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IN VITRO MULTIPLICATION OF *VANGUERIA EDULIS* AS AFFECTED BY CYTOKININS AND MEDIUM TYPE

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ABSTRACT: This work was carried out in the Tissue Culture Laboratory, Horticulture Research Institute, Agricultural Research Center, Giza, Egypt during the period from 2015 to 2017, to investigate the effect of cytokinin type and concentration (BAP, 2-iP and kin, at either 0.0, 1.0, 2.0 and 3.0 ppm) as well as medium type (MS or B5) on micro-shoots multiplication of *in vitro* cultured rare ornamental plant, *Vangueria edulis*.

Results that were significant could be briefed in the following: the first position was obtained by BAP at 1 ppm in regard to survival %, shoot length and shooting %; BAP at 3 ppm for shoot number, leaf number and shoot length; 2-iP at 1 ppm for shooting %; 2-iP at 2 ppm for shoot length and shooting %; and kinetin at 2 ppm for survival %.

Using MS medium gave rise to higher values concerning survival %, shoot number, leaf number, shoot length and shooting % compared to using B5 medium.

The highest position was occupied by the combinations between BAP 1 ppm + MS for survival %, shoot number, shoot length and, shooting %; BAP 2 ppm + MS for shoot number; BAP 3 ppm+B5 for shoot number and leaf number; 2-iP 1 ppm + MS for shooting %; 2-iP 2 ppm + MS for shoot number; kinetin at 1 or 3 ppm + MS for shoot length and kinetin 2 ppm + MS for survival % and shoot length.

It is recommended to treat *in vitro* produced micro-shoots of *Vangueria edulis* with BAP at 1 ppm + MS medium to obtain the highest values during multiplication stage.

Key words: *Vangueria edulis*, multiplication, MS, B5, BAP, kin, 2-iP.

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INTRODUCTION

Vangueria edulis Lam. (syn. *V. madagascariensis* J.F.Gmel.) belongs to fam. Rubiaceae, a tropical evergreen shrub or small tree native to Madagascar and continental Africa. Bears 5 cm fruits with a greenish skin and a flavor that is likened to a tart apple when unripe, to tamarind-like when ripe. Makes an attractive and rare fruiting specimen for the garden. It grows in sandy clay loams. Seed germination is difficult owing to the hard seed coat. (InterNet Site 1-3, 2018).

Cytokinins effect is the most noticeable in tissue cultures where they are used to stimulate cell division and control morphogenesis. When added to shoot culture media, they overcome apical dominance and release lateral buds (George, 1993 and George *et al.*, 2008). Kumar and Loh (2012) stated that one of the major components that have a significant effect on regeneration is the type and concentration of phytohormones in the medium. Optimization of the appropriate phytohormone concentrations in the medium can also be empirically determined in the earlier set of exploratory experiments. Kanwar *et al*. (2013) stated that cytokinins promote shoot proliferation by inducing cell division and enlargement. Lee-Espinosa *et al.* (2008) declared that

cytokinin concentration had a significant effect on the number of shoots regenerated from *Vanilla planifolia* 'Andrews'. Jana *et al.* (2013) mentioned that the addition of BAP with 1/2 MS significantly improved the *Acampe papillosa* shoot growth.

George *et al.* (2008) reported that plant tissues and organs are grown *in vitro* on artificial media, which supply the nutrients necessary for growth. The most commonly used medium is the formulation of Murashige and Skoog (MS). This medium was developed for optimal growth of tobacco callus. Comparing MS to the elementary composition of normal, well-growing plants, they stated that the relatively low levels of P, Ca and Mg in MS are evident. The most striking differences are the high levels of Cl and Mo and the low level of Cu. Plant tissue culture media provide not only inorganic nutrients, but usually a carbohydrate (sucrose is most common) to replace the carbon which the plant normally fixes from the atmosphere by photosynthesis. To improve growth, many media also include trace amounts of certain organic compounds, notably vitamins, and plant growth regulators.

Kumar and Loh (2012) stated that various mineral formulations are available to culture plant tissues. The major media include MS medium (Murashige and Skoog, 1962) and Gamborg's B5 medium (Gamborg and Eveleigh, 1968). Generally, the plant tissue culture media are made up of macro- and micronutrients, vitamins, phytohormones, as well as sucrose. Adjuvants such as activated charcoal may be required for some species that show extreme cases of tissue browning on excision and secretion of polyphenolic substances from the damaged cells.

MATERIALS AND METHODS

This work was carried out in the Tissue Culture Laboratory, Zohriya Garden, Horticulture Research Institute, Agricultural Research Center, Giza, Egypt during the period from 2015 to 2017, to investigate the effect of cytokinin type and concentration (BAP, 2-iP and Kin, at either 0.0, 1.0, 2.0 and 3.0 ppm) as well as medium type (MS or B5) on micro-shoots multiplication of *in vitro* cultured rare ornamental plant, *Vangueria edulis*.

Explant source and preparation:

In the establishment stage, a few branches were collected from *Vangueria edulis* plants found in Zohriya Garden on July 20th, 2015. These branches were cut to small segments, washed under current tap water, soaked and stirred in 3% soap solution for 15 min, rinsed under current tap water for 60 min before being rinsed three times under distilled water. Segments were then taken under hood in axenic conditions. Lateral buds were excised and sterilized by soaking in 20% clorox solution for 20 minutes.

Explants were then rinsed three times with a sterilized distilled water, before being inoculated individually in jars each containing 40 ml of autoclaved (at 121°C for 20 minutes under 1.05 kg/cm^2 pressure) full MS medium supplemented with BA at 3.0 ppm and adjusted to pH 5.8. Jars and were kept in the incubation room at 25/20°C (day/night) ±2°C, 70% relative humidity. Two fluorescent tubes/shelf were installed at 30 cm above explants to provide light intensity of 2200-2400 lux at explant level.

Experimental treatments:

One month later survived explants were used to investigate the effect of two media types (the first factor), i.e. MS and B5 media combined with different cytokinins treatments, BAP, 2-iP and kinetin, at different concentrations in addition to a cytokinin-free treatment as a control (the second factor), in a factorial experiment in a completely randomized block design. These treatments could be arranged as follows:

1. MS medium without cytokinins.
2. MS medium + BAP at 1 ppm.
3. MS medium + BAP at 2 ppm.

4. MS medium + BAP at 3 ppm.
5. MS medium + 2-iP at 1 ppm.
6. MS medium + 2-iP at 2 ppm.
7. MS medium + 2-iP at 3 ppm.
8. MS medium + kinetin at 1 ppm.
9. MS medium + kinetin at 2 ppm.
10. MS medium + kinetin at 3 ppm.
11. B5 medium without cytokinins.
12. B5 medium + BAP at 1 ppm.
13. B5 medium + BAP at 2 ppm.
14. B5 medium + BAP at 3 ppm.
15. B5 medium + 2-iP at 1 ppm.
16. B5 medium + 2-iP at 2 ppm.
17. B5 medium + 2-iP at 3 ppm.
18. B5 medium + kinetin at 1 ppm.
19. B5 medium + kinetin at 2 ppm.
20. B5 medium + kinetin at 3 ppm.

Data recorded one month later were survival %, shoot number, leaf number, shoot length (cm) and shooting %. To statistically test results of these experiments, analysis of variance was carried out, as described by Snedecor and Cochran (1989). The means were compared by Duncan critical range at a probability level of 5% (Duncan, 1955).

RESULTS AND DISCUSSION

Effect of medium type and cytokinin treatments on:

Survival % (Table, 1):

The effect of medium was significant. Using MS medium gave rise to higher survival % compared to using B5 medium (41.13 and 14.44%, respectively).

Cytokinin treatments significantly affected survival % of *Vangueria* shoots. The highest percentages were detected when BAP at 1 ppm or Kinetin at 2 ppm were used (44.50 and 38.94%, respectively). Treatments that induced the second category records were 2-iP at 1 ppm and kinetin at 1 ppm (33.33% for both treatments).

The interaction significantly affected survival %. The highest values were a result of incorporating MS medium with either BAP at 1 ppm or kinetin at 2 ppm (66.78 and 55.67%, respectively). The second position was occupied with values of using MS medium free of cytokinins or augmented with either 2-iP at 1 ppm or kinetin at 1 ppm (44.44, 50.00 and 44.44%, respectively). The lowest record resulted when B5 medium was supplied with BAP at 3 ppm (5.55%). Shoots inoculated on B5 medium either free of cytokinins or fortified with BAP at 2 ppm did not survive at all.

Shoot number (Table, 2):

Medium type significantly affected this character. Using MS medium gave rise to higher shoot number compared to using B5 one (2.2.53 and 0.32 shoots, respectively).

The effect of cytokinin treatments was significant. The highest shoot number was obtained when BAP at 3 ppm was used, followed in the second rank by result of applying 2-iP at 2 ppm (1.75 and 1.42 shoots, respectively). Results of all other treatments occupied the lowest category.

The combined effect of cytokinin treatments and medium significantly influenced shoot number. The highest number was induced by fortifying B5 medium with BAP at 3 ppm, followed in the same category by results of using MS medium supplied with BAP at 1 or 2 ppm; or using 2-iP at 2 ppm (2.2.67, 1.77 and 1.67 shoots, respectively).

Leaf number (Table, 3):

The effect of medium type was significant. Using MS medium gave rise to greater number of leaves compared to using B5 medium (4.53 and 1.23 leaves, respectively).

Cytokinin treatments significantly affected leaf number. The highest number was detected when BAP at 3 ppm was used, followed in the second position by the

Table 1. Effect of cytokinin treatments and medium type on survival % of *Vangueria* micro-shoots.

Cytokinin treatments	Medium type		Mean
	MS	B5	
Control	44.44 bc	0.00 g	22.22 DE
BAP (1 ppm)	66.78 a	22.22 de	44.50 A
BAP (2 ppm)	33.33 cd	0.00 g	16.67 E
BAP (3 ppm)	27.77 de	5.55 fg	16.66 E
2-iP (1 ppm)	50.00 b	16.66 ef	33.33 BC
2-iP (2 ppm)	33.33 cd	22.22 de	27.77 CD
2-iP (3 ppm)	22.22 de	16.66 ef	19.44 DE
Kinetin (1 ppm)	44.44 bc	22.22 de	33.33 BC
Kinetin (2 ppm)	55.67 ab	22.22 de	38.94 AB
Kinetin (3 ppm)	33.33 cd	16.66 ef	25.00 C-E
Mean	41.13 A\	14.44 B\	

Table 2. Effect of cytokinin treatments and medium type on shoot number of *Vangueria* micro-shoots.

Cytokinin treatments	Medium type		Mean
	MS	B5	
Control	1.33 cd	0.00 e	0.67 C
BAP (1 ppm)	1.67 a-c	0.00 e	0.83 C
BAP (2 ppm)	1.77 ab	0.00 e	0.89 C
BAP (3 ppm)	1.50 b-d	2.00 a	1.75 A
2-iP (1 ppm)	1.55 b-d	0.00 e	0.78 C
2-iP (2 ppm)	1.67 a-c	1.17 d	1.42 B
2-iP (3 ppm)	1.50 b-d	0.00 e	0.75 C
Kinetin (1 ppm)	1.33 cd	0.00 e	0.67 C
Kinetin (2 ppm)	1.44 b-d	0.00 e	0.72 C
Kinetin (3 ppm)	1.50 b-d	0.00 e	0.75 C
Mean	1.53 A\	0.32 B\	

Table 3. Effect of cytokinin treatments and medium type on leaf number of *Vangueria* micro-shoots.

Cytokinin treatments	Medium type		Mean
	MS	B5	
Control	2.67 g	0.00 h	1.33 E
BAP (1 ppm)	3.33 fg	0.00 h	1.67 DE
BAP (2 ppm)	3.67 e-g	0.00 h	1.83 C-E
BAP (3 ppm)	6.72 b	10.00 a	8.36 A
2-iP (1 ppm)	5.53 b-d	0.00 h	2.77 C
2-iP (2 ppm)	6.00 bc	2.33 g	4.17 B
2-iP (3 ppm)	5.00 c-e	0.00 h	2.50 CD
Kinetin (1 ppm)	4.33 df	0.00 h	2.17 C-E
Kinetin (2 ppm)	3.75 e-g	0.00 h	1.88 C-E
Kinetin (3 ppm)	4.33 d-f	0.00 h	2.17 C-E
Mean	4.53 A\	1.23 B\	

number induced when 2-iP at 2 ppm was applied (8.36 and 4.17 leaves, respectively). The lowest value in the same request was a result of using no cytokinins at all (1.33 leaves).

This interaction was significant. The highest number resulted when BAP at 3 ppm was used, followed with significant differences by results of applying BAP at 3 ppm, 2-iP at 1 ppm or 2-iP at 2 ppm (6.72, 5.53 and 6.00 leaves, respectively). The lowest value belonged to explants inoculated on cytokinin-free medium (2.67 leaves). Treatments adopting B5 medium and was either cytokinin-free, or supplemented with BAP at 1 or 2 ppm; 2-iP at 1 or3 ppm; or kinetin at 1, 2 or 3 ppm failed to induce leaves at all.

Shoot length (**Table, 4**)**:**

Medium type significantly affected shoot length. MS medium as resulted in longer shoots compared to B5 medium (2.48 and 0.32 cm, respectively).

The effect of cytokinin treatments on shoot length was found to be significant. The highest values were observed when BAP at either 1 or 3 ppm; or 2-iP at 2 ppm was used (1.57, 1.83 and 1.69 cm, respectively). The second position was occupied by kinetin at 1, 2 or 3 ppm (1.40, 1.38 and 1.39 cm, respectively). The lowest record belonged to BAP at 2 ppm (1.5 cm).

This interaction was found to be significant. The longest shoot was induced when MS medium was combined with either BAP at 1 ppm or kinetin at 1, 2 or 3 ppm (3.13, 2.80, 2.77 and 2.78 cm, respectively). Shoots of the second rank were a result of using either cytokinin-free MS medium, or the same medium fortified with 2-iP at 1, 2 or 3 ppm (2.35, 2.58, 2.38 and 2.42 cm, respectively). The shortest shoots were obtained when B5 medium was supplied with 2-iP at 2 ppm was used (2.38 cm). Treatments using either cytokinin-free B5 medium, or B5 medium supplemented with BAP at 1 or 2 ppm; 2-iP at 1 or3 ppm; or kinetin at 1, 2 or 3 ppm failed to induce shoots at all.

Shooting % (**Table, 5**)**:**

MS medium significantly produced higher shooting % than did B5 one (31.65 and 3.07%, respectively).

Cytokinin treatments exerted a significant influence on shooting % of *Vangueria* explants. The highest percentages were produced when BAP at 1 ppm; or 2-iP at either 1 or 2 ppm were used (27.83, 25.00 and 22.17%, respectively). The second position was occupied by kinetin at 2 ppm (19.44%). The lowest records in the same regard were obtained when either BAP at 2 ppm or 2-iP at 3 ppm were applied (9.67 and 9.72%, respectively).

This interaction significantly affected shooting %. MS medium supplied with either 1 ppm of BAP or 2-iP gave rise to the highest percentages (55.67 and 50.00%, respectively). MS fortified with kinetin at 2 ppm induced the second rank in this concern (38.89%). The lowest values were observed when B5 medium was supplied with either BAP at 3 ppm or 2-iP at 2 ppm (16.77 and 13.89%, respectively). Treatments using either cytokinin-free B5 medium, or B5 medium supplemented with BAP at 1 or 2 ppm; 2-iP at 1 or3 ppm; or kinetin at 1, 2 or 3 ppm failed to induce shoots at all.

DISCUSSION

Concerning the effect of medium type the best results were obtained by using MS medium when compared with B5 one. In this regard, two important factors may induce the medium effect, the total concentration of nitrogen in the medium and the ratio of nitrate to ammomium ions. There is a high proportion of NH_4^+ nitrogen in MS medium (ratio of NO_3^- to NH_4^+, 66:34) and the quantity of total nitrogen is much higher than that in the majority of other media (George *et al.*, 2008). The high total N and NO_3^- /NH_4^+ ratio in MS basal salts may have the

Table 4. Effect of cytokinin treatments and medium type on shoot length (cm) of *Vangueria* micro-shoots.

Cytokinin treatments	Medium type		Mean
	MS	B5	
Control	2.35 b-d	0.00 g	1.18 DE
BAP (1 ppm)	3.13 a	0.00 g	1.57 A-C
BAP (2 ppm)	2.10 d	0.00 g	1.05 E
BAP (3 ppm)	1.50 e	2.17 cd	1.83 A
2-iP (1 ppm)	2.58 bc	0.00 g	1.29 C-E
2-iP (2 ppm)	2.38 b-d	1.00 f	1.69 AB
2-iP (3 ppm)	2.42 b-d	0.00 g	1.21 DE
Kinetin (1 ppm)	2.80 ab	0.00 g	1.40 B-D
Kinetin (2 ppm)	2.77 ab	0.00 g	1.38 B-D
Kinetin (3 ppm)	2.78 ab	0.00 g	1.39 B-D
Mean	2.48 A\	0.32 B\	

Table 5. Effect of cytokinin treatments and medium type on shooting % of *Vangueria* micro-shoots.

Cytokinin treatments	Medium type		Mean
	MS	B5	
Control	30.44 cd	0.00 f	15.22 C-E
BAP (1 ppm)	55.67 a	0.00 f	27.83 A
BAP (2 ppm)	19.33 de	0.00 f	9.67 E
BAP (3 ppm)	16.77 e	16.77 e	16.77 C-E
2-iP (1 ppm)	50.00 ab	0.00 f	25.00 AB
2-iP (2 ppm)	30.44 cd	13.89 e	22.17 A-C
2-iP (3 ppm)	19.44 de	0.00 f	9.72 E
Kinetin (1 ppm)	30.55 cd	0.00 f	15.28 C-E
Kinetin (2 ppm)	38.89 bc	0.00 f	19.44 B-D
Kinetin (3 ppm)	25.00 de	0.00 f	12.50 DE
Mean	31.65 A\	3.07 B\	

positive effect on *in vitro* growth of *Vangueria edulis* during multiplication stage.

Concerning the effect of cytokinin type, in the micropropagation of numerous plants, benzylaminopurine was found to be more effective than kinetin, N6-(2-isopentenyl) adenine, and zeatin (Evaldsson and Welander, 1985). Papafotiou *et al.* (2010) mentioned that media supplemented with only BAP (0.5-1.0 ppm) favoured the development of shoots on *Bauhinia variegata* explants. Paudel and Pant (2012) remarked that increasing BAP concentration from 0.5 to 2.0 mg/l induced the maximum number of shoots in *Esmeralda clarkei*. Sheelavantmath *et al.* (2000) revealed that BAP at 1.13 ppm induced multiple shoots of *Geodorum densiflorum* within 4 weeks of culture. Neelannavar *et al.* (2011) remarked that BAP at 1.0 mg/l produced more number of better sized shoots of *Vanilla planifolia*. Nongdam and Chongtham (2011) noticed that shooting of *Cymbidium aloifolium* was best observed in MS medium supplemented with 1 mg/l BAP. Pant and Shrestha (2011) found that maximum number of healthy *Phaius tancarvilleae* shoots was observed on MS with BAP (1.0 mg/l). Gati *et al.* (1991) planted explants of China grass (*Boehmeria nivea*) on MS medium + BA, kinetin or 2-iP, at 0.1-0.7 mg l. They showed that BA at 0.5 mg/l produced the best growth and the largest number of shoots, but the shoots were shorter than that produced in BA at 0.1 mg/l.

Chuenboonngarm *et al.* (2001) successfully propagated shoot tips of *Gardenia jasminoides* in B5 agar medium with 0-10 ppm BAP and 2-iP. The number of shoots in medium with 10 ppm BA was 7 times greater than those in medium without BAP, while the number of shoots in medium with 7.5 ppm 2-iP was 4 times greater than in 2-iP free medium. Mansseri-Lamrioui *et al.* (2011) studied the effect of BAP, 2iP and Kin at 1-8 mg/l on wild cherry (*Prunus avium*). They found that the use of BAP at 2-4 mg/l resulted in the highest percentage of sprouting, number of shoots and ratios of multiplication, followed by 2-iP and then kin.

This increase in multiplication as a result to using BA could be ascribed to the stimulatory effect of BA on cell division and enlargement. BA enabled germinating seeds and many excised tissues to regenerate on synthetic media by promoting cell division and enlargement (Letham *et al.*, 1978 and Dowiadar *et al.*, 1996).

According to this study results, it could be observed that in most cases BAP was better than other cytokinins for *in vitro* multiplication of *Vangueria edulis*. Variation in the activity of different cytokinins can be explained by differences in the uptake rates reported in different genomes (Blakesly, 1991), varied translocation rates to meristematic regions, and metabolic processes in which cytokinin may be degraded or conjugated with sugars or amino acids to form biologically inert compounds as reported by Kaminek (1992).

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الإكثار المعملى للفانجويريا إديولس والمتأثرة بالسيتوكينين ونوع البيئة

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أجريت هذه الدراسة فى معمل زراعة الأنسجة، حديقة الزهرية، معهد بحوث البساتين، مركز البحوث الزراعية، الجيزة، مصر فى الفترة من ٢٠١٥ إلى ٢٠١٧، لدراسة تأثير نوع السيتوكينين وتركيزه (بنزايل آدينين، أيزوبنتينايل أدينين و كينيتين، بتركيزات ٠ ، ١,٠ ، ٢,٠ و ٣,٠ جزء في المليون) وكذلك نوع بيئة الزراعة (موراشيج وسكوج و جامبورج B5) على الزيادة العددية للأفرع الدقيقة الناتجة معملياً لنبات الزينة النادر الفانجويريا.

يمكن تلخيص أهم النتائج المعنوية فيما يلي: جاء فى المركز الأول معاملة ١ جزء في المليون بنزايل أدينين (من حيث صفات النسبة المئوية للبقاء وطول الفرع والنسبة المئوية لتكوين الأفرع)، ومعاملة ٣ جزء في المليون بنزايل أدينين (لصفات عدد الأفرع وعدد الأوراق وطول الفرع)، ومعاملة ١ جزء في المليون أيزوبنتينايل أدينين (النسبة المئوية لتكوين الأفرع)، ومعاملة ٢ جزء في المليون أيزوبنتينايل أدينين (طول الفرع والنسبة المئوية لتكوين الأفرع)، ومعاملة ٢ جزء في المليون كينتين (النسبة المئوية للبقاء).

تسبب إستعمال بيئة موراشيج وسكوج فى الحصول على قيم أعلى لصفات النسبة المئوية للبقاء، عدد الأفرع، عدد الأوراق، طول الفرع، النسبة المئوية لتكوين الأفرع، مقارنة ببيئة جامبورج B5.

شغلت التوليفات التالية المركز الأعلى: (١ جزء في المليون بنزايل أدينين + بيئة موراشيج وسكوج) لصفات النسبة المئوية للبقاء وعدد الأفرع وطول الفرع والنسبة المئوية لتكوين الأفرع، (٢ جزء في المليون بنزايل أدينين + بيئة موراشيج وسكوج) لصفة عدد الأفرع، (٣ جزء في المليون بنزايل أدينين + جامبورج) لصفتى عدد الأفرع وعدد الأوراق، (١ جزء في المليون أيزوبنتينايل أدينين + بيئة موراشيج وسكوج) للنسبة المئوية لتكوين الأفرع، (٢ جزء في المليون أيزوبنتينايل أدينين + بيئة موراشيج وسكوج) لعدد الأفرع، (١ أو ٣ جزء في المليون كينتين + بيئة موراشيج وسكوج) لطول الفرع، (٢ جزء في المليون كينتين + بيئة موراشيج وسكوج) لصفتى النسبة المئوية للبقاء وطول الفرع.

يُنصح بمعاملة الافرع الدقيقة الناتجة معملياً لنبات الفانجريا النادر بـ ١ جزء في المليون بنزايل أدينين + بيئة موراشيج وسكوج خلال مرحلة الزيادة العددية للحصول على أفضل النتائج.