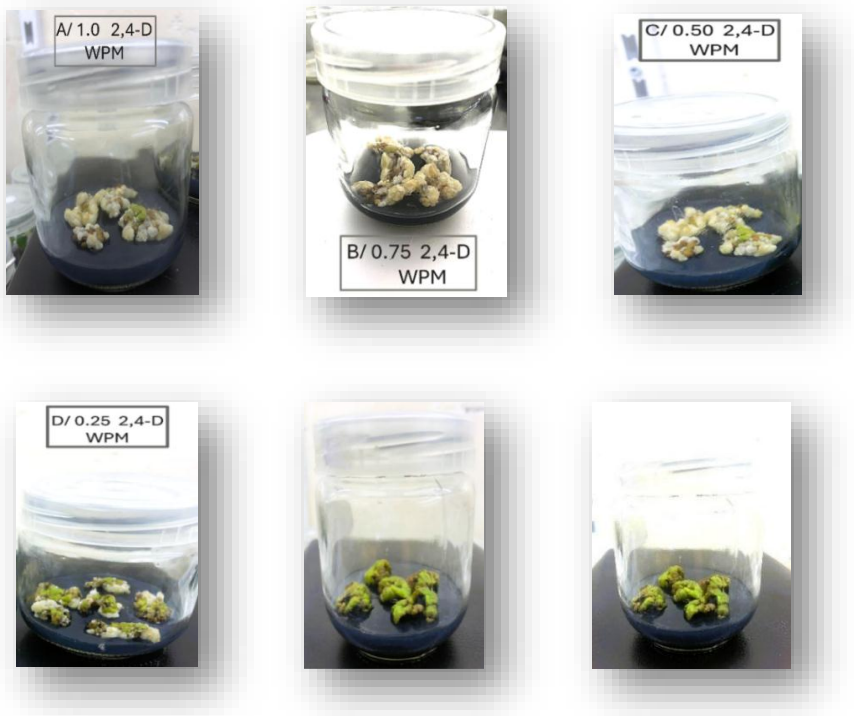


Fig 1: Callus induction from leaf with 2,4-D in the WPM medium.

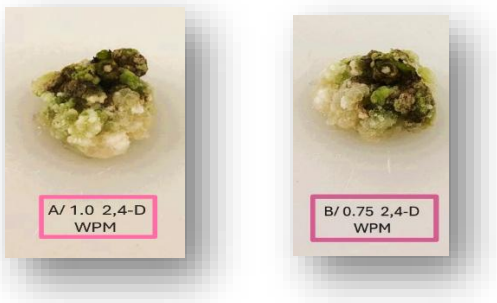


Fig 2: Callus induction from Apical with 2,4-D in the WPM medium.

Fig 3: Industrial Seed.

Conclusions

The effectiveness of sodium hypochlorite at varying concentrations and exposure durations for sterilizing the vegetative tissues of blackberry plants was evaluated. The results showed that different concentrations of the plant regulator 2,4-D had a significant effect on callus formation in terms of response rate, weight, and size. The success of the synthetic blackberry seeds was evaluated in terms of survival rate, storage stability, and shelf life.

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تحفيز الكالس وإنتاج البذور الاصطناعية في نبات بلاك بيري (*Rubus fruticosus* L.) باستخدام تقنيات زراعة الأنسجة.

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الخلاصة
أجريت التجربة في مختبر زراعة الخلايا والأنسجة، قسم البستنة وهندسة الحدائق، كلية الزراعة، جامعة كركوك، العراق. جُمعت العينات من نبات (مورا سينزا، إسبانيا)، من 2024/8/1 الى 2025/3/1.
تم تحفيز انتاج كالس والبذور الصناعية لنبات بلاك بيري بأستخدام تقنية زراعة الانسجة من خلال زراعة خلية نباتية واحدة أو مجموعة من الخلايا النباتية في بيئة منظمة، تستفيد تقنية زراعة الأنسجة النباتية من قدرة هذه الخلايا على التطور إلى نباتات كاملة. زُرِعَت الأجزاء على وسط ***(WPM)*** مُضافًا إليها مُنظِّمات نمو النبات: ***D***- 4,2 بتركيزات (0.0، 0.25، 0.50، 0.75 و1.0) ملغم لتر$^{-1}$ لتحديد أفضل وسط لتكوين الكالس من الاجزاء الأوراق و القمة النامية تُشير البيانات إلى أن أعلى مُعدل استجابة (100%) كان في زراعة الاجزاء الورقية بجميع تركيزات 2,4 ***D***-باستثناء مُعاملة كونترول، بينما كان مُعدل الاستجابة في الاجزاء القمة النامية 100% عند 0.75 ملغم لتر$^{-1}$ من 4,2 ***D***- بالإضافة إلى ذلك، لوحظ أكبر حجم الكالس في زراعة الأجزاء الورقية والاجزاء القمة النامية (47، 27) عند تركيز 1 ملغم لتر$^{-1}$ على التوالي. أظهر التحليل الإحصائي أن أعلى وزن الكالس كان في عينة الأوراق (2.0734) غم عند تركيز 1 ملغم لتر$^{-1}$ من 4,2-***D***, بينما في عينة القمة النامية، كان أعلى وزن للكالس (0.8766) غم عند تركيز 0.75 ملغم لتر$^{-1}$ من 4,2 ***D***-, استخدمنا ألجينات الصوديوم مع كلوريد الكالسيوم لإنتاج بذور اصطناعية بنسبة 1 غم/50 مل من الماء، وتم تخزينها لمدة 40 يوماً.

الكلمات المفتاحية: 2,4- ***D***، كالس، ***WPM***، خارج جسم الحي، بلاك بيري.