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AN ADEQUATE MICROPROPAGATION PROTOCOL FOR *IRIS TINGITANA* BULBLETS PRODUCTION AND CHEMICAL COMPOSITION

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Abstract

The present work was conducted to optimize a micropropagation protocol of *Iris tingitana* using various concentrations of BA and 2ip (0. 0.5, 1, 1.5 and 2.0 mg/l) for *in vitro* shoots multiplication. For more shoot and bulblets proliferation during three repeated subcultures as well as the diameter of obtained bulblets, various concentrations of sucrose concentrations (30, 60, 90 and 120 g/l) alone or with growth inhibitor (3 and 6 mg/l paclobutrazol). The highest number of shootlets per explant were recorded at 1.0 mg/l of BA or 2ip. MS culture medium supplemented with sucrose 30g/l was favored for shootlet multiplication. Increasing sucrose concentration to 90 or 120 g/l supplemented with paclobutrazol at 3mg/l led to the highest diameter of obtained bulblets. Also, using sucrose concentration above 30 g/l in combination with paclobutrazol had a promotion effect on bulblets number that formed during the three repeated subcultures. All plants were successfully survived with high percentage (100%) after 8 weeks from hardening off. The highest percent of carbohydrate were formed for plantlets grown on MS medium supplemented with 120 or 90 g/l sucrose plus 3 mg/l of paclobutrazol. Phenol and tannin were significant increased with 90 g/l sucrose plus 13 mg /l paclobutrazol. Whereas, the flavonoid was maximized value from using 30 g/l sucrose plus 3 mg/l paclobutrazol and 90 g sucrose plus 6 mg/l paclobutrazol.

Key words : Iris, tissue culture, bulblets and chemical composition.

Introduction

Iris plant occupies an important economic position as medicinal and ornamental plant due to its demands increase for local and foreign markets (Ragaa, 2012). *Iris tingitana* that have blue color, is the most commonly grown in Egypt and it is species of the largest and coolest genus of Iridaceae, which comprises more than 300 species (Khalid *et al.,* 2017). Iridaceae family was known as it is rich in secondary metabolic content (Harbone and Williams, 2000). The medicinal importance of Iris species that are used in treatment of cancer, inflammation and viral infections has been documented (Rigano *et al.,* 2006). Some species are valuable to the pharmaceutical and perfume industries (Jevremoviæ *et al.,* 2013). Therefore, a new trend to propagate these species through tissue culture is needed to study the possibility of using them as medical plants or ornamental plants.

The propagation of Iris species through seedlings is difficult because of the very low germination rate. Using tissue culture method is widely used for propagation and biodiversity conservation for plants that are rare and difficult to propagate by conventional methods (Pense, 2013). Also, the propagation of the valuable Iridaceae species through tissue culture technique has been reported by Ascough *et al.,* (2009). Mielk and Anderson (1986) suggested that the associated problems with the adaptation of plantlets to the greenhouse conditions were as being due to bulb size and accordingly, the factors that would increase bulb size *in vitro* were studied.

The aim of present research is to develop a complete micropropagation procedure to proliferate bulblets number using bulbs and further increases the quantity and quality of Iris bulbs *in vitro*.

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Materials and Methods

The experiment was conducted at tissue culture Technique Laboratory- Central Labs - Ornamental Plants and Woody Trees Department, Agricultural and Biological Researches Division, National Research Center (NRC), Tissue Culture & Germplasm Conservation Research Lab., Horticulture Research Institute, Egypt during years 2017 and 2018 to establish an efficient *in vitro* culture protocol for rapid micropropagation and bulblets production of *Iris tingitana.*

Surface sterilization and bulb explant preparing

Bulbs of Iris were collected from commercial nursery, washed in detergent completely under tap water running and surface sterilized in ethanol 70% (v/v) for 30 seconds, then rinsed in Clorox (sodium hypochlorite 15%) for 7 min then, 2% $HgCl_2$ (MC) solution (w/v) for 10 minutes and washed with sterilized distilled water three times.

After sterilization, the bulbs containing the base disk were cut to two or four sections and aseptically cultured on MS culture medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.7% agar. The pH of the medium was adjusted to 5.6-5.8 and autoclaved at 121°C and 15 psi for 15 minutes.

Incubation conditions

The *in vitro* cultures during all stages were placed in the incubation room at 23+2°C under 16 hours photoperiod and 1.5 kilo lux light intensity provided by cool, white, fluorescent lamps.

***In vitro* culture establishment**

After starting stage, the obtained bulblets were separated and subcultured on MS medium supplemented with various concentrations (0. 0.5, 1, 1.5 and 2.0 mg/l) of BA and 2ip for *in vitro* shoots multiplication. The number of shootlets/explant and number of leaves/ shootlet were recorded as characteristic features of regenerated plantlets.

***In vitro* shoots propagation and bulblets development**

Shoot explants were transferred to culture media contained various concentrations of sucrose concentrations (30, 60, 90 and 120 g L^{-1}) alone or with growth inhibitor (3 and 6 mg L^{-1} paclobutrazol) for shoot proliferation and bulblets diameter as well as bulblet number of Iris during three repeated subcultures as well as the diameter of obtained bulblets that were recorded.

Chemical composition

Total hydrolysable carbohydrates were spectrophotometrically determined using phenol- sulphuric acid method (Dubois *et al.,* 1956).

Total phenols, flavonoids and tannins were estimated and assayed using methodology of Tambe and Bhambar (2014).

Hardening off

The bulblets were gradually transferred to pots containing peat moss+sand (1:1), covered with transparent polyethylene pages for six weeks. Survival percentage of plantlets (%) were determined after 8 weeks.

Statistical analysis

The recorded data were statistically analyzed using randomized complete block design with 10 replicates per treatment. LSD test at 5% for comparison among means was used according to methods of Steel and Torrie (1980).

Results and Discussion

***In vitro* culture establishment**

For obtaining *in vitro* culture of *Iris tingitana*, the response of explants to induce multiple shootlets was observed on MS culture medium supplemented with different concentrations of two cytokinins (BA or 2ip) irrespective of their types (Table 1). The highest number of shootlets per explant (2.25) were recorded at 1.0 mg/ l of BA or 2ip. Similar results of proliferated shootlets were indicated with the interaction effect of cytokinin type and concentration whereas, BA at 1mg/l was the favored for more proliferated shootlet (2.5). Meanwhile, the highest number of leaves (4.8 and 5.0) were recorded for MS culture medium supplemented with BA at 1.0 and 1.5 mg/l, respectively. This indicates the superiority of BA at 1mg/l among other treatments.

This finding was confirmed by earlier studies (El-Naggar *et al.,* 2012 and Taha *et al.,* 2018) who found that the highest number of shoots was obtained at 1.0 and 2.0 mg/l of BA. The effect of BA on the formation of adventitious shoots might attributed to its role as synthetic cytokinin in plant growth and development (Takayama and Misawa, 1983 and Maesato *et al.,* 1984).

***In vitro* propagation and bulblets formation**

The effect of sucrose and paclobutrazol at various concentrations supplemented to optimum MS culture medium for shootlet proliferation (enriched with 1mg/l of BA) on micropropagation ability of *Iris tingitan* was tabulated (Table 2). The highest number of shootlets per explant (9.33) was noticed on MS culture medium supplemented with sucrose 30g/l (T1). Adding paclobutrazol at 6 mg/l to the mentioned medium (T1) caused the highest number of leaves (11.67) however,

Table 1: Effect of cytokinin type and concentration on *in vitro* culture establishment of *Iris tingitana.*

Character	Number of shootlets/explant			Number of leaves		
Type (A) / Conc. (mg/1) (B)	BA	2ip	Mean (B)	BA	2ip	Mean (B)
0.0	1.67	1.67	1.67 b	2.25	2.25	2.25 b
0.5	1.75	1.76	1.75 b	3.25	2.0	2.63 b
1.0	2.5	2.0	2.25 a	4.80	2.75	3.78 a
1.5	2.0	1.67	1.83 ab	5.00	3.83	4.42 a
2.0	1.67	1.83	1.75 b	2.17	2.58	2.38 b
Mean (A)	1.92 a	1.79 a		3.49 a	2.68	
LSD at 0.05	A = 0.26 B = 0.42 A*B = 0.59			A = 0.42 B = 0.67 A*B = 0.95		

increasing sucrose concentration to 90 or 120 g/l supplemented with paclobutrazol at 3mg/l led to the highest diameter of obtained bulblets (1.13 and 1.18 cm, respectively). It seems that high concentration of sucrose had inhibition effect on *in vitro* shoot proliferation of *Iris tingitana*. This may attribute to the high levels of sucrose stresses the shoots and exhibited poor development (Barpete *et al.,* 2014). High sucrose concentration caused low photosynthesis due to an increment of osmotic stress resulting in growing weak shoots on explants (Barpete *et al.,* 2015). Takayama and Misawa (1979) mentioned the effect of sucrose on *Lilium auratum* bulb growth that could be mediated by changes of osmotic potential during plants growth. Also, the stimulation effect of high concentration of sucrose and paclobutrazol on bulblet diameter agreed with Ziv (1989) who observed that Paclobutrazol promotes storage organ development when grown in media enriched with sucrose.

In the present study, using 60 g/l of sucrose alone or high concentration of sucrose (120 g/l) supplemented with paclobutrazol at 6mg/l was most effective for the highest percent of rooting (100%). In addition, the longest roots (1.80 cm) was observed with 60 g/l of sucrose but, the highest number of roots (9.0) was obtained with increasing the concentration of sucrose to 120 g/l in MS culture medium. The important role of sucrose and plant growth regulators in multiplication rate and bulblet size in various bulbous crops was mentioned (Lian *et al.,* 2003). Moreover, Hazaika (2003) pointed out that using paclobutrazol caused a Shift in the division of absorption from the leaves to the storage organs and roots, increased chlorophyll, carbohydrates in all parts of seedlings and also increased root respiration, soluble protein and mineral contents in leaf tissues, reduced cell-wall polysaccharides and water loss. The beneficial influence of sucrose on rooting was also recorded by Sinha and Roy (2002).

In vitro bulblets development

The effect of sucrose and paclobutrazol at various concentrations on *in vitro* bulbs development of *Iris tingitana* during three repeated subcultures was indicated in Fig. 1, 2. From these data, it was clear that using sucrose concentration above 30 g/l in combination with paclobutrazol had a promotion effect on bulblets number that formed during the three repeated subcultures. Thus, the highest values were obtained (during the three subcultures) with MS culture media supplemented with

Table 2: *In vitro* propagation ability of *Iris tingitana* under effect of sucrose and paclobutrazol at various concentrations.

Character / Treatment	Number of shootlets	Number of leaves	Bulblet diameter (cm)	Rooting %	Number of roots	Length of roots(cm)
Control (MS + 30 g/l sucrose) (T1)	9.33	5.00	0.48	0.0	0.0	0.0
MS + 60 g/l sucrose (T2)	4.50	4.00	0.76	100	7.00	1.80
MS + 90 g/l sucrose (T3)	3.43	3.00	0.87	60	5.00	1.30
MS + 120 g/l sucrose (T4)	4.17	3.33	0.95	86.33	9.00	1.50
T_1 + Paclobutrazole 3mg/l	5.30	7.00	0.68	40	3.00	0.80
T_2 + Paclobutrazole 3mg/l	3.00	6.33	0.92	83	4.00	1.20
T_3 + Paclobutrazole 3mg/l	3.00	5.67	1.13	71	4.00	1.10
T_4 + Paclobutrazole 3mg/l	2.90	4.00	1.18	66	6.00	1.00
T_1 + Paclobutrazole 6mg/l	4.67	11.67	0.83	60	2.00	0.70
T_2 + Paclobutrazole 6mg/l	2.50	4.33	0.85	66	3.00	1.00
T_3 + Paclobutrazole 6mg/l	2.50	4.67	0.93	77	4.00	0.90
T_4 + Paclobutrazole 6mg/l	2.50	3.67	1.07	100	4.00	0.90
LSD at 0.05	1.35	1.64	0.22	4.42	1.97	0.31

sucrose at 90 g/l + PBZ 3mg/l or the treatment of sucrose 60 g/l + PBZ 6 mg/l. It could also observe that using any concentration of sucrose (30 – 120 g/l) alone without adding PBZ in the culture medium resulted in the lowest values of those formed bulblets during all subcultures. Confirmed results were found by Nagarju *et al*. (2002) who pointed out that the increased growth of tuberous organs needs a relatively high concentration of sucrose (more than 50 g/l) in the medium. Moreover, they found that adding PBZ with sucrose in the medium was beneficial for *in vitro* cormel formation.

Hardening off

When the obtained bulblets were gradually transferred to pots containing peat moss + sand (1:1), all plants were successfully survived with high percentage (100%) after 8 weeks from hardening off (Fig. 3). Also, Thakur *et al.*, (2002) observed the survived Lilium bulblets with highest percent in peatmoss.

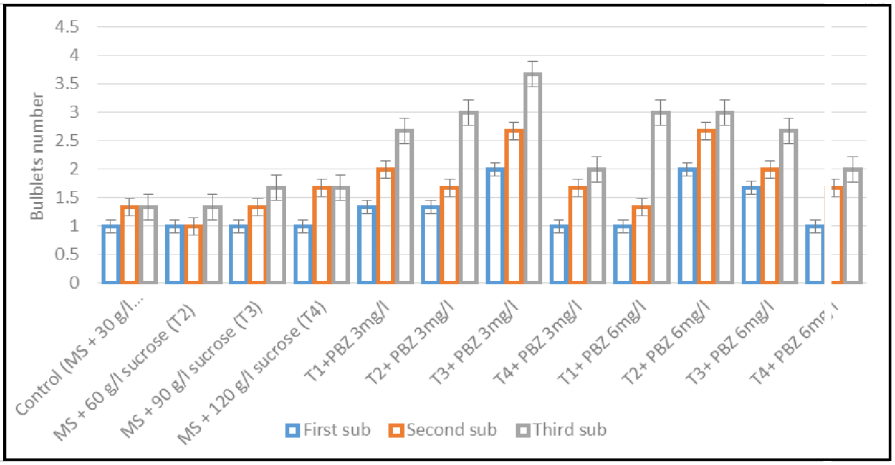

Fig. 1: *In vitro* bulbs development of *Iris tingitana* during three repeated subcultures under effect of sucrose and paclobutrazol at various concentrations.

Chemical composition

The chemical composition of *Iris tingitana* bulblets after grown on MS medium with different concentration of sucrose and paclobutrazol *in vitro* are summarized in Table 3. The highest percent of carbohydrate were formed for plantlets grown on MS medium supplemented with 120 or 90 g/l sucrose plus 3 mg/l of paclobutrazol. Phenol and tannin were significant increased (193.39 and 25.21 mg/g respectively) in treatment with 90 g/l sucrose plus 3 mg/l paclobutrazol. Whereas, the flavonoid was maximized value (19.13 and17.68 mg/g respectively) from using 30 g/l sucrose plus 3 mg/

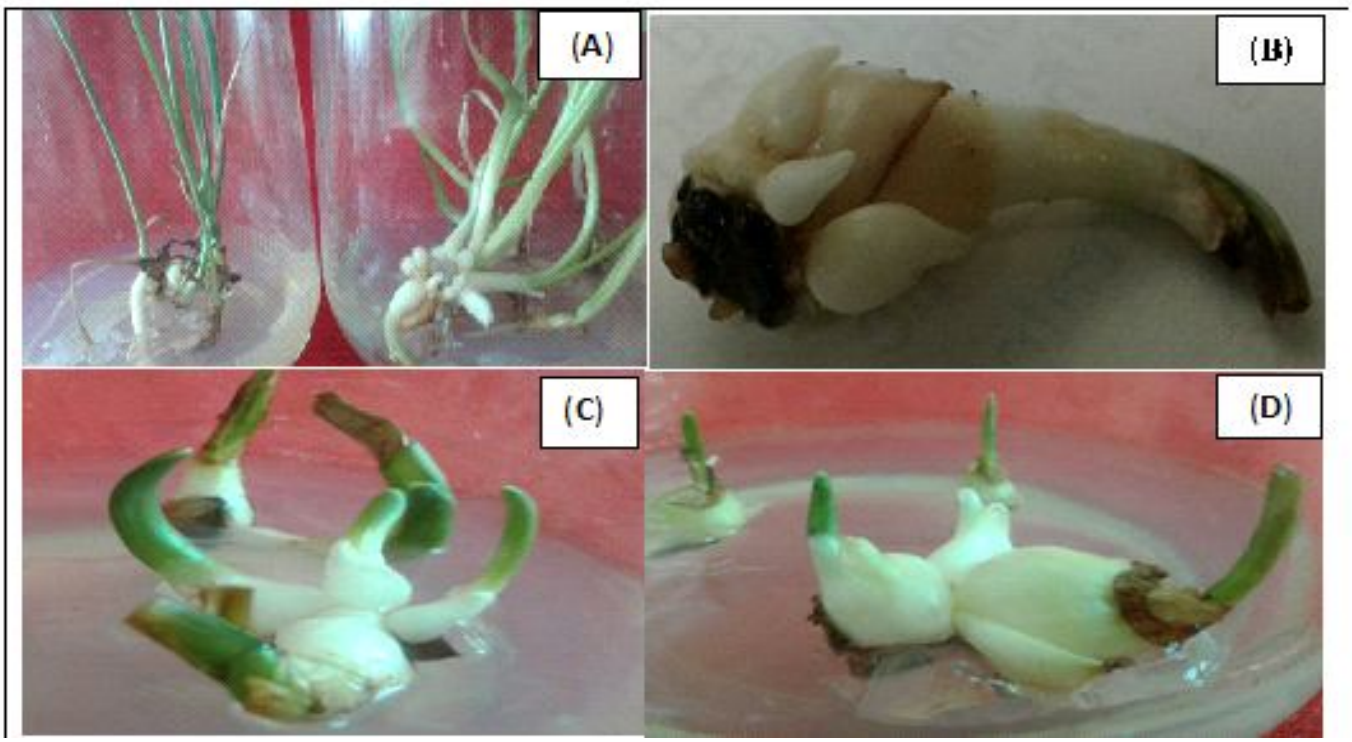

Fig. 2: *In vitro* bulblets development of *tingitana* after second subculture under effect of sucrose and paclobutrazol at various concentrations: (A): Control (MS + 30 g/l sucrose); (B): MS + 120 g/l sucrose; (C): Sucrose 30 g/l + Paclobutrazol 3mg/l and (D): Sucrose 90 g/l + paclobutrazol 3 mg/l.

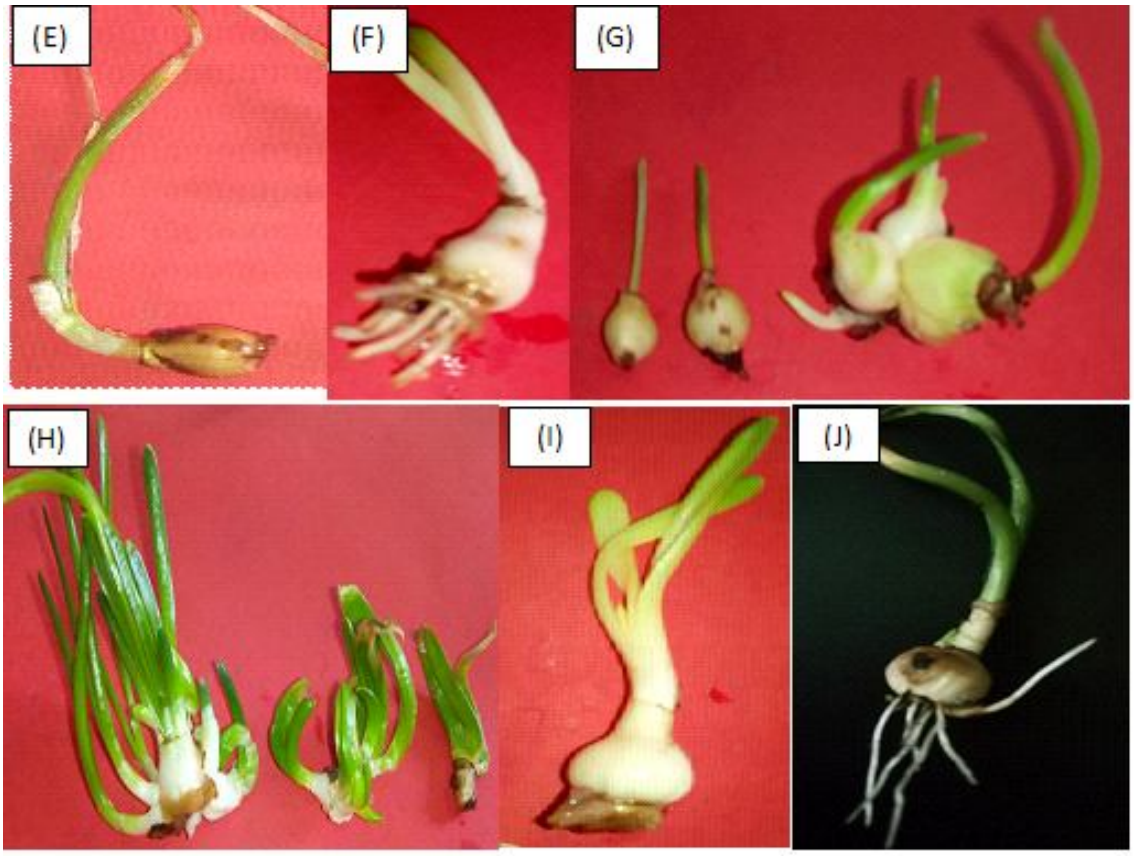

Fig. (3): Acclimatized bulbs of *tingitana* after third subculture under effect of sucrose and paclobutrazol at various concentrations: (E): Control (MS + 30 g/l sucrose); (F): MS + 120 g/l sucrose; (G): Sucrose 30 g/l+ paclobutrazol 3 mg/l, (H): Sucrose 30 g/l+ paclobutrazol 6 mg/l; (I): Sucrose 90 g/l+ paclobutrazol 6 mg/l and (J): Sucrose 120 g/l+ paclobutrazol 6mg/l.

l paclobutrazol and 90 g sucrose plus 6 mg/l paclobutrazol. It seemed that the high concentration of sucrose had a promotion effect on the carbohydrates percent. Confirmed results were found when the chloroplasts of the obtained plantlets on culture medium supplemented with 5% sucrose contained a large deposition of starch as compared to medium containing 3% or 1% sucrose (Capellades *et al.,* 1991). Langens Gerrits *et al*., (2003) found that large bulblets were resulted *in vitro* on medium with a high level of sucrose. The increase in bulblet size was mainly due to an increase in total carbohydrates and starch. Tekalign and Hammes (2005) hypothesize that paclobutrazol may potentially improve accumulation of the carbohydrate in bulbs due to changes in morphogenesis, phytohormonal balance and photosynthetic capacity.

Table 3: Effects of sucrose and paclobutrazol concentration on chemical composition of *Iris tingitana* plantlets.

Treatment	carbohydrate %	phenol (mg/g)	flavonoid (mg/g)	Tannin (mg/g)
30 g/l sucrose	68.59	80.27	12.10	10.39
60 g/l sucrose	71.52	100.92	13.84	11.45
90 g/l sucrose	72.63	125.18	14.4	20.02
120 g/l sucrose	79.83	124.24	14.45	14.95
30 g sucrose +3 mg/l PBZ	72.85	137.73	19.13	18.59
60 g sucrose +3 mg/l PBZ	79.16	171.85	7.03	19.02
90 g sucrose +3 mg/l PBZ	82.55	193.39	7.29	25.21
120 g sucrose +3 mg/l PBZ	85.33	185.03	8.41	18.97
30 g sucrose +6 mg/l PBZ	77.66	84.50	10.77	8.43
60 g sucrose +6 mg/l PBZ	74.66	132.84	14.9	9.19
90 g sucrose +6 mg/l PBZ	81.15	124.48	17.68	18.96
120 g sucrose +6 mg/l PBZ	81.55	122.79	15.16	13.71
LSD at 0.05	3.21	4.70	2.11	1.14

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