

Effect of Thidiazuron and 6-Benzylaminopurine on *in vitro* microtuberization in potato cv. Spunta.

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Abstract

The present study aimed to investigate the effect of TDZ and BAP on *in vitro* microtuberization in potato cv. Spunta. Different concentrations (4, 6, 8, and 10 mg/l) of BAP either alone or in combination with three concentrations (1, 2, and 3 mg/l) of TDZ were used for investigating their influence on microtuberization. The results showed that the individual treatment of BAP at the high level (10 mg/l) recorded the highest value of the average number of the produced microtubers/jar (7.1 microtubers/jar) compared to the other treatments (4, 6, and 8 mg/l). The combinational treatments of BAP and TDZ exhibited an increase compared to the individual corresponding concentration of BAP and this increase reached a significant level with the concentrations (4, 6, and 8 mg/l) of BAP, while a slight increase was recorded with 10 mg/l. Furthermore, the combinational treatment of BAP (6 mg/l) and TDZ (1 mg/l) resulted in the highest average number of the produced microtubers/jar (8.1 microtubers/jar) which represents about 41.1 % increase over the individual treatment of BAP (6 mg/l) with the highest average weight of microtuber (116.94 mg) and the highest average diameter of microtuber (5.3 mm). These results confirm the positive effect of the combinational treatment of BAP and TDZ on enhancing microtuber formation in potato cv. Spunta. Therefore, we report here that an efficient protocol for microtuberization in potato cv. Spunta was established. In addition, these promising results are important for developing advanced technology for microtubers mass production in Egypt.

Keywords: Potato; *Solanum tuberosum*; Microtubers; Thidiazuron, TDZ; 6-Benzylaminopurine, BAP; *in vitro*.

Introduction

Potato (*Solanum tuberosum* L.) is an important vegetable crop belonging to the family Solanaceae. Also, the potato is an important crop for food consumption locally and for trading in the world market, due to its high nutritional value for human nutrition as a source of starch for energy, proteins, mineral nutrients, and vitamins (Koch *et al.* 2020). According to FAO (2021) potato represented one of the most important food crops globally and it ranked fourth after wheat, rice, and maize. Thus, the cultivated area in 2021 was 17.34 million hectares and produced about 370.43 million tons (FAO, 2021).

Also, potato in Egypt ranked the fourth most essential food crop according to its productivity and followed by wheat, rice, and maize (Shabrawy and Ragab, 2019; Ali *et al.* 2020). Thus, the total cultivated area was 175,161 hectares and the production was recorded at 5 million tons (FAO, 2019). Further, most of the production in Egypt is essential for food consumption and the residual for exportation which reached about 350 thousand tons in 2017 (El-Anany *et al.* 2019). Potato is a high input crop for the producers in Egypt, although potato plants exposure to high risk due to the common problems related to the quality of seed tubers used for cultivation, virus diseases, and pests. *In vitro*

production of microtubers derived from potato stems have been used for producing disease-free potato seed tubers. The importance of potato microtubers is because they can be easily stored, transported, and used for producing mini tubers as well as into the field for direct planting. The progress in microtubers research is essential for improving microtuber's technology for producing sufficient amounts of disease-free potato seed tubers to sustain the annual production of potato and to further improve potato breeding and conservation programs. Moreover, potato microtubers have a high potential for commercialization and exchange (Levy *et al.* 1993; Akita and Takayama, 1994; Zaki, 1997; Donnelly *et al.* 2003; Zhang *et al.* 2005; Badoni and Chauhan, 2009; Wang *et al.* 2011; Halterman *et al.* 2012; Radouani and Lauer, 2015; De Morais *et al.* 2018).

Therefore, the aim of this study was to investigate the effect of TDZ and BAP on *in vitro* microtuberization for establishing an efficient protocol for *in vitro* microtuber production in potato cv. Spunta which is one of the important cultivars growing in Egypt to sustain its cultivation by providing a stock of virus-free seed tubers from *in vitro* seed tuber protocol.

Materials And Methods

Plant material and preparation of the explants

In this study, we used the virus-free seed tubers of Potato (*Solanum tuberosum*) cv. Spunta which were obtained from (Potato Research Station, Agricultural Research Centre), Ministry of Agriculture and Land Reclamation, Giza, Egypt. For breaking the dormancy of potato tubers, firstly, the tubers were washed with tap water for 30 minutes followed by 15 minutes in 20% sodium hypochlorite with one drop of Tween-20, then rinsed three times with sterile distilled water and kept in plastic bags for two months at 4 °C and 80 % humidity in darkness. Secondly, the tubers were kept in paper bags for one month at 24 °C to shoot sprouts. Thereafter, the produced shoots were isolated, and surface sterilized by ethanol (70 %) for 1 minute followed by 20% sodium hypochlorite with one drop of Tween-20, then rinsed three times with sterile distilled water. Finally, the sterile shoots were cultured on MS medium (Murashige and Skoog 1962) for shoot multiplication and subcultured every 4 weeks on fresh medium.

Media composition for potato microtuberization

The work in this study contained two stages as follows:

- 1- The first stage was to establish the stock culture of potato shoots to provide the explants for the experiments of microtuberization.
- 2- The second stage was to investigate the effect of different concentrations 4, 6, 8, and 10 mg/l of 6-Benzylaminopurine (BAP) either alone or in combination with three concentrations 1, 2, and 3 mg/l of Thidiazuron (TDZ) on microtuberization.

The composition of the culture medium for establishing the stock culture of potato shoots was the original MS medium containing 3 mg/l BAP + 10 mg/l silver thiosulfate (STS) was freshly prepared according to Perl *et al.* 1988 using AgNO₃, and 3% sucrose in 400 ml glass jars (50 ml/jar), thus, the nodal explants (three nodes/explant) were cultured (6 explants/jar) for 4 weeks under 16h/8h (dark/light) at 22 °C ±2.

Table 1. Composition of the original MS medium (a), the modified MS medium containing a low level of ammonium, and the improved medium for microtuberization in potato (c) the microtubers formation medium; MFM.

Substances	(a) MS	(b) Modified MS	(c) the improved media MFM
	mg/l	mg/l	mg/l
<u>Salts</u>			
MS salts without (NH ₄ NO ₃ , KNO ₃ , and KH ₂ PO ₄)	913.36	913.36	913.36
KNO ₃	1900	1900	4044
NH ₄ NO ₃	1650	1650	825
KH ₂ PO ₄	170	170	510
<u>Amino acids</u>			
Casein hydrolysate	-	-	1000
Glycine	2	2	-
<u>Carbohydrates</u>			
Sucrose	30,000	30,000	80,000
Myo-inositol	100	100	250
<u>Vitamins</u>			
Thiamine.HCl	0.1	0.1	10
Pyridoxine.HCl	0.5	0.5	1
Nicotinic acid	0.5	0.5	1
Pantothenate	-	-	0.5
Biotin	-	-	0.01
Riboflavin	-	-	0.01
Folic acid	-	-	0.01
<u>Plant growth regulators</u>			
6-Benzylaminopurine (BAP)			-
Thidiazuron (TDZ)			-
Gelrite			2,000
pH			5.8

The medium for the second stage was the developed medium used in the previous work (Ibrahim *et al.* 2018) the microtubers formation medium (MFM)

which contains a 60 mM of total nitrogen with a ratio of 5:1 between nitrate and ammonium; in addition to a high concentration of potassium phosphate (3.75

mM) which about 3 times of the MS medium plus some additional vitamins as shown in Table (1). The concentration of sucrose was 8 % and different concentrations of BAP and TDZ were used. The culture media were prepared as double concentrated solutions (2x) then 500 ml (2X) of each culture medium containing BAP and TDZ was filter-sterilized using a sterile filter (0.22 μm ; Millipore-GVWP04700). Then, the 500 ml (2X) filter-sterilized solution of each culture medium was mixed with the 500 ml (2X) Gelrite (which were sterilized by the autoclave for 20 min) and divided into sterile jars 400 ml in volume (50 ml/jar). Thus, the nodal explants (five nodes/explant) were cultured (6 explants/jar) and kept for 10 weeks in full darkness at 17 °C \pm 0.5. Finally, the jars of each treatment were photographed then the diameter and weight of microtubers were recorded.

Statistical analysis

Analyses of variance (ANOVA) of the completely randomized design (CRD) were performed on the collected data using SPSS software. The least significant difference test; L.S.D. was used at the level ($p \leq 0.05$) to compare the mean values of the treatments; mean \pm SE of 20 replicates; $n = 20$, (Snedecor and Cochran, 1980).



Fig. 1. The effect of silver ions (Ag-STS) on the growth of potato shoots. On the left side; the culture medium containing 10 mg/l silver thiosulphate (STS) and on the right side; without (STS).

The effect of BAP and TDZ on microtuberization in potato cv. Spunta

In the second stage, the experiments were conducted to investigate the effect of the inclusion of different concentrations of 4, 6, 8, and 10 mg/l BAP either alone or in combination with three concentrations of 1, 2, and 3 mg/l TDZ in MFM on microtuberization. The results in (Table 2 and Fig. 2&3) showed that the individual treatment of BAP at the high level (10 mg/l) recorded the highest value of the average number of the produced microtubers/jar (7 microtubers/jar) compared to the other treatments (4, 6 and 8 mg/l). The combinational treatments of

Results And Discussion

The effect of silver ions (Ag-STS) on the growth of *in vitro* potato shoots

In the first stage of this study the stock culture of potato shoots was successfully established during the first year, hence, the shoot explants of potato cv. Spunta were cultured on MS medium containing 3 mg/l BAP, 10 mg/l STS, and 3% sucrose in 400 ml glass jars compared to the same medium without STS. The growth of potato shoots exhibited a sharp variation between the medium containing STS and the medium without STS. Thus, the cultured shoots on MS medium without STS produced new shoots have slim stems and very small leaves compared to the produced shoots on the medium containing STS as shown in (Fig. 1). Therefore, the medium containing STS was used to produce the required explants to perform the experiments of microtuberization. Potato is a very sensitive plant to ethylene, and the accumulation of ethylene in the vessels of *in vitro* culture can strongly inhibit the growth of plants and lead to producing slim stems and very small leaves. The low concentration of ethylene (0.1 $\mu\text{l/l}$) or less caused strong growth inhibition for potato plantlets (Jackson *et al.* 1987).

BAP and TDZ exhibited an increase compared to the individual corresponding concentration of BAP and this increase reached a significant level with the concentrations (4, 6, and 8 mg/l) while a slight increase was recorded with 10 mg/l. Furthermore, the combinational treatment of BAP (6 mg/l) and TDZ (1 mg/l) resulted in the highest average number of the produced microtubers/jar (8.1 microtubers/jar) which represents about 41.1 % increase over the individual treatment of BAP (6 mg/l) with the highest average weight of microtuber (116.94 mg) and the highest average diameter of microtuber (5.3 mm).

Table 2. Effect of different concentrations of 6-Benzylaminopurine (BAP) either alone or in combination with Thidiazuron (TDZ) on the average number of the produced microtubers/jar, the average weight of microtuber (mg), and the average diameter of microtuber (mm).

Treatments	BA mg/l	TDZ mg/l	Avg. No. of microtubers/jar	Avg. weight of microtuber (mg)	Avg. diameter of microtuber (mm)
A1	4	0	4.9 ±0.05	71.26 ±1.12	3.3 ±0.03
A2	4	1	6.1 ±0.07	89.08 ±1.34	4.0 ±0.3
A3	4	2	6.5 ±0.07	98.13 ±1.45	4.3 ±0.04
A4	4	3	6.4 ±0.07	96.74 ±1.39	4.6 ±0.04
B1	6	0	5.7 ±0.06	82.53 ±1.16	4.3 ±0.3
B2	6	1	8.1 ±0.09	116.94 ±1.64	5.3 ±0.04
B3	6	2	7.6 ±0.08	113.15 ±1.55	5.1 ±0.04
B4	6	3	7.3 ±0.08	105.76 ±1.49	5.0 ±0.03
C1	8	0	6.2 ±0.07	91.56 ±1.31	4.3 ±0.04
C2	8	1	7.8 ±0.08	111.22 ±1.58	4.6 ±0.04
C3	8	2	7.4 ±0.08	105.34 ±1.46	5.0 ±0.05
C4	8	3	7.1 ±0.07	98.89 ±1.44	4.6 ±0.04
D1	10	0	7.0 ±0.07	95.16 ±1.41	5.0 ±0.6
D2	10	1	7.6 ±0.08	112.47 ±1.72	5.2 ±0.5
D3	10	2	7.2 ±0.07	111.18 ±1.53	5.0 ±0.5
D4	10	3	6.9 ±0.07	109.52 ±1.50	4.6 ±0.04
L.S.D 0.05			0.78	12.8	0.55

The mean (±SE) of twenty replicates

In addition to the high level of sucrose which represents the milestone in the culture medium for microtuber induction in potato, the plant growth regulator in particular the cytokinins proved to be essential for enhancing microtuberization in different potatoes genotypes. Many studies were used BAP for increasing the production of microtubers in potato (Aboshama *et al.* 2006; Momena *et al.* 2014; Dhaka and Nailwal, 2015; Mohapatra *et al.* 2016; Emaraa *et al.* 2017; Khalil *et al.* 2017; Moreno *et al.* 2017; Salem And Hassanein, 2017; Ali *et al.* 2018; Vural *et al.* 2018; Borna *et al.* 2019; Hossain *et al.* 2019; Naqvi *et al.* 2019; Khorsandi *et al.* 2020; Refaie *et al.* 2020; Sembiring *et al.* 2020; Sota *et al.* 2020; Andriani *et al.* 2021).

So far, a few studies were used TDZ either alone or in combination with other growth regulators for microtuberization in potato (Kefi *et al.* 2000; Lajayer *et al.* 2011; Kianmehr *et al.* 2012; Kepenek *et al.* 2017; Yagiz *et al.* 2020), and only one report was found on the effect of TDZ and BAP on microtuberization in potato (Abd Elaleem *et al.* 2015).

The results of the present study are partially in accordance with several reports using BAP or TDZ due to the differences in the genotypes of potato and the concentrations or combinations of plant growth regulators used in these studies; for example, Kefi *et al.* (2000), reported that TDZ at concentration 0.1 mg/l promoted microtuberization like kinetin at concentration 2 mg/l in MS medium containing 25 g/l sucrose for potato Atlantic cultivar under short-day conditions at 25 °C light and 22 °C dark.

On the other hand, our results disagree with those obtained by Abd Elaleem *et al.* (2015) thus, they reported that the use of TDZ and BAP (5 and 8 mg/l) either alone or in combination and sucrose at (30, 60, and 80 g/l) for microtuberization in two potato cultivars Almera and Diamant under dark and light conditions at 25 °C, resulted in the production of the highest number of microtubers for Almera (6 microtubers/jar) and for Diamant (3 microtubers/jar) only with 80 g/l sucrose without growth hormones under dark conditions.

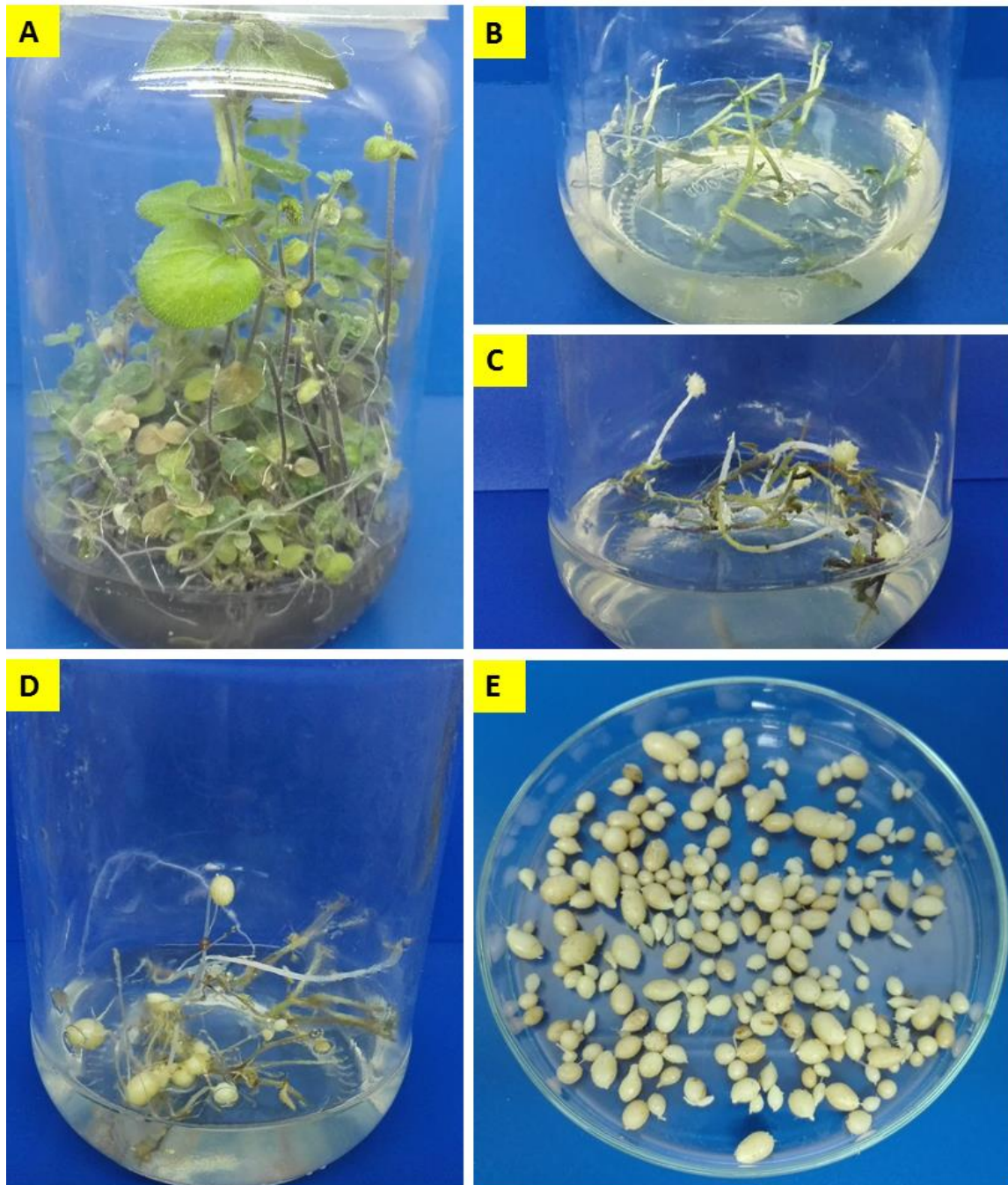


Fig. 2. The effect of BAP and TDZ on microtuberization in potato cv. Spunta. (A) The shoots of potato were used for microtubers experiments. (B) The shoot explants were cultured on microtubers formation medium (MFM) containing BAP and TDZ. (C) Initiation of microtubers after two weeks on MFM + BAP and TDZ. (D) Production of microtubers after 10 weeks on (MFM + BAP 6 mg/l + TDZ 1 mg/l). (E) Harvest of microtubers after 10 weeks of culture (MFM + BAP 6 mg/l + TDZ 1 mg/l).

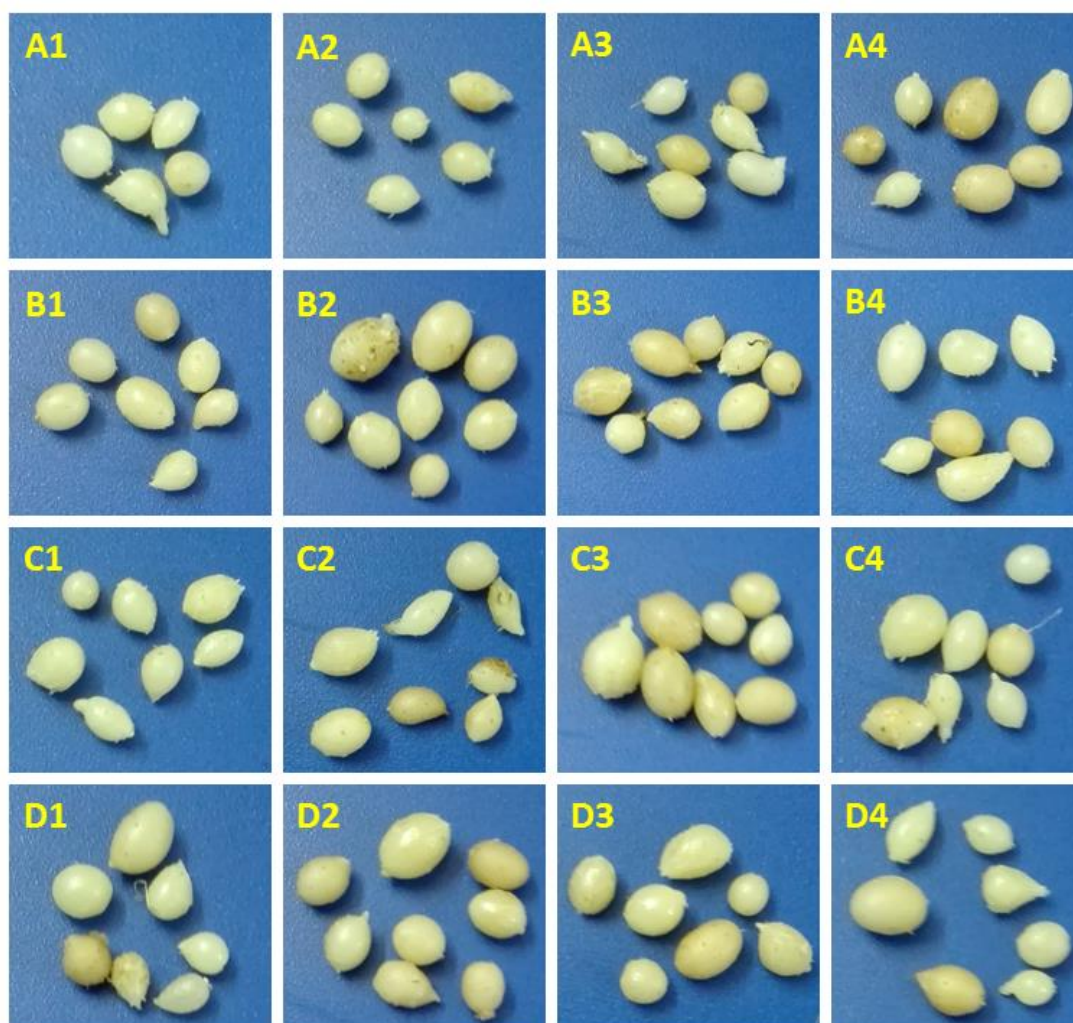


Fig. 3. The effect of different concentrations of BAP (4, 6, 8 and 10 mg/l) and TDZ (1, 2 and 3 mg/l) on microtuberization in potato cv. Spunta. A1 (4 mg/l BAP), A2 (4 mg/l BAP + 1 mg/l TDZ), A3 (4 mg/l BAP + 2 mg/l TDZ) and A4 (4 mg/l BAP + 3 mg/l TDZ). B1 (6 mg/l BAP), B2 (6 mg/l BAP + 1 mg/l TDZ), B3 (6 mg/l BAP + 2 mg/l TDZ) and B4 (6 mg/l BAP + 3 mg/l TDZ). C1 (8 mg/l BAP), C2 (8 mg/l BAP + 1 mg/l TDZ), C3 (8 mg/l BAP + 2 mg/l TDZ) and C4 (8 mg/l BAP + 3 mg/l TDZ). D1 (10 mg/l BAP), D2 (10 mg/l BAP + 1 mg/l TDZ), D3 (10 mg/l BAP + 2 mg/l TDZ) and D4 (10 mg/l BAP + 3 mg/l TDZ).

Our results indicated that the combination of BAP 6 mg/l and TDZ 1 mg/l resulted in a synergistic effect and increased the microtubers production.

The synergistic effect of the phenylurea compound TDZ, when added to BAP, may be attributed to the role of TDZ as an inhibitor to the cytokinin oxidase/ dehydrogenase; CKX, EC 1.5.99.12, (Chatfield and Armstrong 1986; Hare and Van Staden 1994, Nisler *et al.* 2016) through preventing the catabolism of purine compounds such as zeatin, dihydrozeatin and N6-isopentyladenosine (Chatfield and Armstrong, 1986, Laloue and Fox, 1989) consequently, increasing the final concentration of the endogenous cytokinin in the tissues and in addition to the exogenous BAP in the medium. Several reports indicated that the biosynthesis and storage of purines are increased by TDZ, and this may occur due to over synthesis or inhibition of catabolism (Capelle *et al.* 1983; Murthy *et al.* 1998; Victor *et al.* 1999; Murch *et al.* 2001;

Casanova *et al.* 2004; Zhang *et al.* 2005; De Melo Ferreira *et al.* 2006; Jones *et al.* 2007). Furthermore, some synthetic cytokinins (N6-benzyladenine, and kinetin) are reported to be resistant to degradation by the cytokinin oxidase (McGaw and Horgan, 1983, Whitty and Hall, 1974). Moreover, TDZ exhibits a wide range of activity associated with the metabolism of different plant growth regulators. The role of TDZ as cytokinin is actually dependent on the regulation of biosynthesis and catabolism of endogenous cytokinin in plant cells. Also, the plant tissues treated with TDZ recorded an increase in endogenous hormones; auxin, ethylene, and ABA (Yip and Yang, 1986; Suttle 1986; Murthy *et al.* 1995; Murthy *et al.* 1998). In addition, the concentration of endogenous Indole amine compounds; melatonin, and serotonin (the auxin-related compounds) were increased and accumulated in plant tissues treated with TDZ and considered to be important for plant regeneration (Murch *et al.*

1997; Murch and Saxena, 2001; Murch *et al.* 2001; Jones *et al.* 2007). The exposure of plant tissues to TDZ can cause oxidative stress and melatonin can mitigate the oxidative stress in plant cells (Mundhara and Rashid, 2006). Under oxidative stress the plant cell can utilize the reactive oxygen species in signal transduction cascades as second messengers for different physiological processes; cell division, morphogenesis, and senescence (Foyer and Noctor, 2005). Therefore, besides the known roles of TDZ in plant cells, the response of plant cells to TDZ during the differentiation; shoot regeneration, or embryogenesis may be attributed also to the change and the regulation of oxidative stress in the cells.

Conclusions

We report here that the combinational treatment of BAP (6mg/l) and TDZ (1mg/l) led to an increase in the production of potato microtubers (8.1 microtubers/jar) and this increase represents about 41.1 % over the individual treatment of BAP (6 mg/l) with the highest average weight of microtuber (116.94 mg) and the highest average diameter of microtuber (5.3 mm). Therefore, we report here that an efficient protocol for microtuberization in potato cv. Spunta was established. In addition, these promising results are important for developing advanced technology for microtubers mass production in Egypt.

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تأثير الثيديازورون (TDZ) والبنزويل امينو بيورين (BAP) على تكوين درنات البطاطس الدقيقة صنف سيونتا معملياً

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الهدف من هذه الدراسة هو دراسة تأثير الثيديازورون (TDZ) والبنزويل امينو بيورين (BAP) على تكوين درنات البطاطس صنف سيونتا معملياً. وتم استخدام تركيزات مختلفة (4، 6، 8 و 10 ملجم / لتر) من BAP إما منفردة أو بالتداخل مع ثلاث تركيزات (1، 2 و 3 ملجم / لتر) من TDZ لمعرفة تأثيرها على تكوين الدرنات. أظهرت النتائج أن المعاملة الفردية لـ BAP عند المستوى المرتفع (10 ملجم / لتر) سجلت أعلى قيمة لمتوسط عدد الدرنات المنتجة (7.1 درنة دقيقة / جار) مقارنة بالمعاملات الأخرى (4، 6 و 8 ملجم / لتر) BAP. وأظهرت المعاملات التداخلية لـ BAP مع TDZ زيادة مقارنة بالتركيز المقابل الفردي لـ BAP ووصلت هذه الزيادة إلى مستوى معنوي بتركيزات (4 و 6 و 8 ملجم / لتر) من BAP بينما تم تسجيل زيادة طفيفة مع 10 ملجم / لتر BAP. علاوة على ذلك، نتج عن المعاملة BAP بتركيز (6 ملجم / لتر) مع TDZ بتركيز (1 ملجم / لتر) أعلى متوسط عدد من الدرنات الدقيقة المنتجة / جار (8.1 درنة دقيقة / جار) والتي تمثل زيادة بنسبة 41.1% تقريباً عن المعاملة الفردية لـ BAP بتركيز (6 ملجم / لتر) و أعلى متوسط وزن للدرنات (116.94 ملجم) وأعلى متوسط لقطر الدرنات (5.3 ملليمتر). تؤكد هذه النتائج التأثير الإيجابي للتداخل التوافقي لـ BAP و TDZ في تعزيز تكوين الدرنات الدقيقة للبطاطس صنف سيونتا. لذلك هذه النتائج تؤكد التأثير الإيجابي لتداخل كلا من TDZ و BAP في تشجيع تكوين درنات البطاطس معملياً صنف سيونتا للمساهمة في تأسيس نظام كفاء لتكوين درنات البطاطس معملياً لإكثار البطاطس في مصر.