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In Vitro Propagation and Enhancement of Quercetins and Isorhamnetin Production in Wild *Paronychia argentea* L.

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ABSTRACT

Paronychia argentea is a herbal plant that grows wildly in Jordan and frequently used in folk medicine for treatment of many ailments, which exposed this plant to over collection and decline. Till now, there are few scientific researches on *Paronychia argentea* in terms of *in vitro* propagation and medicinal properties. This study aimed to experiment the possibility of propagating this plant via tissue culture approach, and to asses accumulation of quercetins, and isorhamnetin in the resulted microshoots. To achieve this aim, nodal segment from pre-established microshoots were transferred into different types of shoot multiplication media for shoot proliferation, while the resulted microshoots were inoculated into rooting media to test their rooting abilities. Meanwhile, amounts of querceins and isorhamnetin as affected by shooting media type were determined in the *in vitro* grown microshoots. Successful shoot proliferation was obtained in most treatments, while maximum number of microshoots (3.5 shoots/ explant) was recorded in cultures grown in Murashige and Skoog (MS) medium plus 1.5 mg/L TDZ. Meanwhile, best rooting (5.9 roots/ explant) was recorded in cultures grown in MS medium plus 0.5 mg/L IBA. Moreover, maximum amounts of quercetins and isorhamnetin (1.867 and 1.376 ppm) were extracted from microshoots pregrown on MS media plus 0.5 mg/L TDZ. Generally, our results indicated that 0.5 mg/L TDZ was the best growth regulator to be added to the growth media of *Paronychia argentea* L. as it yielded a reasonable number of microshoots containing maximum amounts of quercetins and isorhamnetin as well. Our results can open the gate for more research on other *in vitro* approaches that can be applied to enhance production of such valuable compounds in this plant.

Keywords: Isorhamnetin, *Paronychia argentea*, Quercetins, Tissue culture.

INTRODUCTION

Paronychia argentea L. is a wild herb that belongs to Caryophyllaceae family, and is commonly named as (Rejelel- Al-Hamama) or (Shoishet el-raei) (Abuhamdah, et al., 2013). This herbal plant grows wild in waste places, uncultivated fields margins and field boarders (Braca, et al., 2008) of many countries such as, Jordan, Palestine, Syria, Egypt (Gad, et al., 2012), Libya (Mukassabi, et al., 2012), Spain, Algeria, (Gonz´alez-Tejero, et al., 2008) and Portugal (Ferreira, et al., 2006). *P. argentea* is widely used in folk medicine for the treatment of several disorders such as kidney stones, urinary tract infections, diseases of gastro-intestinal disorders in addition to cold and fever (Noubani, et al., 2006). Also, it was reported to possess an antimicrobial effect against gram positive bacteria (Abuhamdah, et al., 2013).

Meanwhile, due to the great medicinal value of *P. argentea*, this herb is currently exposed to over collection and decline. Setting a protocol for massive production of (*P. argentea)* plants is a great need to insure a sustainable

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supply of *P. argentea* to be utilized later on as a resource for more progressive medicinal research on this plant. Al Qudah et al. (2014) reported that microshoots had developed successfully from *P. argentea* buds grown *in vitro*, but no previous studies had reported a full micro-propagation protocol of this plant.

Flavonoids play major roles in plants such as plant coloring and protection from pathogen and light stress (Crozier, et al., 2006; Pereira, et al., 2009). The best known types of flavonoids are quercetin and isorhamnetin. In spite of the huge research data about these two flavonoids extracted from many medicinal plants in terms of their curative abilities, studies on the these flavonoids produced from *P. argentea* grown i*n vivo* are extremely rare.

However, successful enhancement of production of many types of secondary metabolites including flavonoids has been reported in many medicinal herbs grown under *in vitro* conditions (Malik, et al., 2013). Therefore, this study was conducted to introduce *P. argentea* to tissue culture systems for micropropagation purpose (*in vitro* shooting and rooting) and to investigate accumulation of quercetin, and isorhamnetin in the resulted microshoots as affected by cytokinin type (growth regulator) level in the growth media.

2. MATERIALS AND METHODS

2.1 Micropropagation

2.1.1 Shoot Multiplication:

For shoot multiplication, nodal segment were excised form microshoots of *P. argentea* established previously by (Al Qudah, et al. 2014), and subcultured into Erlenmeyer flasks containing growth media consisted of (4.4 g\ L) of MS (Murashige and Skoog, 1962) premix salts plus 30 g\L sucrose in addition to different sources of cytokinins (BAP (6-Benzylaminopurine), Kinetin (6-Furfurylaminopurine), 2ip (6-(gamma,gamma-Dimethylallylamino) purine (2iP) or TDZ (thididiazuron)) added at different rates (0.0, 0.1, 0.5, 1.0, 1.5 or 2.0 mg/L), (Duhoky, 2009) in combination with 0.1 mg/L NAA **(**alpha-Naphthaleneacetic acid Free acid). The cultures were grown under growth room conditions of growth room with daily regime of 16- hr light, 8- hrs dark and 24 ± 1°C. Data was collected after five weeks for the number of proliferated shoots and shoot height.

2.1.2 Rooting:

Rooting was experimented by subculturing one microshoot from the plant material resulted from section 2.1.1 into a test tube containing rooting media of MS salts (4.4 g/L) + 30 g/L sucrose and rooting hormones (IBA, NAA, or IAA) at different concentrations (0.0, 0.1, 0.5, 1.0, 1.5, or 2.0 mg /L) (Marković, 2013; Al Qudah et al., 2011, Al Hawmdeh et al., 2014). After subculturing to the rooting media, cultures were incubated at 24 ± 1°C under growth room conditions.

2.2 Determination of quercetin, and isorhamnetin content:

2.2.1 Sample preparation and extraction

To evaluate the effect of media type on quercetin and isorhamnetin content in the microshoots, samples from microshoots pregrown in each media types described in section 2.1.1 were oven dried at 50 Cº for 2 days, then grounded using mortar and pistil. Next, 2.0 g were taken from the dried matter of each treatment and soaked in 20 ml of pure ethanol with 5% of glacial acetic acid. The samples were then placed on a shaker at a slow speed (140 rpm) for at least 72 hours at room temperature (Hussain, et al., 2012). The plant extract was purified using nano filtration, and centrifuged at 10,000 rpm for 10 min, and the resulting supernatant was pooled, filtered, and dried in a rotary evaporator at 70 Cº. Next, the dried extract was resuspended in the mobile phase for HPLC analysis.

To compare between *in vitro* grown microshoots and wild type (*P. argentea)* in terms of quercetins, and isorhamnetin content, samples from wild type plants of (*P. argentea)* were collected from Jerash (32°16′20″N 35°53′29″E), north Jordan in April, 2014 then dried, extracted and purified as described before.

2.2.2 Preparation of quercetins and isorhamnetin

stock solutions and working standards.

Quercetins stock solution at concentration of 600 ppm was prepared by weighing 3.0 mg of quercetins in 5 ml volumetric flask, then dissolved and completed up to volume by methanol HPLC grade. The prepared stock solution was stored at 4ºC in dark. Working solutions were prepared by serially diluting stock solutions using the mobile phase at concentrations of 25, 12.5, 6.25, 3.125, 1.5, 0 .78, 0.39, 0.19, 0.09, 0.049 ppm. Isorhamnetin stock solution at concentration of 1000 ppm was prepared by weighing 5 mg of isorhamnetin in 5 ml volumetric flask, then dissolved and completed up to volume by methanol HPLC grade. The prepared stock solution was stored at 4ºC in dark. Working solutions were prepared by serially diluting stock solutions using the mobile phase at concentrations of 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.78, 0.039 ppm. Calibration curves were constructed for quercetins and isorhamnetin before starting chemical analysis as shown in Fig. 1 and 2.

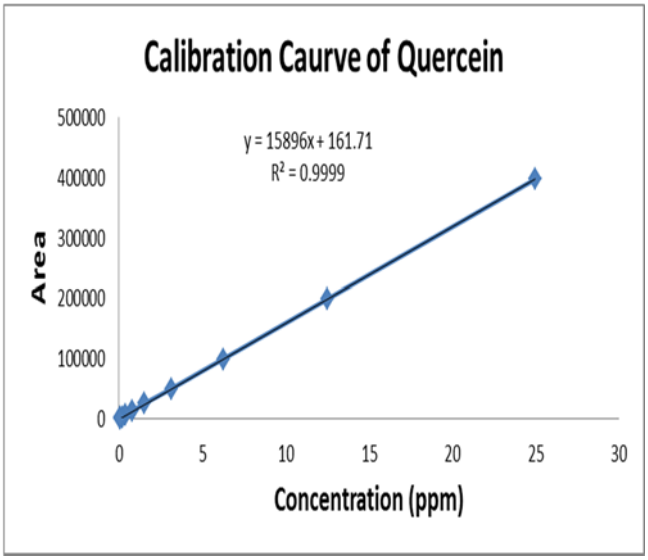

Figure 1: Quercetins standard calibration curve

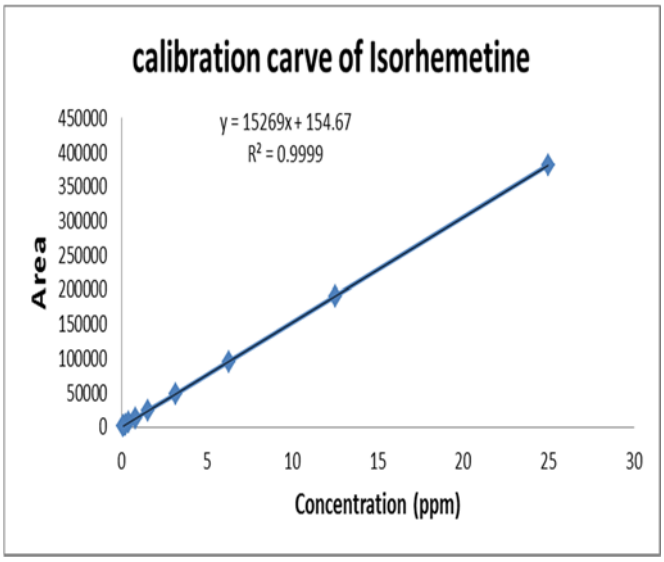

Figure 2: Isorhamnetin standard calibration curve.

2.2.3 Analysis of (quercetins and isorhamnetin) using (Reversed-phase chromatography)

The samples were eluted through reversed phase C18 column using an isocratic mobile phase comprising 40% from (0.5%) phosphoric acid: 60% acetonitrile+ 5.0 ml of pH 2.4, at room temperature with a sustainable flow rate. The samples were injected and were carried out at 210 nm as described by Hussain et al., (2012). The peaks appear at 3.3 min. for quercetins and 4.1 min for isorhamnetin.

2.3 Statistical Analysis

Treatments in each experiment were arranged in a completely randomized design (CRD). In shoot multiplication experiments, each treatment was replicated five times with two shoots/ replicate; while in rooting experiments each treatment was replicated 20 times. For quercetins isorhamnetin determination, three replicates with 2 samples per replicate (sample dry weight= 3.0 g) were taken from each treatment. The collected data were statistically analyzed using SPSS analysis system. Analysis of variance (ANOVA) was used, and means were

separated at probability level of 0.05 according to the Tukey's HSD.

3. RESULTS

3.1 *In Vitro* Shoot Multiplication:

Shoot multiplication results varied with type and level of cytokinin added to the media. For example, the nodal segments responded positively to addition of BAP to the culture media compared to those grown in the control media (hormone free MS media) (Table 1, Figure 3 B). The maximum number of new microshoots (3.2 microshoots/ explants) was obtained in nodal segments grown in MS media plus 1.0 mg/L BAP (Table 1, Figure 3 B). On the other hand, maximum shoot height (3.65 cm) was obtained in the control treatment compared with other treatments which imply that BAP has a negative impact on shoot height (Table 1).

Meanwhile, shoot multiplication results was poor in Kinetin treated explants, where maximum value of shoots (1.7/ microshoot) was recorded Kinetin level of (0.1 mg/L) (Table 1). On the other hand, a remarkable increase in with shoot height data was recorded in response to kinetin at all levels compared to the control treatment, which proves the negative relationship between shoot number and height.

Moreover, TDZ induced shoot multiplication in *Paronychia argentea* L. microshoots (Table 1). The highest number of new shoots (3.5) was obtained at TDZ level of 1.5 mg/L, while maximum value (3.65 cm) for shoot height was recorded in the control treatment, as shoot height tended to decrease significantly due to exposure to TDZ (Table 1). Moreover, adding 2iP to the media had resulted in a significant increase in shoot number (2.8) at level of 1.5 mg/L compared to the other 2iP levels and the control (Table 1). On the other hand, 2ip had a negative impact on shoot height at 2ip levels higher than 0.1 mg/L, as shoot height tended to decrease with increasing 2ip level in the media (Table 1).

Table 1. Effect of different cytokinin types and concentrations on number of shoots and shoot height in the *in vitro* grown *Paronychia argentea* L. explants.

Cytokinin type		
BAP		
Concentration (mg/L)	**Number of new shoots/explants**	**Shoot height of new shoots(cm)**
Control	1.50 * e ± 0.09	3.650 a ± 0.03
0.1	2.30 d ± 0.06	2.581 b ± 0.01
0.5	2.50 c ± 0.02	2.151 c ± 0.01
1.0	3.20 a ± 0.01	1.790 d ±0.008
1.5	2.80 b ±0.01	1.600 e ±0.007
2.0	2.40 c ± 0.03	1.290 f ± 0.007
Kinetin		
Control	1.50 b ± 0.096	3.650 e ± 0.032
0.1	1.70 a ± 0.010	6.750 a ± 0.038
0.5	1.40 c ± 0.006	6.500 b ± 0.005
1.0	1.40 c ±0.010	6.302 c ± 0.033
1.5	1.30 d ± 0.008	6.210 c ±0.027
2.0	1.20 e ±0.036	5.240 d ± 0.063
TDZ		
Control	1.50 e ± 0.096	3.650 a ± 0.032
0.1	2.50 d ± 0.085	b ± 0.011 1.902
0.5	3.00 b ± 0.011	1.900 b ± 0.010
1.0	3.00 b ± 0.030	1.582 c ± 0.079
1.5	3.50 a ± 0.025	1.321 d ± 0.056
2.0	2.80 c ± 0.019	0.960 e ± 0.056
2iP		
Control	1.50 c ± 0.096	3.650 e ± 0.032
0.1	1.50 c ± 0.010	6.510 a ± 0.042
0.5	1.90 b ± 0.056	5.271 b ± 0.019
1.0	1.90 b ± 0.450	4.190 d ± 0.520
1.5	2.80 a ± 0.019	4.671c ± 0.01
2.0	1.90 b ± 0.056	3.190 f ± 0.022

*Values represent means ± standard error. [x] Control : represents free hormone MS media + 0.1 M sucrose. Means with different letters are significantly different according to Tukey HSD range test at P≤ 0.05.

Figure 3: A: *Paronychia argentea* L., growing wildly in Jerash north Jordan (32°16′20″N 35°53′29″E), (April, 2014). B: *In vitro* grown microshoots of *Paronychia argentea* L.

3.2 *In Vitro* Rooting

Microshoots were rooted successfully on MS media supplemented with different concentrations of auxins (Table 2). In IAA experiment, full rooting rates (100%) were recorded in the control and IAA treatments (Table 1). Moreover, IAA had resulted in increasing number of new roots to reach a maximum value of (3.2 roots/explant) at 1.0 mg/L IAA (Table 2).

In IBA experiment, it was observed that production of new roots was significantly enhanced by adding IBA to the media to reach a maximum value of (5.9 roots/explant) at (0.5 mg/L) IBA compared to only (1.2) new roots developed in the control (Table 2), while length of the newly developed roots was negatively affected by adding IBA at elevated levels to record a minimum value of (2.57 cm) at 2.0 mg/L IBA.

Meanwhile, full rooting was obtained in all NAA treatments, while maximum number of new roots (4.8 roots/explant) was obtained in explants treated with 1.5 mg/L NAA (Table 2). However, root length tended to decrease as NAA was added to the media at all levels compared to the control (Table 2)

Table 2. Effect of different auxin types and concentrations on rooting percentage, number of roots and root length in *in vitro* grown microshoots of *Paronychia argentea* L.

Auxin type			
IAA			
Concentration (mg/L)	**Rooting%**	**Number of new roots/explants**	**Root length (cm)**
Control	100	1.20* e ±0.011	4.820 a ± 0.015
0.1	100	1.80 c ±0.009	1.590 b ±0.005
0.5	100	2.50 b ±0.010	1.401 c ± 0.042
1.0	100	3.20 a ±0.020	1.470 c ±0.011
1.5	85	2.60 b ±0.024	1.280 d ± 0.007
2.0	70	1.30 d ±0.005	1.270 d ±0.010
	IBA		
Control	100	1.20 e ± 0.011	4.820 a ±0.015
0.1	100	4.90 c ± 0.014	3.570 b ± 0.019
0.5	85	5.90a ± 0.006	2.901 c ± 0.006
1.0	60	5.90 a ± 0.016	2.870 c ± 0.014
1.5	60	5.70 b ± 0.024	2.772 d ± 0.01
2.0	50	4.30 d ± 0.01	2.570 e ±0.016
	NAA		
Control	100	1.20 e ± 0.410	4.820 a ±2.440
0.1	100	1.70 c ± 0.921	2.400 b ± 0.520
0.5	100	1.70 c ±0.760	2.161 c ± 0.305
1.0	100	2.60 b ± 0.881	2. 067 c ± 0.330
1.5	100	4.80 a ± 1.842	1.501 d ±0.304
2.0	100	1.30 d ± 0.511	1.330 e ± 0.166

*Values represent means ± standard error. [x]Control : represents free hormone MS media + 0.1 M sucrose. Means with different letters are significantly different according to Tukey HSD range test at P≤ 0.05.

3.3 Quercetins and Isorhamnetin Content

Maximum values for quercetins and isorhamnetin were obtained from the wild type plant samples compared to

amounts extracted from tissue cultured samples (Table 3). Meanwhile, microshoots content of quercetins and isorhamnetin varied greatly with Cytokinin type and level in the media. For example, BAP at level of 0.10 mg/l was able to increase quercetins amount significantly in the *in vitro* grown microshoots (1.838 ppm) compared to the control (hormone free media) results (0.196 ppm), while quercetins concentrations decreased sharply at BAP levels higher than (0.5 mg/l) (Table 3). Meanwhile, isorhamnetin concentration was maximized (2.169 ppm) in explants grown in MS media supplemented with 0.5 mg/L BAP, while it declined significantly at higher BAP levels (Table 3).

Results of Kinetin experiment revealed that, quercetins and isorhamnetin contents extracted from the wild type plant were the highest in all treatments (Table 3). Meanwhile, adding 0.1 mg/L Kinetin to the culture media had improved quercetins and isorhamnetin concentrations compared to the control treatment (1.838, 0.998 ppm) compared to the amounts of both compounds extracted from the hormone free control, while both compounds tended to decrease significantly at higher Kinetin levels (Table 3).

On the other hand, microshoots pregrown in MS media plus (0.5 mg/L) TDZ had the highest quercetins and isorhamnetin content (1.867, 1.3762 ppm) compared to the control and other TDZ treatments (Table 3), while both flavonoids declined dramatically at higher TDZ levels (Tables 3).

Meanwhile, 2iP had a negative effect on quercetins concentration; as it tended to decrease significantly at all 2iP levels compared to the control (Table 3). On the other hand, isorhamnetin was maximized (1.362 ppm) in extracts of microshoots pregrown in 2iP concentration of (0.5 mg/L) which was much higher than those extracted from other *in vitro* grown microshoots samples, but still less than amounts extracted from the wild type plant (Table 3).

Table 3. Effect of different cytokinin types and concentrations on quercetins and isorhamnetin content (ppm) in the wild type plants and *in vitro* grown microshoots of *Paronychia argentea* L.

Cytokinin Concentration (mg/L)	Quercetins (ppm)	Isorhamnetin (ppm)
BAP		
Wild type [y]	1.9235 a ± 0.004	2.368 a ± 0.007
Control[x]	0.196 d ± 0.009	0.074 e ± 0.0009
0.1	1.838 b ± 0.010	1.184 c ± 0.0004
0.5	0.587 c± 0.0034	2.169 b ± 0.0004
1.0	0.076 e ± 0.0004	0.094 d ± 0.0006
1.5	0.00 f ± 0.0000	0.063 f ± 0.001
2.0	0.002 f ± 0.0004	0.062 f ± 0.0046
Kinetin		
Wild type [y]	1.9235 a ± 0.004	2.368 a ± 0.007
Control[x]	0.196 e ± 0.009	0.074 e ± 0.001
0.1	0.389 b ± 0.002	0.998 b ± 0.005
0.5	0.335 c ± 0.004	0.849 c ± 0.002
1.0	0.3130 c ± 0.003	0.482 d ± 0.005
1.5	0.2305d ± 0.003	0.498 d ± 0.005
2.0	0.2270 d ± 0.004	0.044 f ± 0.001
TDZ		
Wild type [y]	1.935 a ± 0. 0041	2.368 a ± 0.007
Control[x]	0.196 d ± 0.009	0.074 f ± 0.0009
0.1	0. 376 c ± 0.0003	0 .543 c ± 0. 0011
0.5	1.867 b ± 0.015	1.3762 b ± 0.012
1.0	0. 025 e ± 0. 0001	0. 275 d ± 0. 0025
1.5	0.023 e ± 0.0004	0.267 d ± 0. 002
2.0	0. 0037 f ± 0.0003	0. 198 e ± 0. 0003
2iP		
Wild type [y]	1.935 a ± 0.004	2.368 a ± 0.08
Control[x]	0.196 b ± 0. 009	0.071 f ± 0.003
0.1	0.172 c ± 0.002	0.360 d ± 0.12
0.5	0.098 d ± 0.001	1.362 b ± 0.003
1.0	0.091 d ± 0.001	0.487 c ±0.03
1.5	0.007 e ± 0.035	0.086 e ± 0.003
2.0	0.005 e ± 0.015	0.025 g ± 0.006

*Values represent means ± standard error. [x]Control : represents free hormone MS media + 0.1 M sucrose. [y] wild plant: collected from Jearsh (32°16′20″N 35°53′29″E),

north of Jordan in April, 2014). Means with different letters are significantly different according to Tukey HSD range test at P≤ 0.05.

4. DISCUSSION

4.1 Shoot Multiplication

Cytokinins were able to enhance shoot multiplication in all treatments, particularly in explants treated with TDZ; where maximum shooting was obtained at level of 1.5 mg/L (Table 1). The main aspect of using cytokinins in growth media is for micropropagation reasons to stimulate formation of the adventitious shoot. Meanwhile, enhancement of shooting is governed by the specific Cytokinin type and level added to the culture media in addition to plant species and explant type (Al-Mahmood, et al., 2012).

According to our results, BAP had a significant positive impact on shoot multiplication (Table1), which agrees with many other related studies for micropropagation of different medicinal plant species in Jordan such as, *Zizifora tinnure*, *Thymbra spicata* and *Silybum marianum* (Al- Baba, et al., 2016; Tahtamouni, et al., 2016; Al-Hawamdeh, et al., 2014). However, our data showed that increasing BAP had affected adversely shoot height (Table1) which was in agreement with Chandra et al. (2013) study on caper plant, as they mentioned that high concentrations of BAP (1.35 and 1.8 mg/l) had reduced shoot height.

In Kinetin experiment, it was noticed that Kin had slightly improved shoot multiplication compared to the control (Table 1), while shoot height had increased significantly to record (6.75) at 1.5 mg/L Kin (Table 1). However kinetin was reported to enhance growth by increasing shoot length rather than expansion, which was proven by (Naeem, 2004) who reported that that kinetin promotes shoot elongation by blocking shoot extension.

Meanwhile, TDZ was generally very efficient in enhancing microshoot propagation; as maximum shooting was obtained at level of 1.5 mg/L (Table 1). TDZ was recommended to stimulate shoot multiplication in many plant species grown under tissue culture conditions. For example, in Capelle et al. study (1983) TDZ was found to trigger biological activities involved in cell division and growth, and to stimulate the biosynthesis of other cytokines hormone that would promote production of new shoots. TDZ hormone had also played a key role in successful shoot multiplication in many plants such as, *Camellia sinensis, Hordeum vulgare, oryza sativa* and *Solanum villosum* (Sajid, et al., 2009; Tahtamouni, et al., 2017). Also, (Tefera and Surawit, 2006) found in their study on Korarima (*Aframomum corrorima*) that, TDZ increased remarkably shoot multiplication rate.

In 2iP experiment, it was found that 1.5 mg/L 2iP gave the maximum number of shoots (Table 1). Using 2iP for shoot proliferation *in vitro* was reported by Catapan et al. (2000), as high number of shoots were produced in nodal segment of *Phyllanthus caroliniensis* using MS medium supplemented with (0.5 – 1.0) mg/l of 2iP. Also Sharaf (2010) reported that the best multiplication parameters and growth performance of *Artemisia herba-alba* were obtained at (1.0 mg/l) 2iP. Meanwhile, 2iP at level of (0.25 mg/l) failed to induce multiple shoots in *Psoralea corylifolia* L. (Baskaran and Jayabalan, 2008).

However, according to our results, TDZ at level of (1.5 mg/ L) seems to have the most power full effect on enhancement of shooting in *Paronychia argentea* L. cultures.

4.2 *In Vitro* Rooting

Our data indicated that, all experimented auxin types had encouraged rooting in *Paronychia argentea* L. microshoots (Table 2). Auxins were always described to act as key factors in rooting process, as they stimulate plant cells to differentiate into roots (Taiz and Zeiger, 2002). However, it can be indicated from our results that IBA seems to be the best rooting enhancer, especially at level of (0.5 mg/L) (Table 2), which indicated that the effect of auxins on root enhancement varies with auxin type in addition to concentration.

4.3 Quercetins and Isorhamnetin content

According to our results, quercetins and isorhamnetin amounts in wild type plant were much higher than those found in the tissue cultured microshoots (Table 3). This could be attributed to high moisture content of the *in vitro*

grown cultures as a result of the continues supplement of water, which might adversely affected synthesis of flavonoids in grown microshoots. The negative correlation between secondary metabolites production and moisture content of the *in vitro* grown plants was reported in many related studies. For example, Tahtamouni et al. (2016) confirmed this negative correlation, as they compared content of volatile oil extracted from *in vitro* grown *Thymbra spicata* L. var. *spicata* microshoots with data reported by (Saifan, et al., 2009) for volatile oil extracted from aerial parts of the wild type plant collected from the wild of Kufranja (northern Jordan).

Also, our data revealed that quercetins and isorhamnetin were affected by type and level of cytokinins. For example, adding BAP at level at of 0.10 mg/l was able to increase significantly quercetins amount in the *in vitro* grown microshoots (1.838 ppm) compared to the control. Meanwhile, maximum amount of isorhamnetin obtained from the wild type plants (2.368 ppm) (Table 3). Adding BAP to the culture media was recommended in many other related studies for the enhancement of the production of many types of secondary metabolites in various species of *in vitro* grown plants. For example, Al- Ashoush (2017) and Udomsuk et al., (2009) reported that, BAP had a positive effect on amounts of some types of secondary metabolites in addition to total isoflavonoids extracted from *in vitro* grown on callus cultures of (*Lantana camara* and *Pueraria candollei* var. *mirifica*.), respectively. Also Taveira et al. (2009) found that, increasing BAP concentration to 2.0 mg/L had resulted in increasing phenolic compounds level in *Brassica oleracea*.

Moreover, our data revealed that adding 0.1 mg/L Kinetin to the culture media had improved quercetins and isorhamnetin concentrations remarkably compared to the control treatment (0.389, 0.998 ppm) (Table 3).This was in agreement with Akula and Ravishankar (2011), who found that adding (0.1 or 0.2 mg/L) Kinetin in *Daucus carota* had yielded superior amounts of flavonoids. Moreover, Kinetin was reported to influence polyphenol group such, as quercetin and isohrmerin by affecting synthesis of nucleic acids that might influence the production polyphenols (Shah, et al., 1976).

In TDZ experiment, increasing TDZ concentration to 0.5 mg/l in the media had maximized values of both quercetin and isohrmerin (1.867, 1.376 ppm) compared to the control and other TDZ treatments (Table 3). This finding agreed completely with Al-Ashoush (2017) and Udomsuk, et al., (2009), who reported that TDZ at level of 0.5 mg/ L was the best treatment for obtaining maximum amounts of flavinoids in *Lantana camara L.*, in addition to isoflavonoids in callus cultures of *Pueraria candollei* var. *mirifica*.

Meanwhile, in 2iP experiment our data indicated that amounts of quercetins increased with increasing 2iP to reach a maximum level (0.172 ppm) at 0.1 mg/L 2iP, while isorhamnetin was maximized (1.362 ppm) ppm) at 2iP concentration of (0.5 mg/L) which was much higher than those extracted from *in vitro* grown microshoots (Table 3). 2iP was also reported for enhancing production of some flavonoids in tissue cultured microashoots of *Silybum marianum* L., as (1.0 mg/l) of 2iP was found to be most effective (Al- Hawmdeh, et al., 2013).

Generally, our results indicated 0.5 mg/L TDZ was the best growth regulator to be added to the growth media of *Paronychia argentea* L.as it yielded a reasonable shoot number that yielded maximum amounts of quercetins and isorhamnetin as well (Tables 1, 3). This can be applied as a part of protocol for massive propagation of plant material that contains extra amounts both flavonoids types.

CONCLUSION

In this study, an efficient *in vitro* propagation protocol was successfully achieved for *Paronychia argentea*. Applying this propagation protocol can hopefully participate in saving this valuable plant from threats of extinction and decline, and insuring a constant supply of plant material for scientific research and phyto- industry. Also, it can be concluded from our data that, 0.5 mg/L TDZ was the best growth regulator to be added to the growth media of *Paronychia argentea* L., as it yielded a

reasonable number of microshoots containing maximum amounts of quercetins and isorhamnetin compared to the other treatments. However, more research is still needed for better enhancement of quercetins and isorhamnetin production in this plant using other biotechnological approaches such as, cell culture and bioreactors.

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الإكثار الدقيق وتعزيز إنتاج مادتي كيورستين وايزورمنتين في نبات رجل الحمام البري

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ملخص

يعتبر نبات رجل الحمام نباتا بريا عشبيا مهملا ينمو في مناطق مختلفة بدون أي أبحاث عن زراعته وإكثاره. وتستخدم هذه العشبة عادة في الطب الشعبي بسبب خصائصها العلاجية. بالإضافة لذلك لغاية الآن هنالك القليل من الأبحاث عن القيمة الطبية لنبات رجل الحمام. لذلك فإن هذا البحث تم إجراؤه للحصول على بروتوكول لإكثار هذا النبات باستخدام تقنية زراعة الأنسجة النباتية و لتقييم وجود المواد الطبية كيورستين وايزورمنتين في السويقات الناتجة من النبات داخل الأنابيب بعد معاملتها بأنواع و تراكيز مختلفة من منظمات النمو الستيوكينين باستخدام تقنية فصل السوائل بتقنية عاليه (HPLC). تم الحصول على عدد من السويقات (3.5 ساق/ نبات) باستخدام بيئة موراشيغ وسكوج (MS) مزودة ب 1.5 مغ/لتر من هرمون ثيوديزورون (TDZ). بالإضافة لذلك تم الحصول على عدد من الجذور (5.9 جذر/ نبات) على بيئة موراشيغ و سكوج (MS) مزودة ب 0.5 مغ/لتر من هرمون اندول بيوتريك اسيد (IBA). بالإضافة لذلك تم الحصول على أعلى كمية من المواد الطبية كيورستين وايزورمنتين (1.867 و 1.376 جزء لكل مليون) من السويقات المزروعة على بيئة موراشيغ و سكوج (MS) مزودة ب 0.5 مغ/لتر من هرمون ثيوديزورون (TDZ).

الكلمات الدالة: ايزورمنتين، رجل الحمام، كيورستين، زراعة الأنسجة.

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