DJS Vol. 48(1)(2024) –pp. 1-12 ISSN: 1012-5965

Delta Journal of Science

Available online at
https://djs.journals.ekb.eg/

Research Article

BOTANY

Impact of PEG Combined with 2,4- Dichlorophenoxyacetic Acid on Improving Vitality and Friability of the Induced Callus in Spunta and Cara Potato Cultivars

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Received: 24/11/2023 **Accepted:** 6/1/2024

KEY WORDS

Explant,
Gibberellin,
Fresh weight,
Osmoticum,
Sprouting

ABSTRACT

Potatoes are the principal and widespread crop used to fill the nutritional gap. Potatoes as a food crop is considered an axis of multiple manufacturing operations. Unfortunately, it is a crop susceptible to fungal and bacterial infections that threaten productivity. The constant search for alternative methods is an urgent need for potato propagation to ensure the production of virus-free seedlings. Therefore, tissue culture represents the best technique for this purpose. This current study aiming investigation the best conditions for obtaining potato callus with high vitality and fragility. The present investigates two certificated potato cultivars (Spunta and Cara) for sprouting and callus induction by using suitable phytohormones like 0.1 g/L gibberellin (GA) and 3mg/L of 2, 4-dichlorophenoxyacetic acid (2, 4-D) with an osmoticum like PEG6000 of 50g/L for improving callus fragility. The results showed that GA remarkedly stimulated sprouting in both cultivars. While 2, 4-D hormone-induced potato callus on potato callus medium modified from Murashige and Skoog (MS), responsively. The fresh weight of the produced calli significantly increased by PEG application in the successive subcultures. The translucent calli is converted into fragility by PEG, enabling friable callus subsequent subcultures. Sprouting stimulation by GA may be due to its effect in improving carbohydrate metabolism and releasing potato dormancy. Also, 2, 4-D has a role in callus induction on callus induction media.

The study ensures using an osmoticum like PEG for obtaining a vital callus during successive subcultures to improve callus fragility and vitality as expressed by the fresh weight increase.

Introduction

The potato is an American native and a perennial member of the Solanaceae family. Potato world production increased by 1.2% to 376 million tons in 2021. China produces the most potatoes (21.8%), according to **FAOSTAT (2022)**, while India ranks second globally with 14.3% of production. Algeria surpassed Egypt to become Africa leading producer of potatoes in 2013, having doubled its output in just five years **(FAOSTAT, 2022)**.

Potatoes are the main staple meal in most countries **(Hameed *et al*., 2018)**. Also, potatoes are a consistently nutritious crop and have a remarkable role in malnutrition reduction/ elimination in underdeveloped nations **(Dévaux *et al*., 2020)**.

Tissue culture has evolved as a biological tool with a high rate of multiplication that presents an intriguing option for improving crop quality and yield **(Mohapatra and Batra, 2017)**. Plant tissue culture deals with growing plant cells, tissues, or organs from the mother plant using artificial feeding media with a predetermined composition and aseptic conditions **(Muthoni and Kabira, 2014)**. Usually, the potato sprouting needs induction by the suitable plant hormone for dormancy release. Gibberellin (GA) is an ideal phytohormone that regulates tuber sprouting during peak physiology, affecting potato dormancy **(Hu *et al*., 2023; Prathama *et al*., 2023)**. The mechanism of GA in affecting dormancy is going through regulating carbohydrate metabolism by preventing starch resynthesis by inhibiting AGPase and GBSS expression and stimulating BAM and UGPase expression, contributing to starch degradation and sucrose biosynthesis. GA is an antagonist of ABA-regulated seed germination **(Hu *et al*., 2023; Prathama *et al*., 2023**).

Tissue culture methods can generate novel plant genotypes by growing explants and induction of calli and plantlets on culture media such as Murashige and Skoog (MS) Medium **(Murashige and Skoog, 1962)**.

Murashige and Skoog medium needs some modification to suit potato for tissue culture purposes. Thus, the potato propagation medium is a Murashige and Skoog-modified medium with alterations that aid in species compatibility **(HIMEDIA, 2017)**.

Tan *et al*., (2016) characterized the callus as a growing mass of haphazardly arranged plant parenchyma cells that potentially grow into a whole plant. Embryogenic callus (EC) is a source of planting material used to regenerate new

plants **(Mohajer *et al*., 2012; Das *et al*., 2018).** The ideal concentrations of auxins and cytokinins, separately or in combination, are essential to induce potato callus **(Shirin *et al*., 2007)**. Phytohormones like 2, 4-dichlorophenoxyacetic acid (2, 4-D) are crucial for callus induction in explants of most potato cultivars, according to **Metwali *et al*., (2020)**. Furthermore, the concentration of 3.0 mg/L 2, 4-D was the most efficient concentration for inducing callus internodal and leaf explants in potato cultivars **(Shirin *et al*., 2007)**. Polyethylene glycol (PEG 6000) commonly generates osmotic stress due to its enormous molecular weight, preventing water absorption **(Abdel-Rahman and Widholm, 2010; Yang *et al*., 2019).** The PEG causes increase in total soluble sugars, which can function as an osmoticum or provide respiratory substrates **(Elmaghrabi *et al*., 2013)**. Callus growth rose in rice genotypes when PEG was present compared to controls **(Biswas *et al*., 2002)**.

This study investigated how far the PEG can improve the translucent potato callus induction when combined with a 2, 4-D hormone-enriched potato callus induction medium during successive subcultures.

Materials and Methods

Plant Materials

This research work has employed two commercially available and approved cultivars (Spunta and Cara) of potatoes (*Solanum tuberosum* L.), 2n= 4x = 48. The provider of the recruited cultivars was the Abu El-Matamir Agriculture Research Station in the EL-Behaira governorate, Egypt. The investigated potato cultivars have pure line pedigree, known as Spunta and Cara. England is the country of origin of Cara, characterized by a late maturity date. The Netherlands is the country of origin of Spunta, characterized by a medium early maturity date.

For explant sterilization, tubers brushing and washing under running water to remove dirt or muck is necessary. The immersed tubers in 0.1 g/L GA solution for one to two hours **(Hu *et al*., 2023; Prathama *et al*., 2023)** have been washed and stored at 24°C in a tight paper bag until little sprouts grew.

Sprouts were sterilized by washing tubers under running water and 25% (v/v) Clorox liquid bleach for 20 minutes. The tubers were sprayed with 70% alcohol and dried with a fresh towel. The 0.5–1 cm sprout had been removed from the tubers under sterilized conditions at a laminar flow. Sprout surface was sterilized by submerging them in 70% alcohol for one minute and then washing

them three times with sterilized distilled water.

The sprouts were rinsed five-times with sterilized distilled The sprouts were rinsed five-times with sterilized distilled water after submerging in a 25% (v/v) sodium hypochlorite solution for twenty minutes. The de-infested-sprouts were kept on sterile filter paper in sterile-Petri dishes to be ready as explants for inoculation **(Khalafalla *et al*., 2010**).

The Experimental Media

A modified-potato medium from Murashige and Skoog 1962 (MS) medium described by **HIMEDIA (2017)**, supplemented with seven g/L agar as a gelling agent and 30 g/L of sucrose. The pH of the medium was adjusted to 5.8 by 1N NaOH/ HCl, then autoclaved for 20 minutes at 121°C. A callus induction medium consists of potato medium supplemented with three mg/L of 2, 4-D was suitable for explants cultivation in harmony to **Khalafalla *et al*., (2010)** and **Shirin *et al*., (2007)**. Sterilized sprouts were cultivated aseptically on a potato callus induction medium of 25–30 milliliters in volume poured at 10 cm diameter Petri plates.

For inducing callus, the explants were kept for three to six weeks at 25°C on potato callus induction medium. For further growth and maintenance, the calli were subcultured on fresh callus-inducing medium with 21-day intervals in between each cycle **(Khalafalla *et al*., 2010; Shirin *et al*., 2007).** Calculations were achieved after the first subculture as the percentage of induced calli or not produced calli then after each subculture the percentages of alive and dead (brown) calli for both Spunta and Cara cultivars was calculated.

PEG Treatment

Both Spunta and Cara potato cultivars translucent (watery) calli inoculation on the same potato callus induction medium supplemented with 50 g/L PEG 6000 (PEG; Sigma, Poole, United Kingdom) was attained for three cycles of 21-days intervals. Calculations of the average and total fresh weight (g) of calli before and after each cycle of PEG treatment were achieved **(Abdel-Rahman and Widholm, 2010).**

Results

Callus Induction with 2, 4-D

During trials of callus induction in subcultures of 21-day intervals in between each cycle, the best callus activity was detected after seventeen subcultures in Spunta, and six subcultures in Cara. The alive (yellowish and highly proliferating calli) and dead (brown calli with no developing growth features) calli count of both Spunta and

Cara cultivars showed a significant difference during the successive subcultures on callus induction potato medium (Table, 1).

Table (1): ANOVA analysis for count between, within and total groups of live and dead (brown) calli for both Spunta and Cara cultivars after successive subcultures of 21-day intervals in between each cycle on potato callus induction medium

Calli Groups			Sum of Squares	*df	Mean Square	**F	Significance
Spunta	Alive	Between	2781.27	16	173.8	31.794	0.000
		Within	7244.179	1325	5.467		
		Total	10025.455	1341			
	Dead	Between	232.091	16	14.506	13.641	0.000
		Within	1408.980	1325	1. 063		
		Total	1641.071	1341			
Cara	Alive	Between	742.195	5	148.439	23.803	0.000
		Within	1128.757	181	6.236		
		Total	1870.952	186			
	Dead	Between	14.182	5	2.836	5.155	0.000
		Within	99.593	181	0.550		
		Total	113.775	186			

*dF: degree of freedom **F: ANOVA value

After each subculture, the Spunta callus clumps number increased (Fig. 1a). The overall callus clumps count ranged from 196 in the first subculture to 1647 in the final subculture. In total, there were 16,624 live callus clumps out of 17,319. While there were 695 dead (brown) callus clumps out of 17,319. The overall percentage of both alive and dead (brown) calli of the total successive subcultures with 21-day intervals in Spunta cultivar has collected in Fig. (1b). However, the total alive and dead (brown) calli percentages for Spunta and Cara cultivars for each individual subculture is shown in (Fig. 1c and d) and (Fig. 2, c and d), respectively. The highest alive calli percentage in Spunta (99.6%) was in the fourth subculture (Fig. 1c). The first subculture showed the least percentage of calli induction in Spunta, as 24% of explants couldn't produce calli during this subculture (Fig. 1d). The count of Cara callus clumps increased after each subculture (Fig. 2a). The overall callus clusters counted thirty in the first subculture and reached 1047 in the last subculture. Cultivar Cara totally generated 2525 callus clumps with 2445 live ones opposed to 80 dead

(brown). The Cara alive and dead (brown) calli overall percentage after the total subcultures was collected in Fig. (2b).

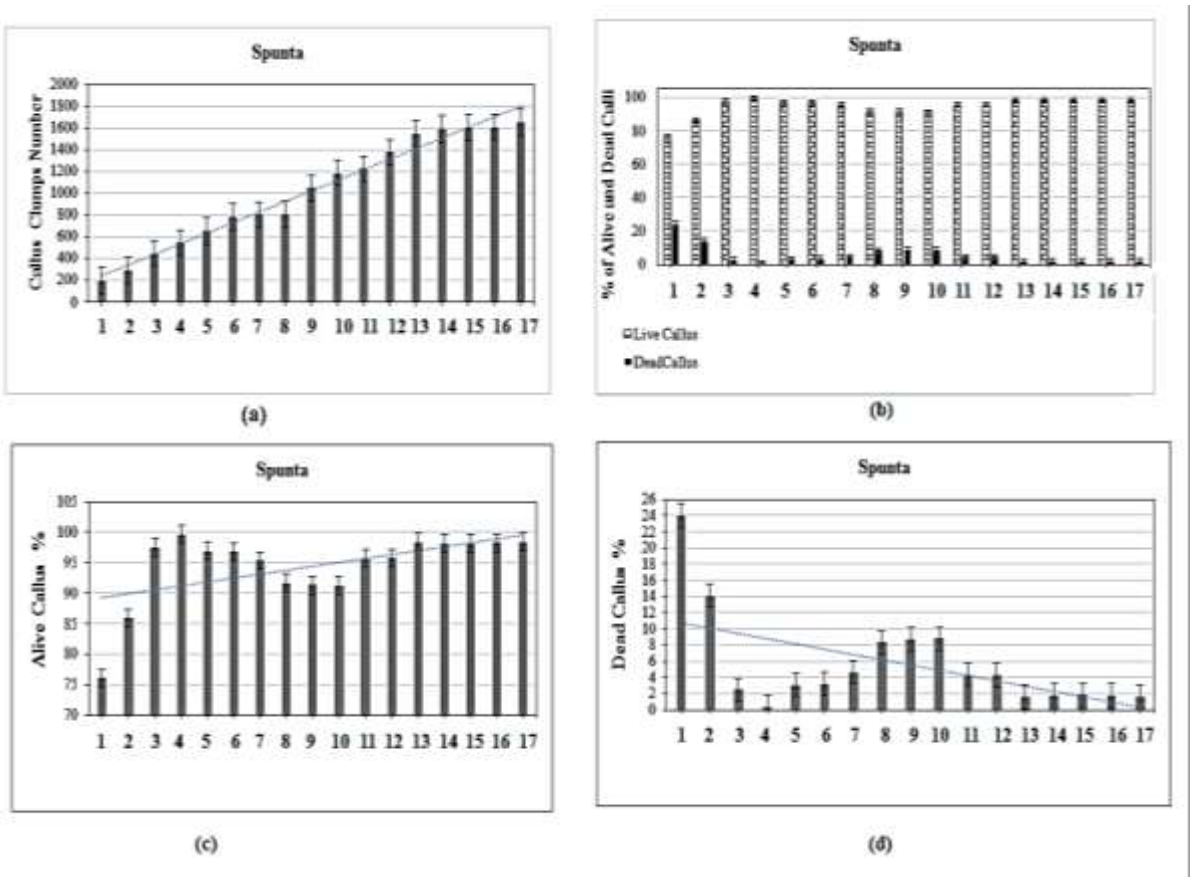

Fig. (1): The total number of callus clumps **(a)**, the overall percentage of both alive calli induction and dead (brown) calli of the total subcultures **(b)**, the percentage of alive calli induction **(c)** and the percentage of the induced calli that became dead **(d)** Numbers from 1 to 17 refer to each individual subculture with 21-day intervals in between each cycle on potato callus induction medium for Spunta cultivar.

Calculations of Cara alive and dead (brown) calli percentages was attained after each individual subculture. Cara recorded 100% of living calli in the first subculture and 98.7% in the fifth subculture (Fig. 2c). In contrast, the fourth subculture exhibited the highest percentage of dead (brown) calli of 6.8% (Fig. 2d).

Callus Response to PEG Treatment

The application of fifty g/L PEG 6000 on the potato callus induction medium for three cycles at 21-day intervals improved the physical state of the translucent Spunta calli into a friable one (Fig. 3a, 3b, 3c, 3d). Inoculation of Cara translucent calli on the same callus induction potato medium containing 50 g/L PEG 6000 for three cycles spaced around 21-days apart converted them to more friable calli (Fig. 3e, 3f, 3g, 3h).

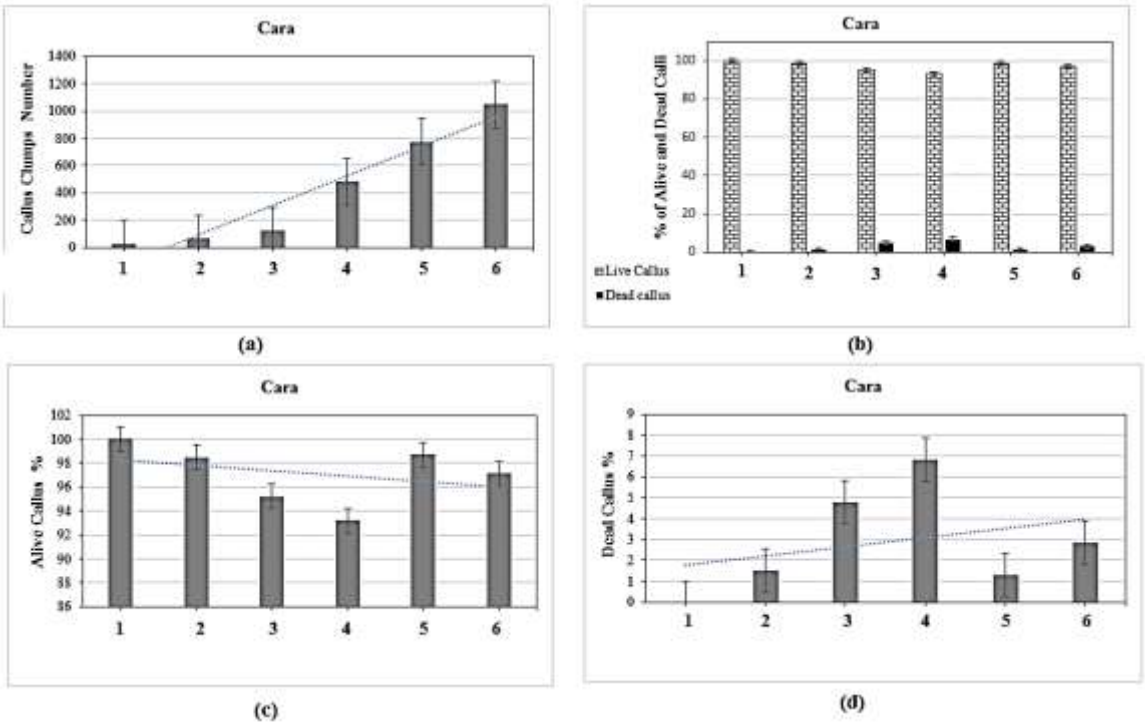

Fig. (2): The total number of callus clumps **(a)**, the overall percentage of both alive calli induction and dead (brown) calli of the total subcultures **(b)**, the percentage of alive calli induction **(c)** and the percentage of the induced calli that became dead **(d)**. Numbers from 1 to 6 refer to each individual subculture with 21-day intervals in between each cycle on potato callus induction medium for Cara cultivar.

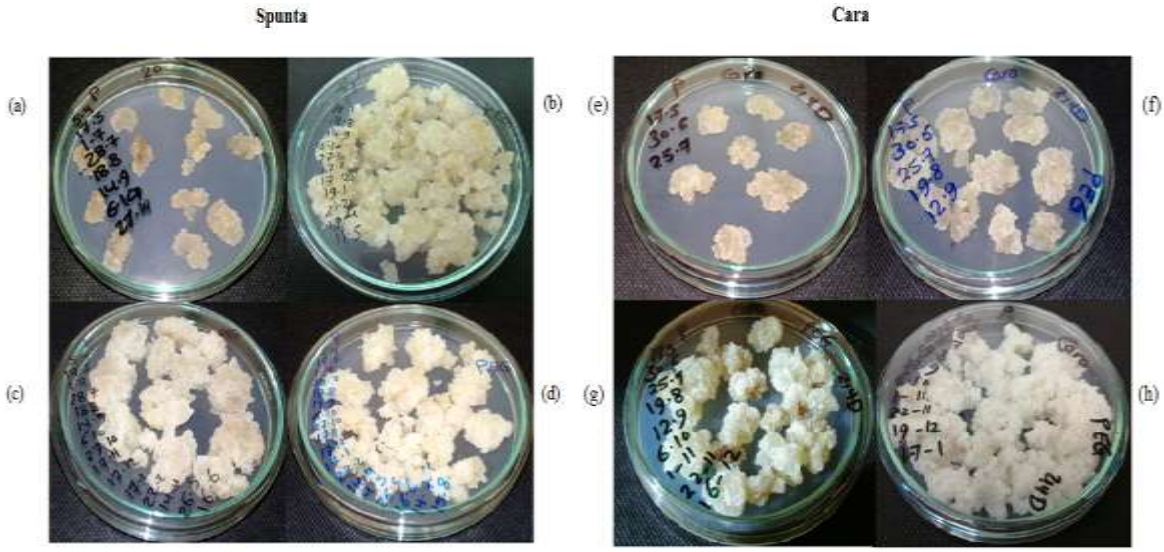

Fig. (3): The different growth stages for callus production on callus induction potato medium. Callus after 27 weeks with zero PEG **(a)**, against callus on a callus induction potato medium containing 50g/L PEG; **(b)** 53 weeks, **(c)** 57 weeks, **(d)** 65 weeks. Callus after nine weeks with zero PEG **(e)** against calli on callus induction potato medium containing 50g/L PEG; **(f)** sixteen weeks, **(g)** 28 weeks, **(h)** 32 weeks

After each subculture, there was a significant increase in both the total and average embryogenic calli fresh weight in both Spunta and Cara (Table, 2).

Table (2): ANOVA analysis for count between, within and total groups for the difference in fresh weight of both Spunta and Cara callus clumps after three subcultures of 21-day intervals in between each cycle on potato callus induction medium supplemented with 50 g/L PEG 6000

Fresh weight of the groups		Sum of Squares	*df	Mean Square	**F	Significance
Spunta	Between	88.783	2	44.392	5.612	0.004
	Within	2879.511	364	7.911		
	Total	2968.295	366			
Cara	Between	137.791	2	68.895	8.382	0.002
	Within	164.389	20	8.219		
	Total	302.179	22			

*dF: degree of freedom **F: ANOVA value

This significant increase in embryogenic calli total and average fresh weight of Spunta after each subculture is represented in Fig. (4). The total callus clumps' fresh weight of Spunta was 415 g after the first subculture, then reached 863 g after the second subculture, and 1203.6 g by the end of the third subculture (Fig. 4a). The average fresh weight of Spunta was 5.9 g, 6.5 g, and 7.2 g after the first, second, and third subcultures, respectively (Fig. 4b).

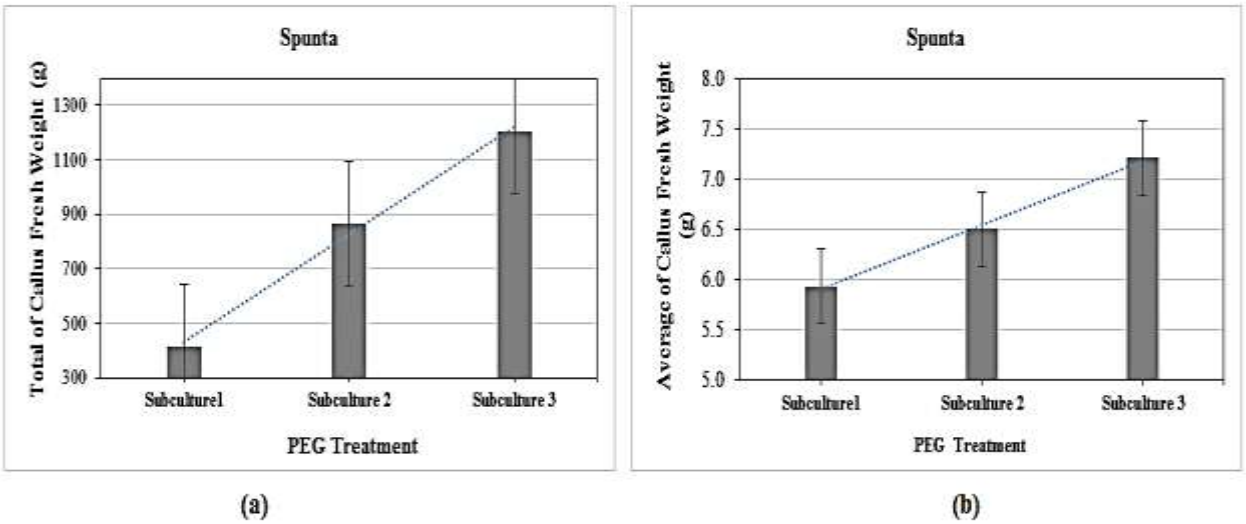

Fig. (4): Total **(a)** and average **(b)** fresh weight of Spunta callus clumps through three subcultures for Spunta cultivar. Numbers from 1 to 3 refer to each individual subculture with 21-day intervals in between each cycle on potato callus induction medium supplemented with 50 g/L PEG 6000

total fresh weight of the Cara callus clumps increased from 61.1 g after the first subculture to 96.8 g after the second one (Fig. 5). The increase in Cara callus clumps continued until reached approximately 127.8 g by the end of the third subculture (Fig. 5a). After the first, second, and third subcultures, the callus clumps' average fresh weights recorded 8.7 g, 13.8 g, and 14.2 g, respectively (Fig. 5b).

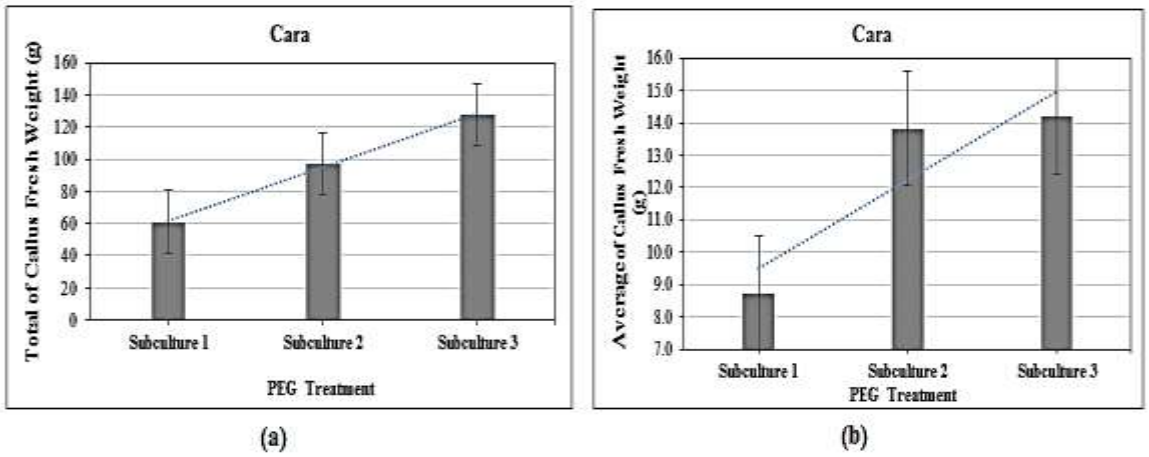

Fig. (5): Total (a) and average (b) fresh weight of Spunta callus clumps through three subcultures for Cara cultivar. Numbers from 1 to 3 refer to each individual subculture with 21-day intervals in between each cycle on potato callus induction medium supplemented with 50 g/L PEG 6000

Discussion

Based on the previous results, the 2, 4-D hormone at three mg/L significantly affected callus induction. For both the Spunta and Cara potato cultivars, the number of callus clumps increased after subsequent subcultures on callus induction potato medium containing three mg/L 2, 4-D. Total number of callus clumps in Spunta varied from 196 at the beginning of culture to 1647 at the end. In contrast, the callus clump count increased from thirty in the first subculture to 1047 in the final in Cara.

The results agreed with the finding of **Metwali *et al.*, (2020)** that 2, 4-D is a crucial plant hormone for causing callus in explants of various potato cultivars. Our result endorses those of **Shirin *et al.*, (2007)** that the concentration of three mg/L 2, 4-D is the most efficient concentration for inducing callus in internodal and leaf explants of potato cultivars. Our findings are consistent with those of **Laboney *et al.*, (2013)**, who found that adding 2, 4-D to MS

medium improved the callus induction percentage in the Granola potato cultivar. Moreover, the auxin 2, 4-D has commonly been used alone or in combination with cytokinins to enhance callus induction and maintenance **(Castillo *et al*., 1998)**.

The total and average fresh weight of callus clumps increased after PEG treatment in both Spunta and Cara calli. These results agreed with **Biswas *et al*., (2002)** regarding the increase of callus proliferation in the presence of PEG compared to controls in rice genotypes. This increase may be due to the impact of PEG by raising the soluble sugars quantity and proline buildup, consequently increasing calli fresh weight **(Elmaghrabi *et al*., 2013).**

The PEG-treated cells displayed higher growth rate, indicating a healthy and proliferating culture compared to the controls shown in our results. The increased vitality and fragility of the PEG-treated callus may be due to the activation of defense systems in accordance to results of **Balestrazzi *et al*., (2011)**. Balestrazzi denoted that PEG-treated cells continued survival may also be due to the activation of DNA repair. Regarding the embryogenic callus, **De Schutter *et al*., (2007)** explained that PEG increases the expression of the cell cycle checkpoint gene *WEE1* kinase to participate in normal cell size regulation and growth under osmotic stress. **De Schutter *et al*., (2007**) added that when single or double-strand DNA breaks occur, *WEE1* kinase, a DNA replication checkpoint, can be activated to repair the damage, as it does in *Arabidopsis thaliana.*

Conclusions

The study concluded remarkable sprouting induction by GA in Spunta and Cara potato cultivars. Also, potato callus induction was responsive to the application of 2, 4-D hormone for both cultivars. However, PEG improves the vitality and physical state remarkedly of the translucent calli of both Spunta and Cara, causing more fragility. In addition to the significant increase of the calli fresh weights by PEG application.

Acknowledgment

STDF (2418) funded this work. Scientific Research Academy (55z).

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تأثير PEG مع ٢،٤- حمض ثنائي كلوروفينوكسي أسيتيك على تحسين حيوية وقابلية تفتيت الكالى المستحث في صنفي البطاطس سبونتا وكارا

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تعد البطاطس هي المحصول الرئيسي والاكثرانتشارا و المستخدم لسد الفجوة الغذائية. كما تعتبر البطاطس محصول غذائي محوري وهام لعمليات تصنيعية متعددة. ولسوء الحظ، فالبطاطس محصول عرضة للإصابة بالعدوى الفطرية والبكتيرية التي تهدد انتاجيته. ولهذا فإن البحث الدائم عن طرق بديلة لإكثار البطاطس لضمان إنتاج شتلات خالية من الفيروسات اصبح ضرورة ملحة. وتمثل زراعة الأنسجة التقنية الأفضل لهذا الغرض. تهدف الدراسة الحالية إلى التعرف على أفضل الظروف للحصول على كالى بطاطس ذات حيوية عالية ويسهل فصله. و يهدف هذا البحث لدراسة كيفية تكوين براعم لدرنات صنفين من البطاطس المعتمدين (سبونتا وكارا) باستخدام ٠.١ جم/لتر جيبريلين والحث على تكوين الكالى باستخدام ٣ ملجم/لتر من ٢،٤- حمض ثنائي كلوروفينوكسي أسيتيك (٢، ٤-D) وكذلك دور البولى ايثيلين جليكون فى تحسين الصفات الفيزيائيه للكالى عند اضافته بمقدار ٥٠ جم/لتر لتحسين هشاشة الكالى. أظهرت النتائج أن الجيبريلين له القدره على تحفيز الإنبات وتكوين البراعم في سبونتا وكارا بشكل ملحوظ. في حين أن ٢،٤- حمض ثنائي كلوروفينوكسي أسيتيك حث على تكوين كالى البطاطس بواسطة ميديا البطاطس المعدله من ميديا MS. كما أدى استخدام ال PEG الى زيادة الوزن الطازج للكالي المنتج بشكل ملحوظ وكذلك تحسين الكالى الشفاف وزيادة هشاشته ، قد يكون تحفيز الانبات بواسطة GA بسبب تأثيره في تحسين التمثيل الغذائي للكربوهيدرات وإطلاق سكون البطاطس.