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Clonal propagation of *Tetragonolobus palaestinus* Bioss: A Jordanian medical plant

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Clonal propagation of *Tetragonolobus palaestinus* Bioss: A Jordanian medical plant

Abstract: *Tetragonolobus palaestinus* Bioss (Aljalaton) is one of the Jordanian medicinal plants that can be used to treat stomach pain and some infections. This study was done in order to establish optimal *in vitro* propagation method for *T. palaestinus*. Factors of *in vitro* shooting, rooting, and acclimatization of the *in vitro Tetragonolobus palaestinus* seedlings were studied using different growth regulators. For *in vitro* shooting, different cytokinins including benzylamino purine (BAP), kinetin, TDZ, and zeatin were used in increasing concentrations (0.0, 0.3, 0.6, 0.9, 1.2, 1.5, and 2.0 mg l^{-1}). Using benzylamino purine (BAP produced a maximum of 2.0 shoots/explants on Murashige and Skoog (MS) medium supplemented with 0.3 mg l^{-1}. Moreover, the effect of different concentrations of IBA (indole-3-butyric acid), IAA (indole-3-acetic acid), andnaphthalene acetic acid (NAA) was evaluated for *in vitro* rooting. The highest number of roots (4.06 roots/explant) was obtained on MS medium supplemented with 0.3 mg l^{-1} IBA. All of the plants (100 %) were grown normally after the acclimatization process. Based on these results simple protocol of *T. palaestinus in vitro* culture was optimized for the first time which can be utilized to do more studies on cell culture and production of active secondary metabolites.

Key words: acclimatization;, *in vitro*; shoot multiplication;, rooting

Klonsko razmnoževanje vrste *Tetragonolobus palaestinus* Bioss: jordanske zdravilne rastline

Izvleček: Vrsta *Tetragonolobus palaestinus* Bioss (Aljalaton) je jordanska zdravilna rastlina, ki se lahko uporablja za blaženje bolečin v želodcu in zdravljenje nekaterih okužb. Namen raziskave je bil vzpostaviti optimalen način *in vitro* razmnoževanja te rastline. Preučevani so bili dejavniki *in vitro* gojenja (vkoreninjenja, tvorbe poganjkov) in aklimatizacije sadik te rastline z uporabo različni rastnih regulatorjev. Za *in vitro* tvorbo poganjkov so bili uporabljeni različni citokinini in sicer benzilamino purin (BAP), kinetin (TDZ) in zeatin v naraščajoči koncentraciji (0,0; 0,3; 0,6; 0,9; 1,2; 1,5 in 2,0 mg.l^{-1}). Uporaba benzilamino purina (0,3 mg l^{-1}) je dala pri gojenju na Murashige in Skoog (MS) gojišču največ poganjkov, dva na izseček. Učinek različnih koncentracij rastnih regulatorjev (IBA-indol-3-maslene kisline, IAA -indol-3-ocetne kisline in naftalen ocetne kisline NAA) je bil ovrednoten pri *in vitro* vkoreninjenju. Največje število korenin (4,06 korenin/izseček) je bilo dobljeno na MS gojišču, z dodatkom 0,3 mg l^{-1} IBA. Vse rastline (100 %) so po obdobju aklimatizacije rastle normalno. Na osnovi teh rezultatov je bil prvič optimiziran enostaven protokol za *in vitro* gojenje te vrste, ki bi se lahko uporabil v nadaljnih raziskavah na celičnih kulturah in produkciji aktivnih sekundarnih metabolitov.

Ključne besede: aklimatizacija; *in vitro*; namnoževanje poganjkov; vkoreninjanje

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1 INTRODUCTION

Tetragonolobus palaestinus is a wild plant from the Fabaceae family. Its natural habitat can be found in the northern parts of Jordan on the rocks and meadow areas (Afifi & Abu-Irmaileh, 2000). *T. palaestinus* is a herbaceous plant, it starts to grow after seasonal rainfall in the winter, and has a red flower and a small fruit (pod) which can be served as fresh or boiled to eat, it is well-known among the people as Aljlatoun (Al-Karaki, 2000). Most Jordanian wild plants are becoming endangered due to the expansion of urban and rural settlements, uncontrolled deforestation, illegal collection, industrial pollution, and low level of environmental awareness (Al-Bakri et al., 2011). Therefore, to solve such problem, alternative methods for massive plant propagation like plant tissue culture techniques and other biotechnological approaches are used for producing medicinal plants, isolating medicinal secondary products, conserving and rapid propagating valuable, rare, and endangered plant species (Arafeh et al., 2006; Al-Mahmood et al., 2012; Qrunfleh et al., 2013; Shatnawi , 2013). Propagation methods of *T. palaestinus* using seeds are not preferred due to the low germination percentage (Al-Karaki, 2000).

In vitro culture of *T. palaestinus* can solve propagation problems as it guarantees mass production of plant material without compromising the natural resources and it also improves and conserves this plant (Alenizi et al., 2020; Ebrahim et al., 2007; Shatnawi 2006, Shibli et al., 2018). The use of *in vitro* culture technique is the best solution to overcome *T. palaestinus* propagation problems and can also enhance mass production without threatening the natural resources (Shibli et al., 2003; Makhadmeh & Shatnawi, 2008; Shatnawi et al., 2011, Al Qudah et al., 2011). Also, it is an important tool in both basic and applied studies and for commercial applications (Arafeh et al., 2006; Ahmad et al., 2010). Using micropropagation; the plant developed from this technique is true to type or genetically uniform with the mother plant (Shibli et al., 2003). Production of a large number of genetically uniform disease-free plants is known to be a reliable technique system. The conventional method of propagation is done by vegetative methods through root suckers or terminal cutting which is classified as very slow (George et al., 2008). *In vitro* propagation plays a major role in the rapid production of disease-free planting material of newly improved varieties year rounded basis (Ebrahim et al., 2007; Shatnawi 2006, Shatnawi et al., 2011). Shoot tip culture is a relatively simple *in vitro* technique for the rapid propagation of selected pathogen-free plant materials. Therefore, many simple protocols have been developed for the rapid multiplication of newly released commercially important genotypes through apical meristem cultures. Successful commercial micropropagation protocol depends on successful rooting and acclimatization of *in vitro* derived plantlets (Ebrahim et al., 2007; Shatnawi 2006, Shatnawi et al., 2011). Till now; there are no available data on the *in vitro* propagation of *T. palaestinus*. So, this study was initiated to develop an applicable and simple protocol for in vitro establishment, multiplication, rooting, and acclimatization of *T. palaestinus*.

2 MATERIAL AND METHODS

2.1 ESTABLISHMENT OF *IN VITRO* CULTURE

Plant seeds of wild *T. palaestinus* were collected in mid of April in north Jordan – "Al-Sareeh, Irbid" (32.3306° N latitude and 35.8951° E Longitude). Firstly, surface sterilization of seeds was done by washing seeds with tap water for 5 min. After that, seeds were immersed in (4 %) sodium hypochlorite for 15 min. The following sterilization steps were performed under sterile conditions in a laminar air flow chamber; the seeds were washed 3 times in sterile distilled water, then soaked in 70 % ethanol solution for 30 s and washed several times with sterile distilled water. Seeds were cultured on the surface of hormone free Murashige and Skoog (MS) medium (1962) inside Petri dishes (five seeds/ Petri dish). Murashige and Skoog (MS) medium was supplemented with vitamins and 30 g $l^{-1}$of sucrose. After the final volume of the MS media was adjusted to 1 l and the pH to 5.75, 8 g of agar was added to the media mixture with constant stirring and heating until the agar was completely dissolved. After that, 100 ml of medium was poured into *Erlenmeyer flask*s. Then flasks were plugged and autoclaved at 121 °C for 15 min. After that cultures of seeds were kept in a growth room in the dark and moderate temperature 24 ± 2 °C for four weeks until full germination. The germinated seedlings were transferred to light conditions in the growth room under the light regime (16/8 h (light/dark) and a light intensity of 50 µmol $m^{-2}s^{-1}$. Afterward, cultures were transferred to the new medium and further grown. Then, cultures were transferred to MS medium provided with growth regulators, i.e. 0.3 mg l^{-1} benzyl amino purine (BAP) and 0.05 mg l^{-1} naphthalene acetic acid (NAA) with 30 g l^{-1} sucrose, to increase the growth of the cultures.

2.2 SHOOT PROLIFERATION

Microshoots of 10 mm in length, were treated with different concentrations of cytokinins for shoot proliferation experiments. MS media were supplemented with 0.0, 0.3, 0.6, 0.9, 1.2, 1.5 or 2.0 mg l^{-1} of BAP, Kinetin, Thidiazuron (TDZ) or Zeatin. Five replications with three microshoots were used for each treatment. Data were collected after five weeks for the microshoots growth parameters as shown in Table 1.

2.3 ROOT FORMATION OF *IN VITRO* CULTURES

Microshoots, 10 mm in length, were treated with different concentrations of auxins. For root formation MS media were supplemented with 0.0, 0.3, 0.6, 1.2, 1.5 or 2.0 mg l^{-1} of indole-3-butyric acid (IBA), indole acetic acid (IAA) or naphthalene acetic acid (NAA). Ten replications were used with one microshoot / replicate. Data were collected for the number of axillary shoots/ explant, shoot length, root length, and rooting (%) after five weeks.

2.4 ACCLIMATIZATION

The fully *in vitro* rooted microshoots were hardened gradually from *in vitro* tubes. Firstly, the tubes plugs were removed for three days and the cultures were left in the growth room. After that microshoots were gently transferred from test tubes and washed until all agar residues were removed and grown in plastic pots that have a suitable mixture of (1 peat : 3 perlite). Cultures were covered with perforated plastic bags for 3 days with continuous wetting with sterile distilled water. After that, plastic bags were removed and the pots were left for extra 2 weeks under growth room conditions with continuous wetting. At the end of the acclimatization experiment, the survival percentage of the acclimatized plants was registered.

2.5 EXPERIMENTAL DESIGN

The completely randomized design (CRD) was used with all the experiments. Data were analyzed in SPSS Software with Tukey HSD Multiple Range test at $p \leq 0.05$. Means and standards error of means were calculated.

3 RESULTS AND DISCUSSION

In this study, significant differences in the shoots growth parameters were obtained using BAP, Kinetin, TDZ, or zeatin at different concentrations. At 0.3 mg l^{-1} of BAP, maximum shoots numbers (2.0 shoot per explants) were obtained (Table 1 and Fig. 1). Additionally, the length of shoots and the number of leaves on average increase up to two- fold (30.0 mm and 12.2 leaves, respectively) in comparison with controls (18.0 mm, 7.0 leaves, respectively). At 0.9 mg l^{-1} BAP, a maximum fresh mass of 112.0 mg was obtained and it was 1.7-fold higher compared to controls (66.0 mg). Shoot length increased up to 30 mm and 26 mm at the lowest and the highest concentrations of BAP (0.3, 2.0 mg l^{-1}; respectively), comparing with the control (18.0 mm). But; at 1.2 and 1.5 mg l^{-1} BAP the length of the shoots was significantly smaller compared to the length of shoots in medium with 0.3 mg l^{-1} BAP. Furthermore; BAP at (0.3 and 0.6 mg l^{-1}) concentrations resulted in the highest number of leaves 12 (leaves). One of the best cytokinins that can be used to induce *in vitro* shoot formation is 6-benzylaminopurine (Singh et al., 2019). Thảo et al. (2013) reported the highest shoot induction in common bean (*Phaseolus vulgaris* L.) when BAP was used with NAA in media. Similarly, in *Securidaca longipedunculata* (Fresen) the combinations between BAP and IBA at (1.5 mg and 0.1 mg l^{-1}; respectively) produced a better short number and length per explant than other growth regulator combinations (Lijalem and Feyissa, 2020). Besides that, BAP has been reported in many previous studies for shoot multiplications. For example; *Trichosanthes dioica* Roxb. was established from nodal explants on MS medium containing 1.0 mg l^{-1} BAP (Tiwari et al., 2010). BAP also; gave the best results for *Prosopis cineraria* (L.) Druce in vitro establishment (Kumar and Singh, 2010). BAP gave the best outcome for shoot induction in the in vitro grain legume, *Phaseolus vulgaris* (Malik and Saxena, 1992)

Similarly, kinetin at 0.3 mg l^{-1} had increased the shoot length up to 32.0 mm which was 1.7-fold longer than control (18.0 mm) (Table 1, Fig 1). Moreover, maximum dry and fresh mass (140 and 114 mg; respectively) of *in vitro Tetragonolobus palaestinus* explants were obtained at 0.3 g l^{-1} of kinetin. Increasing concentrations of kinetin inhibited the growth of the shoots in length and their appearance was swelling and short. Kinetin induced expansion of growth by swelling rather than elongation, this was confirmed previously by Naeem (2004). Ahmadi et al. (2011) reported that using kinetin at 2.0 mg l^{-1} increased the *in vitro* shoot induction in

Matthiola incana (L.) W.T.Aiton. In *Moringa stenopetala* (Baker f.) Cufod.**;** maximum number of shoots per explant (3.43 ± 1.41) and 7.97 ± 4.18 leaves per explant were obtained on MS medium containing 0.5 mg l^{-1} kinetin with 0.01 mg l^{-1} NAA. **(**Adugna et al., 2020**).**

The addition of 0.3 mg l^{-1} TDZ to MS medium resulted in longer shoots (32.0 mm) compared to controls (18.0 mm), and the highest number of leaves per explant (14.8 leaves per explant) was obtained on MS medium supplemented with 1.2 mg l^{-1} TDZ. The Growth regulator TDZ had been used in previous studies in order to promote *in vitro* propagation of different plants species of the Fabaceae family; such as, *in vitro Psophocarpus tetragonolobus* (L.) D.C. (Singh et al., 2014); and common bean (Veltcheva et al., 2005). The results from the present work demonstrated that TDZ at low concentration was effective compared to other cytokinins (Table 1). However, it was found to be effective at low concentration. Low concentrations of TDZ (0.01 mg l^{-1}) were the most appropriate for shoot regeneration in *Abelmoschus moschatus Medik* (Sharma & Shahzad, 2008). The effect of TDZ on growth parameters is not entirely clear, and more studies are needed to understand its role in plant tissue cultures. (Ugandhar et al., 2012). TDZ in combination with NAA produced relatively shorter shoots when used with *Securidaca longipedunculata* (Fresen) (Lijalem and Feyissa, 2020). Furthermore; TDZ had been reported to have an adverse effects with *Vitex trifolia* L. (Ahmed and Anis, 2012).

When zeatin was used, a maximum number of shoots (1.4 shoots per explant) was obtained on MS medium supplemented with 0.3, 0.9, and 1.2 mg l^{-1} Zeatin (Table 1). While the maximum shoot length (28.0 mm) was produced on MS medium supplemented with 0.9 mg l^{-1} Zeatin. On the other hand, Vikram et al. (2012) reported that Zeatin at 1.2 mg l^{-1} produced a maximum number of multiple shoot formation in *Lycopersicum esculentum* L.. In addition, a highly efficient organogenesis protocol for in vitro regeneration of eggplant was developed using zeatin (García-Fortea et al., 2020). This may be due to that, zeatin suppress apical dominance which leads to increase numbers of multiple shoots and reduce the length of the shoot.

3.1 IN VITRO ROOTING

The in vitro rooting of T. palaestnius was significantly induced at a concentration of 0.3 mg l^{-1} of IBA with (4.06 roots/microshoot). The rooting percentage was 40 % with 3.33 mm/root long at 0.3 mg l^{-1} of IBA. Meanwhile; control and other concentrations of IBA showed lower in vitro rooting; as we can show in (Table 2 & Fig 2). Low concentrations of IBA (1.0 mg l^{-1}) also resulted in the highest in vitro rooting in Cicer microphyllum Benth. (Singh et al., 2019) and in common bean (Phaseolus vulgaris L.) (Thảo et al., 2013).

For rooting with IAA growth regulator; the maximum number of roots per microshoots was (2.14 roots/explant) obtained at 1.2 mg l^{-1} IAA with a maximum root length of 2.94 mm. The maximum root percentage (30 %) was also recorded on media supplemented with 1.2 mg l^{1} IAA. Using 0.3 mg l^{-1} NAA resulted in 0.5 developed root length of 1.94 mm (Table 2). No callus occurred at microshoots bases.

The current study showed that auxin is essential for the induction of root formation of in vitro T. palaestnius cultures. This is because auxin exerts a primary role in root formation by its involvement in successive and interdependent phases (Mineo, 1990). The rooting of leguminous species is dependent on the auxin type (Dewir et al.,

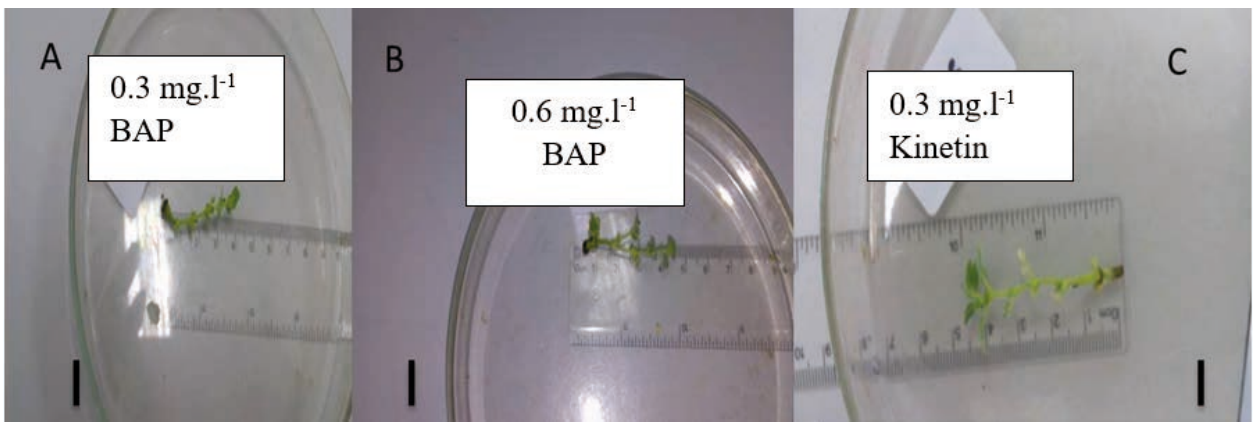

Figure 1: In vitro shoot formation of *Tetragonolobus palaestinus* after five weeks. A) MS with 0.3 mg l^{-1} (BAP). B) MS with 0.6 mg l^{-1} (BAP). C) MS medium with 0.3 mg l^{-1} Kinetin. Bars represent 1.0 cm

2016). Using IBA or NAA at a low concentrations such as 0.3 mg l^{-1} increased cell division and root primordial formation and proved to enhanced rooting percentage, number of roots per rooted explants and, root length as compared to IAA or IBA. Some studies found that NAA was the best rooting auxin in Vigna mungo (L.) Hepper (Mony et al., 2010). Furthermore; different legumes of the Fabaceae family have been in vitro rooted using different auxins types. For example; the in vitro rooting in Clitoria ternatea L. which is known as the butterfly pea plant was obtained by the addition of 1.50 mg l^{-1} NAA with the highest number of adventitious roots (12.86 ± 2.14) (Lee et al., 2021). While IBA had been used in the in vitro rooting of Lotononis bainesii Baker (Fabaceae) at the concentration of 0.049 µM IBA (Vidoz et al., 2012). Also, 83 % of in vitro rooting in Thermopsis turcica Kit Tan, Vural & Küçük. (Fabaceae) was attained on pulsed-IBA treated shoots (Cenkci et al., 2008). The growth regulator IAA, was used at 0.005–0.01 mg l^{-1} to induce in vitro rooting in Psoralea corylifolia L.(Fabaceae) which is

Table 1: The effect of different concentrations of the cytokinins on *in vitro* grown *Tetragonolobus palaestinus* after five weeks of incubations. *Values represent means ± standard error. *Means within the column for each growth regulator having different letters are significantly different according to Tukey HSD at $p \leq 0.05$

Concentrations mg L^{-1}	Number of axillary shoots/explant	Shoot length (mm)	Number of leaves/ explant	Fresh mass/five explants (mg)	Dry mass/five explants (mg)
Control 0.0	1.0 ± 0.0 b	18.0 ± 1.22 c	7.0 ± 0.32 c	66.0 ± 2.0 b	34.0 ± 2.4 c
			BAP		
0.3	2.0 ± 0.32 a	30.0 ± 3.1 a	12.2 ± 0.74 a	66.0 ± 2.0 b	34.0 ± 2.4 c
0.6	1.6 ± 0.20 ab	28.0 ± 2.0 ab	12.0 ± 0.44 a	90.0 ± 2.0 ab	56.0 ± 5.0 b
0.9	1.6 ± 0.40 ab	26.0 ± 2.4 ab	11.2 ± 0.37 ab	112.0 ± 22.0 a	106.9 ± 15.0 a
1.2	1.0 ± 0.24 b	22.0 ± 2.0 b	10.8 ± 0.37 b	92.0 ± 4.0 ab	52.0 ± 5.0 b
1.5	1.6 ± 0.00 ab	22.0 ± 2.0 b	11.4 ± 0.51 ab	52.0 ± 0.0 c	20.0 ± 5.0 d
2.0	1.8 ± 0.20 ab	26.0 ± 2.4 ab	11.8 ± 0.37 ab	88.0 ± 17.6 ab	62.0 ± 5.0 b
			Kinetin		
0.3	1.2 ± 0.20 a	32.0 ± 3.7 a	12.4 ± 1.12 a	140.0 ± 24.4 a	114.0 ± 25.0 a
0.6	1.2 ± 0.20 a	22.0 ± 0.0 b	11.8 ± 0.48 a	96.0 ± 14.7 ab	64.0 ± 9.21 ab
0.9	1.2 ± 0.2 a	24.0 ± 2.4 ab	11.8 ± 0.48 a	92.0 ± 3.7 ab	66.0 ± 5.12 ab
1.2	1.0 ± 0.0 b	15.0 ± 0.0 d	8.0 ± 0.20 c	44.0 ± 4.0 d	20.0 ± 3.1 c
1.5	1.4 ± 0.24 a	20.0 ± 0.0 b	10.2 ± 0.37 b	88.0 ± 2.0 b	66.0 ± 4.0 ab
2.0	1.0 ± 0.0 b	15.0 ± 0.0 d	4.0 ± 0.24 d	30.0 ± 0.0 d	14.0 ± 2.0 c
			TDZ		
0.3	1.6 ± 0.24 a	32.0 ± 2.0 a	10.8 ± 0.37 b	84.0 ± 4.4 b	58.0 ± 7.3 c
0.6	1.8 ± 0.37 a	20.0 ± 0.0 ab	12.8 ± 0.58 ab	90.0 ± 4.4 ab	64.0 ± 5.1 b
0.9	1.2 ± 0.20 b	22.0 ± 2.0 b	10.8 ± 0.37 b	84.0 ± 5.1 b	85.0 ± 5.8 ab
1.2	1.8 ± 0.37 a	30.0 ± 4.4 a	14.8 ± 0.24 a	136.0 ± 26.1 a	112.0 ± 24.1 a
1.5	1.6 ± 0.24 a	24.0 ± 2.4 ab	10.6 ± 0.40 b	92.0 ± 2.0 ab	60.0 ± 5.4 c
2.0	1.6 ± 0.24 a	22.0 ± 2.0 b	11.8 ± 0.41 b	90.0 ± 6.7 ab	66.0 ± 6.7 b
			Zeatin		
0.3	1.4 ± 0.25 a	26.0 ± 2.45 a	15.4 ± 0.81a	66.0 ± 1.8 b	106.9 ± 24.0 a
0.6	1.2 ± 0.20 ab	20.0 ± 0.0 ab	10.4 ± 0.24 ab	92.0 ± 2.0 ab	56.0 ± 4.8 b
0.9	1.4 ±0.40 a	28.0 ± 3.74 a	15.4 ± 1.02 a	112.0 ± 22.0 a	74.0 ± 5.1 ab
1.2	1.4 ± 0.24 a	24.0 ± 2.44 ab	10.0 ± 0.95 ab	92.0 ± 5.0 ab	52.0 ± 1.9 b
1.5	1.0 ± 0.00 b	11.0 ± 1.00 c	7.6 ± 0.51 c	52.0 ± 2.0 c	20.0 ± 6.0 d
2.0	1.2 ± 0.20 ab	20.0 ± 00 ab	11.60 ± 0.88 ab	88.0 ± 4.0 ab	62.0 ± 3.0 b

a rare and endangered herbaceous medicinal plant (Rout et al., 2010). We can see from this, that in vitro rooting depend on plant species and the type of auxin used.

3.2 ACCLIMATIZATION

The acclimatization process of *Tetragonolobus palaestinus* microshoots was proved its ability to the production of healthy acclimatized micro shoots. The mixture of (Peatmoss: Perlite) was suitable for roots growth and new leaves were formed after 2 weeks (Fig 3). All of the rooted plantlets were survived after acclimatization process after acclimatization process. No variations were observed visually among the acclimatized plantlets as shown in Fig (3). A lower survival rate of 65 % was obtained in *C. microphyllum* using different potting culture mixture (garden soil, vermiculite, and vermicompost (1:1:1) (Singh et al., 2019)

Plantlet needs an acclimatization period and this could be due to the effect of tissue differentiation, growth, and development (Quisen, 2013). The acclimatized plantlet may be affected by the change in environmental conditions during acclimatization, which may be due to the short acclimatization period evaluated in this study (Shatnawi., 2013). Different plants of the Fabaceae family have been successfully in vitro rooted and acclima-

Figure 2: The effect of 0.3 mg l^{-1} indole-3-butyric acid (IBA) on *in vitro* growth of *Tetragonolobus palaestinus* after five weeks growth. The bar represents 1.0 cm

Table 2: The effect of different auxin concentration on *in vitro* root formation of *Tetragonolobus palaestinus* after five weeks growth period. ˙Values represent means ± standard error. *Means within the column for each growth regulator having different letters are significantly different according to Tukey HSD at $p \leq 0.05$

Concentrations mg.l^{-1}	Number of axillary shoots/explant	Shoot length (mm)	Number of roots / explant	Root length (mm)	Rooting %
Control 0.0	1.01 ± 0.23* b	18.07 ± 7.46 ab	1.50 ± 0.48 b	2.00 ± 0.70 ab	20%
		IBA			
0.3	1.16 ± 0.32 a	19.0 ± 9.5 a	4.06 ± 0.67 a	3.33 ± 0.90 a	40%
0.6	1.09 ± 0.18 a	17.06 ± 7.06 ab	3.17 ± 0.52 b	1.44 ± 1.33b	20%
1.2	1.04 ± 0.15 a	12.28 ± 4.21 b	2.67 ± 0.78 ab	2.94 ± 1.86 a	30%
1.5	1.04 ± 0.16 a	13.33 ± 7.13 b	2.06 ± 0.78 ab	1.94 ± 2.83 b	30%
2.0	1.06 ± 0.17 a	12.04 ± 5.29 b	2.94 ± 0.81 ab	3.17 ± 0.86 a	20%
		IAA			
0.3	1.00 ± 0.22 b	13.78 ± 4.41 ab	0.56 ± 0.24 b	0.89 ± 0.40 b	20%
0.6	1.22 ± 0.18 a	12.61 ± 3.45 b	0.50 ± 0.20 b	2.61 ± 0.84 a	27%
1.2	1.06 ± 0.16 ab	19.33 ± 4.02 a	2.14 ± 0.17 a	2.94 ± 1.50 a	30%
1.5	1.09 ± 0.12 ab	12.28 ± 4.09 b	0.57 ± 0.60 b	1.83 ± 1.54 ab	26%
2.0	1.02 ± 0.13 b	12.61 ± 3.38 b	1.39 ± 0.41 ab	2.39 ± 1.11 a	20%
		NAA			
0.3	1.00 ± 0.22 b	17.00 ± 4.41 ab	0.56 ± 0.24 a	0.89 ± 0.40 c	30%
0.6	1.06 ± 0.18 ab	18.01 ± 3.45 a	0.50 ± 0.20 a	1.61 ± 0.84 ab	27%
1.2	1.01 ± 0.16 b	16.03 ± 4.02 b	0.44 ± 0.17 b	1.94 ± 1.50 a	20%
1.5	1.09 ± 0.12 ab	17.08 ± 4.09 ab	0.17 ± 0.60 b	1.83 ± 1.54 a	26%
2.0	1.02 ± 0.13 b	17.01 ± 3.38 ab	0.39 ± 0.41 ab	1.39 ± 1.11 b	30%

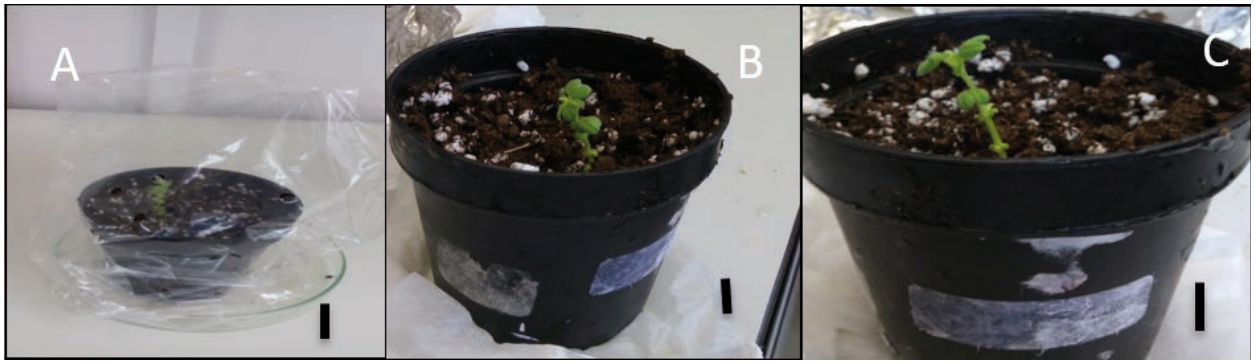

Figure 3: Acclimatization process of *Tetragonolobus palastinus* microshoots. Plantlets after: A) one week B) three weeks and C) five weeks of the Acclimatization process. Bars represent 1.0 cm

tized. For example, the regenerated plantlets of licorice (*Glycyrrhiza glabra* L.; Fabaceae) were acclimatized, with a survival rate of 77.7 %, when transferred to ex vitro conditions and showed no morphological abnormalities (Shaheen, 2020). While, the in vitro seedlings of *Caesalpinia ferrea* Mart. were acclimated without the presence of roots in different types of the substrate with 73.4 % surviving plantlets after 30 days of growth (Silva et al., 2018). On the other hand, in *A. leiocarpa* (L.A.S.Johnson ex G.J.Leach) K.R.Thiele & Ladiges plantlets the substrate composition did not affect the survival or growth of in vitro rooted plantlets during acclimatization (Haygert-Lencina it., 2017). We can notice from this, that acclimatization is a critical process and it depends on plant species and the successful adjustment of the environment of the acclimatized plants.

4 CONCLUSION

The current results indicat that *in vitro* propagation method of *Tetragonolobus palastinus* was successful, with full survival percentage for the first records of this plant species *in vitro*. The optimum *in vitro* propagation method of *Tetragonolobus palastinus* was obtained at 0.3 mg l^{-1} of Benzylamino purine (BAP) with 2.0 shoots/explants and 0.3 mg l^{-1} IBA with (4.06 roots/explants) and this method is recommended for *in vitro* clonal propagation in *T. palaestinus*.

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