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Enhance *in vitro* propagation of date palm (*Phoenix dactylifera* L.) Barhee cv. by using Nano-bio fertilizers (Lithovit)

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ABSTRACT
This work aimed to study the effect of Lithovit as a Nano bio-fertilizer on *in vitro* propagation of date palm (*Phoenix dactylifera* L.) cultivar Barhee, during multiplication and rooting stages, to obtain full intact plantlets, that can be successfully transferred to the acclimatization stage. Lithovit added at different concentrations (0.0, 0.25, 0.50, 0.75, and 1.0 g/L.) to culture media of proliferated somatic embryo clusters during the multiplication stage and elongated shoot proliferation during rooting stages. Data collected for vegetative growth during both studied stages, moreover the biochemical constituents of date palm cv. Barhee explants evaluated during the rooting stage. The results indicated that using Lithovit at 0.25g/ L caused a significant increase in all studied vegetative growth parameters during the multiplication stage comparing to the control treatment. And for the rooting stage, data indicated that using Lithovit at 0.75 g/L concentration gave the highest values of vegetative growth parameters. Also, the results showed that Lithovit at 0.75 g/l in rooting medium gave a significant increase in biochemical constitutes of the total sugars (mg /1g), amino acids, total chlorophyll, total indole (mg /1g), and total phenols (mg /1g) comparing to the control treatment. In conclusion, the obtained results recommended using Lithovit as growth stimulators, for enhancing the multiplication stage and, improving the rooting stage, in date palm cultivar Barhee, to optimize successful micropropagation protocol.

***Keywords*:** micropropagation, shoot multiplication, rooting stage, biochemical constituents, Lithovit, Nano-bio fertilizers, *Phoenix dactylifera* L.

Introduction

Date palm (*Phoenix dactylifera* L.) is extensively grown around the Arab world. The Middle East and North African region's main growing area of date palm trees. Date palms are of cultural, social, and environmental significance, mainly the fruits have great nutritional value; moreover, the many practical advantages of the whole tree (Ghazzawy *et al.,* 2017; Gantait *et al.*, 2018). Ground offshoots commonly propagate date palm; however, a female date palm produces only 10-20 offshoots in its entire life (Zaid and deWet, 1999), which is a restrictive factor for the propagation of commercial cultivars. One of the main difficulties in date palm cultivation, that avoids rapid crop enhancement, is the deficiency of an acceptable technique of asexual proliferation. Date palm propagation through tissue culture technique was the most successful path for the mass development of true-to-type plantlets (Bekheet, 2013; Mohammed *et al.*, 2019). Giving a high amount of plants within a short time through two protocols, either indirect somatic embryogenesis (callus phase) (Khan *et al.* , 2004; Fki *et al.* , 2011; Naik, and Al-khayri, 2016; Zayed, 2017) or direct adventitious shoots (Esmaeil *et al.* , 2015; Mazri *et al.* , 2016; Abahmane, 2013; Mohammed *et al.* , 2019). The core target of tissue culture studies in plant clonal propagation is to achieve high-frequency shoot regeneration and to produce a strong roots system, which is a requirement for an effective transfer to the external acclimatization level (Haque *et al.*, 2017). Some researchers focused on factors influencing shoot elongation and root development, including the type and concentrations of exogenous growth regulators and medium-growth basal salts, carbon source, activated coal, and light intensity (El-Dawayati, 2000; Abul-Soad and Jatoi, 2014; Abahmane, 2013; Mazri, 2014; Mazri *et al.* , 2016).

Nanotechnology is an inventive scientific methodology, that involves the use of equipment and material, able to improve the chemical, as well as, physical properties of a material at molecular stages

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and can be a hopeful field of interdisciplinary study, so it opens up an enormous range of opportunities in various fields like pharmaceutical, medical field, agriculture and biotechnology (Fakruddin *et al.*, 2012; Prasad *et al.*, 2014).

Lithovit 100 % organic (nano $CaCO_3$) structure is a natural calcium carbonate fertilizer supplemented with calcium which delivers fine particles that can simply be adsorbed directly during the stomata of plant leaves. Lithovit with the micronutrients provided an influence on cell wall formation, plant metabolism, and plant photosynthetic activity, (Thorn and Rogan, 2015), that it works as an enzyme activator, a constituent of many enzymes, and a carrier of phosphorus in the plant, (Marschner, 1995; Allison *et al.* , 2001 and Haq and Mallarino, 2005). Besides, Lithovit contains one of the important elements for plant growth (nano-Iron), which plays an essential role in photosynthetic reactions. Iron activates several enzymes and contributes to RNA synthesis and increases the performance of photosystems as reported by (Malakouti and Tehrani, 2005). Lithovit also contains nano-Mg, magnesium is an essential nutrient for plant growth and plays an important role in many plant physiological processes such as photosynthesis (Mg conceder as one of the essential elements of the chlorophyll molecule), sugar synthesis, starch translocation (Marschner, 1995). It was reported that Lithovit could establish the plant oils and fats, control of nutrient uptake, increase iron utilization, and assisted nitrogen fixation in legume nodules (Allison *et al.*, 2001 and Haq and Mallarino 2005). Lithovit has a very significant positive influence on plant growth in the open filed (Moisă, 2015). A significant results achieved in *Zia maize* (Azevedo Neto *et al.* , 2005), *Koelreuteria paniculat* (Sabina 2013), *Triticum aestivum* (Maswada and Abd El-Rahman 2014); Hayward kiwi fruit (Thorn and Rogan 2015), and *Lycopersicon esculentum* (Moisă, 2015).

There is no report of using Nano-bio fertilizers during the tissue culture of the date palm to enhance shoot elongation and root formation of shoot clusters. The present study carried out to study the biological effect of using Nano-bio fertilizers, at low concentrations of Lithovit, during the *in vitro* multiplication and rooting stages of date palm propagation, to enhance the growth of the cultures. And to obtain well full intact plantlets that can be successfully transferred to the acclimatization stage.

Material and Methods

Shoot tip meristems of date palm offshoots of Brahe cultivar, established and sterilized, for in vitro propagation, through indirect somatic embryogenesis as described by (El-Dawayati et al 2018a) for establishing the embryonic callus cultures, somatic embryos differentiation, then the secondary embryos and the new shoot clusters conversion, Figure (1), which used as the primary explants material, in this study.

Two experiments were conducted to study the effect of the addition Lithovit as Nano bio-fertilizer to the nutrient medium of the growing explants of date palm cultures, during multiplication and rooting stages.

1. Experiment 1: Effect of Lithovit concentrations on the shoot clusters proliferation during the multiplication stage.

1.1. Explant material:

The proliferated somatic embryo cluster consists of a repeated secondary embryo (20-25 secondary embryo) and (4-5 new proliferated shoots at 1-2 cm length)/ explant, as followed by (El-Dawayati et al 2014).

Explants were cultured on multiplication nutrient media of MS (Murashige and Skoog), (MS) vitamins, 40 g/L sucrose, and the other basic components of the date palm multiplication medium, 0.05 mg/L Benzyl adenine (BA), 0.1 mg/L Naphthaleneacetic acid (NAA), 100 mg/L myoinositol, 170 mg/L Potassium Phosphate Monobasic $KH_2PO_4.2H_2O$, and 0.4 mg/L thiamine HCl. (Zayed et al, 2017), Lithovit compound was added at different separate concentrations (0.25, 0.50, 0.75 and 1.00) to test its effect on the vegetative growth parameters during the multiplication stage. The pH of all media was adjusted to 5.8 before agar addition at 6 g/L. All media were autoclaved at 121°C for 20 min.

The application of Lithovit combinations was as follows:

T_1. 0.00 g/L Lithovit (Control).
T_2. 0.25 g/L Lithovit.
T_3. 0.50 g/L Lithovit.
T_4. 0.75 g/L Lithovit.

T_5. 1.00 g/L Lithovit.
The quantitative composition of Lithovit as Nano Biofertilizer presents in Table (1) as reported by (Carmen *et al.* , 2014).

Table 1. Quantitative composition of Lithovit.

Ingredient	Content	Ingredient	Content
Calcium carbonate ($CaCo_3$)	79.2 %	Sulphate ($SO4^2$)	0.33 %
Magnesium carbonate ($MgCo_3$)	4.6 %	Copper (Cu)	20 mg kg^{-1}
Alumina ($Al2O_3$)	1.0 %	Iron (Fe)	13 mg kg^{-1}
Silica (SiO_2)	11.4 %	Manganese (Mn)	140 mg kg^{-1}
Sodium monoxide (Na_2O)	0.6 %	Zinc (Zn)	57 mg kg^{-1}
Phosphate (P_2O_5)	0.01 %	Nickel (Ni)	4.9 mg kg^{-1}
Potassium oxide (K_2O)	0.2%		

All cultures of all studied treatments were incubated at 27±2°C under a photoperiod of 16 h using cool white fluorescent lamps (Toshiba 40 W tubes) irradiate 1500 Lux. Data collected for vegetative growth parameters, as (Secondary embryos formation degree, the shoot number, the shoot length, the verification degree and the growth vigor), for three subcultures with 8 weeks' interval period. Secondary embryos formation degree, verification degree and growth vigor degree were scored visually, (such as, 0 = no change; 1 = below average; 2 = average; 3 = above average; 4 = high; 5 = very high), following the recommendation of El-Dawayati *et al.,* (2018b).

Fig.1: Indirect somatic embryogenesis micropropagation protocol (a) sterilized shoot tip segment, cultured on callus medium induction. (b) Embryonic callus production (c) differentiated somatic embryos. (d) secondary embryos and the new shoot clusters conversion described protocols by (Zayed ,2017).

2. Experiment 2: Effect of Lithovit concentrations on the elongated shoots during rooting stage

In this experiment elongated shoots received previously on control multiplication medium (6-8 cm in length) cultured on rooting medium of date palm consist of 3/4 strength of (MS) salts, (MS) vitamins, 170.0 mg/L (KH2PO4), 100 mg/L myo-inositol, 2 mg/L Calcium dpantothenate (Ca.P), 0.4 mg/L thiamine. HCl, 0.1 mg/L glycine, 0.2 mg/L biotein and 0.2 mg/L arginine, 40 g/L sucrose and the growth regulators were 1.0 mg/L NAA ,1.0 mg/L Indole-3-butyric acid (IBA) and 0.4 mg/L Paclobutrazol (Pbz), (Abd Elzaher *et al.* 2019). Lithovit compound added at different separate concentrations (0.25, 0.50, 0.75 and 1.00) as mentioned above, to test their effect on the vegetative

growth parameters during the rooting stage. The pH of all media was adjusted to 5.8 before agar addition at 6 g/L. All media were autoclaved at 121°C for 20 min. All cultures of all studied treatments, during rooting stage, incubated at 27±2°C under a photoperiod of 16 h using cool white fluorescent lamps (Toshiba 40 W tubes) irradiate 3000 Lux.

The data was recorded after two subcultures (8 weeks' intervals) on the vegetative growth (shoot length cm, growth vigor, root number, and root length cm).

The growth vigor degree during the rooting stage, scored visually, as maintained above.

3. The chemical analysis of the elongated shoots of date palm during the rooting stage under the effect of different concentrations of Lithovit.

At the end of the rooting stage, data about the biochemical determination were carried out by using (fresh leaves) 1 gm from each sample of each treatment studied of the elongated shoots explants for each treatment, chemically analyzed for, the contents of (total sugars were followed by a procedure according to Shales and Schales (1945), the amino acids were determined on extracted samples according to Mc. Grath (1972), determination of plant chlorophyll was extracted and evaluated according to Wettstein (1954). Total Indoles were determined according to Larsen *et al.* , (1962), and Phenols were determined by the colorimetric method as described by Snell and Snell (1953).

Finally, the full intact plantlets from all treatments were transferred to the acclimatization stage to observe their growth in the greenhouse.

Statistical analysis

The collected data were statistically analysed according to Gomez and Gomez (1984), using Statistical Analysis Software, Release 9.4 (SAS Institute, North Carolina, USA) and the Duncan Multiple Range Test was applied to determine the least significance difference between the means (Waller and Duncan, 1969).

Results and Discussion

1. Effect of Lithovit concentrations on the shoot clusters proliferation during multiplication stage:

Data in Table 2 determined the vegetative growth (shoot number, shoot length, secondary embryos formation degree, verification degree and growth vigor degree of the date palm embryo clusters explants cv. Barhee, which cultured on the shoot proliferation medium with different concentrations of Lithovit for three subcultures indicated a statistically significant variation ($P \leq 0.05$) among different treatments. The highest value of shoots number per explant of date palm cv. Barhee, was by (T_2) 0.25 g/L Lithovit followed by (T_3) 0.50 g/L Lithovit (53.17 and 49.81), respectively while the control treatment was 32.41.

Table 2: Effect of Lithovit concentrations on the shoot clusters proliferation during multiplication stage for 3 subcultures.

	Shoot number	**Shoot length (cm)**	**Sec. Embryos formation degree**	**Verification appearance degree**	**Growth vigor degree**
T_1 (Lithovit 0.00)	32.41d (±0.08)	5.153c (±0.02)	4.21c (±2.90)	3.10c (±0.00)	4.18c (±0.02)
T2 (Lithovit 0.25)	53.17a (±0.01)	7.340a (±0.01)	5.27b (±0.03)	3.12c (±0.01)	4.80a (±0.01)
T3 (Lithovit 0.50)	49.81b (±0.02)	7.220a (±0.01)	5.18a (±0.01)	3.64b (±0.05)	4.77a (±0.03)
T4 (Lithovit 0.75)	43.97c (±0.38)	7.007b (±0.16)	4.98a (±0.01)	3.66b (±0.06)	4.57b (±0.02)
T5 (Lithovit 1.00)	32.48d (±0.03)	5.213c (±0.01)	4.92b (±0.01)	4.13a (±2.76)	4.15c (±0.02)
LSD 0.05	**0.5893**	**0.149**	**0.1427**	**0.1259**	**0.0402**

There is a statistically significant difference if the variation between means is higher than the LSD value (*P≤0.05*). Figures in brackets showed standard error within replicates.

The maximum shoot length value was observed when 0.25 g/L Lithovit followed by 0.50 g/L Lithovit (7.34 and 7.22). However, the minimum shoot length was 5.15 cm in (T_1) 0.00 g/L Lithovit. Secondary Embryos formation degree gave the highest value when the explants treated with (Lithovit 0.25) T_2 followed by (0.50 g/L Lithovit) and T_4 (5.27 & 5.18). Data regarding verification degree in Table 2, showed significant difference ($P \leq 0.05$) among various means of different treatment of Lithovit as Biofertilizer (0.0, 0.25, 0.50, 0.75 and 1.0 g/L), (T_1& T_2) 0.00 & 0.25 g/L Lithovit recorded lowest values (3.10 & 3.12), followed by (T_3 & T_4) 0.50 and 0.75 g/L Lithovit then T_5 (1.00 g/L) recorded the highest values verification degree 4.13. Whereas, the best result of growth vigor degree was recorded with (T_2) 0.25 g/L Lithovit followed by the results of (T_3) 0.50 g/L Lithovit (4.80 & 4.77). In this regard, Abd El-Aal and Eid (2018) founded that, application with growth stimulators as Lithovit at 500 mg/l as fertilizer material could be recommended in soybean cultivation for improving its growth, productivity, and quality (vegetative growth characteristics) i.e., the number of leaves, stem diameter, plant higher and leaf area per plant. Also, (El-Atabany, 2015) founded that spraying snap bean plants with micronized calcium carbonate significantly increased all measured growth aspects expressed as plant height, the number of leaves plant, fresh and dry weights as well as leaf area/plant compared with the control. Also, (Sadak *et al.* , 2015) reported that application of amino acids are well-known biostimulant which has positive effects on plant growth of faba bean plant besides they play a key role in secondary metabolism in plants, (Hildebrandt *et al.* , 2015), (Zewail, 2014) who demonstrated that application with amino acids at 4 ml/l increased estimated growth characteristics i.e., plant height, stem diameter, number of branches and leaves/ plant, total leaf area /plant, dry weight of shoots and specific growth rate of the common bean plant. (Maswada and Abd El-Rahman, 2014) Elevated CO_2 concentrations generally increase plant growth through increased carbon assimilation, biomass, and leaf area of plants because higher CO_2 can suppress ribulose-1, 5-bisphosphate (RuBP) oxygenase activity; decrease photorespiration, and increase carbon assimilates for plant growth. Among different combination treatments, Lithovit as Biofertilizer (0.0, 0.25, 0.50, 0.75 and 1.00 g/L). The effective concentration of Lithovit gave the highest values of vegetative growth of date palm shoots clusters on multiplication stage was 0.25 g/L (T2) Figure (2), followed by T3 (0.50 g/L Lithovit) when supplemented to multiplication medium compassion expect parameter verification appearance degree recorded the maximum value (4.13) when treated with T5.

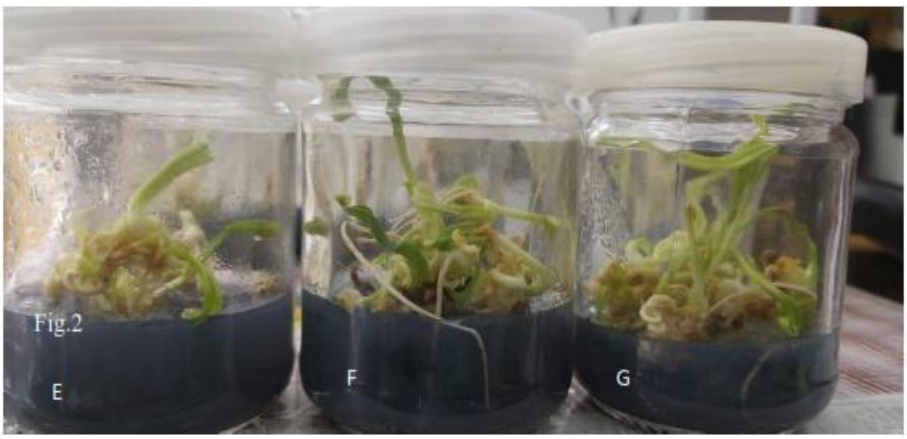

Fig. 2: The effect of lithovit treatments during multiplication stage (e) the lowest significant, secondary somatic embryos degree, shoot number, shoot length and vigour growth degree, on multiplication MS medium, with control treatment. (f,g). The highest significant secondary somatic embryos degree, shoot number, shoot length and vigour growth degree, on multiplication MS medium treatment with lithovit at (0.25 g/L)

2. Effect of Lithovit concentrations on the elongated shoots during the rooting stage:

Data in Table 3 showed the vegetative growth (shoot length, growth vegetative, root number, and root length) of the date palm shoot clusters explants cv. Barhee, which cultured on the rooting medium with different concentrations of Lithovit for tow subculture indicated a statistically significant variation ($P \leq 0.05$) among different treatments. The highest significant value of Shoot length was 16.80 cm of T_4

(0.75 g/L Lithovit) Figure (3), followed by (T_5) 1.00 g/L Lithovit (16.72 cm). The same trend was found with growth vigor, root number, and root length. The maximum values of growth vigor were (5.03), 8.27 of root number, and 5.84 cm of root length, respectively. However, minimum values of the same parameters were recorded with control treatment T_1 (0.00 g/L Lithovit) as follows (3.66, 5.32 and 5.55).

Table 3: Effect of Lithovit concentrations on the elongated shoots of date palm during rooting stage for 2 subcultures.

	Shoot length (cm)	Growth vigor	Root number	Root length(cm)
T_1 (Lithovit 0.00)	12.29c (±0.01)	3.66c (±0.01)	5.32c (±0.05)	5.55b (±0.01)
T2 (Lithovit 0.25)	13.75b (±0.03)	4.26b (±0.02)	6.50b (±0.01)	5.54b (±0.03)
T3 (Lithovit 0.50)	13.81b (±0.01)	4.32b (±0.02)	6.49b (±0.02)	5.59b (±0.01)
T4 (Lithovit 0.75)	16.80a (±0.06)	5.03a (±0.03)	8.27a (±0.02)	5.84a (±0.04)
T5 (Lithovit 1.00)	16.72a (±0.02)	4.33b (±0.06)	8.21a (±0.01)	5.87a (±0.01)
LSD 0.05	**0.0933**	**0.1142**	**0.0911**	**0.0781**

There is a statistically significant difference if the variation between means is higher than the LSD value (*P≤0.05*). Figures in brackets showed standard error within replicates.

Fig. 3: The best date palm shoots on rooting (MS) medium supplemented with lithovit at (0.75 g/L)

The obtained results are in agreement with (Abo Basha and El-Aila, 2015) reported that spraying the radish plants with fertilizer had a statistically effect on fresh & dry weights of root length, shoot, and root and diameter as well as nutrients content and uptake. This is maybe due to Lithovit particles that can enhance the growth of crops through increasing natural photosynthesis. Such results are connected with those reported by (Maswada and Abd El-Rahman, 2014; Agrawal and Deepak, 2003 and Wang *et al.* , 2013).

3. The chemical analysis of the elongated shoots of date palm during the rooting stage under the effect of different concentrations of Lithovit.

As shown in Fig (4-a), the application of T_4 (0.75 g/L Lithovit) was recorded the highest values (9.49) followed by (T_5) 1.00 g/L Lithovit (7.74) of total sugars, however, the minimum value was observed in the control treatment (T_1) 0.00 g/L Lithovit (6.91). The same observation was detected in amino acids, total chlorophyll, total indole, and total phenols (7.29, 37.79, 0.24 and 65.44), as shown in Fig (4-a), (4-b), (4-c), (4-d), and (4-e), respectively. (Maswada and Abd El-Rahman, 2014) showed that Lithovit treatment significantly increased total chlorophyll and total carotenoids of the wheat plant. But (T_1) 1.00 g/L of Lithovit reduced all the values of the same parameters above and were record (4.77, 29.31, 0.15 and 36.34). The presence of some of the compounds which enter as nutrients and in the vital processes needed by the plant increases and improves the ability of plants to form leaves and roots with an increase in length due to the increase of total sugars and increase of auxin with the lack of composition of phenolic compounds, of the efficiency and rapid development of plants in the of the rooting stage, the formation of adventitious roots was achieved using nutrient medium involving 40 g / l sucrose and full-strength MS basal salts within 4 subcultures and AC was applied in the last 2

subcultures. An average number of trees, length of trees, and leaves. The width was increased and reached up to 3-4 leaves/plantlet, 18-24 cm in length, and 3.5-6.9 mm in width. Also, most of the plantlets were able to grow 4-6 root/plantlet adventitious roots, which ranged to 6-8 cm with a thickness of 1.3-1.4 mm as reported by (Abul-Soad and Jatoi., 2014).

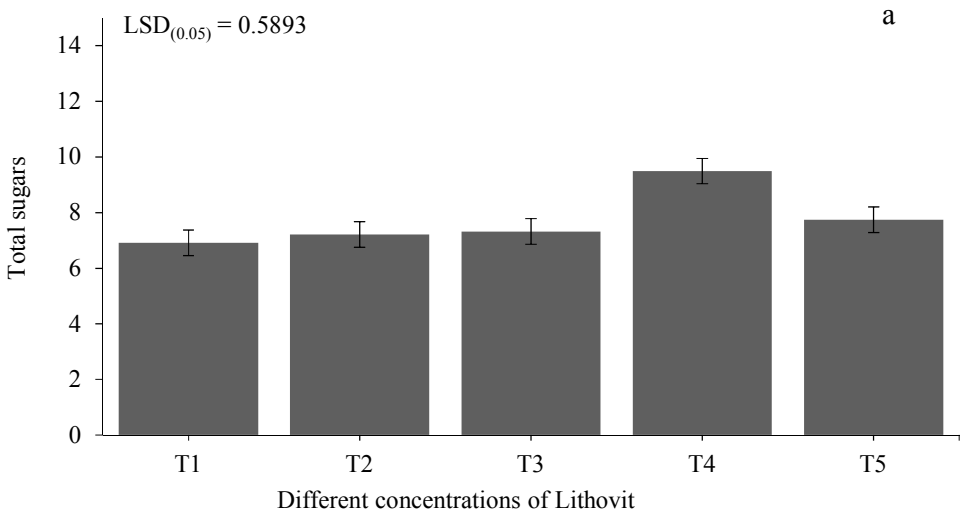

Fig. 4-a: Effect of different concentrations of Lithovit (0.0, 0.25, 0.50, 0.75and 1.0 g/L) on the total sugars of date palm cv. Barhee after 8 weeks. LSD was calculated at 5% probability and Y-bars indicated the sample variation.

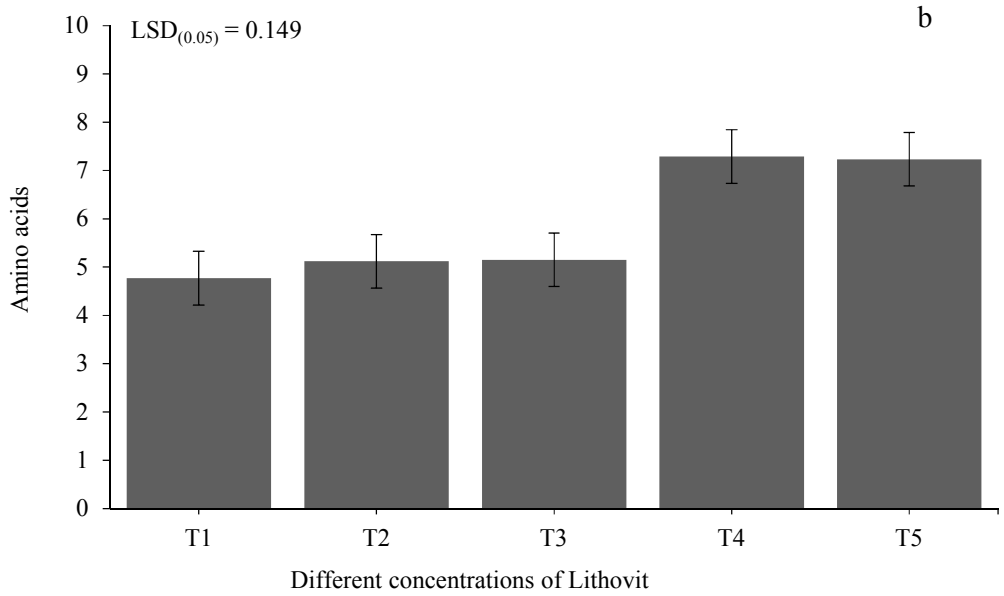

Fig. 4-b: Effect of different concentrations of Lithovit (0.0, 0.25, 0.50, 0.75and 1.0 g/L) on the amino acids of date palm cv. Barhee after 8 weeks. LSD was calculated at 5% probability and Y-bars indicated the sample variation.

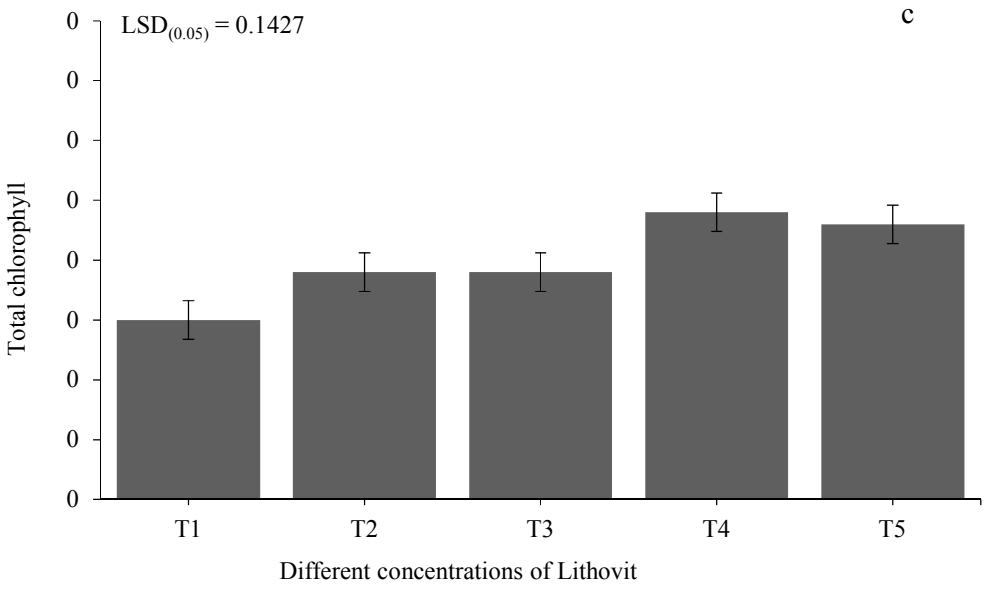

Fig. 4-c: Effect of different concentrations of Lithovit (0.0, 0.25, 0.50, 0.75and 1.0 g/L) on the total chlorophyll of date palm cv. Barhee after 8 weeks. LSD was calculated at 5% probability and Y-bars indicated the sample variation.

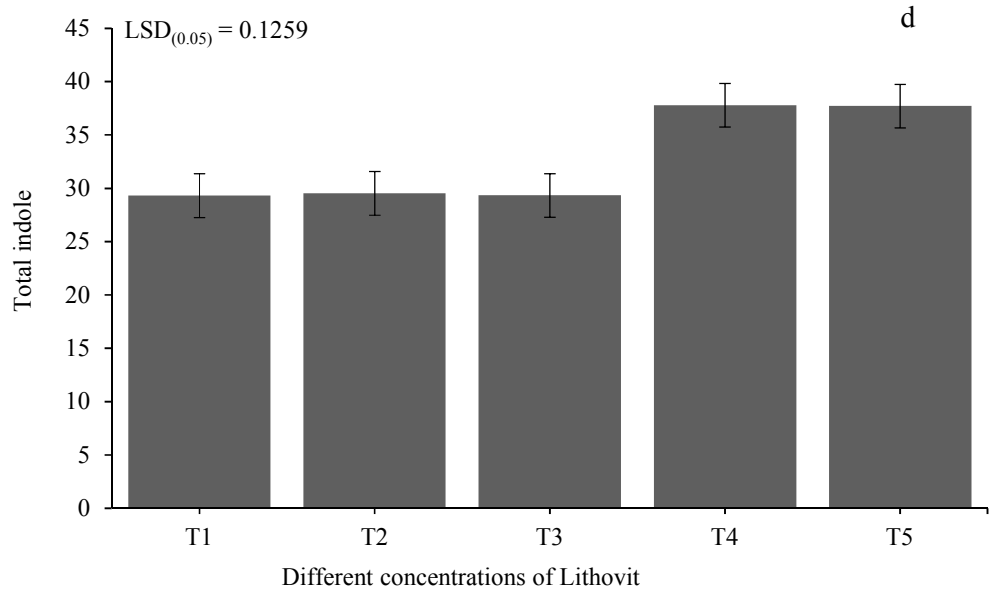

Fig. 4-d: Effect of different concentrations of Lithovit (0.0, 0.25, 0.50, 0.75and 1.0 g/L) on the total indole of date palm cv. Barhee after 8 weeks. LSD was calculated at 5% probability and Y-bars indicated the sample variation.

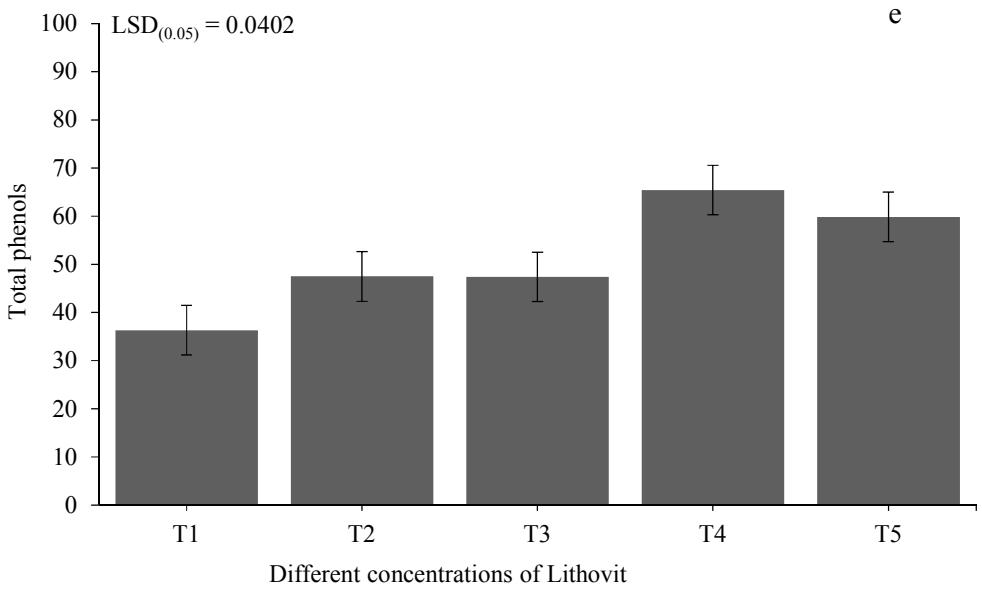

Fig. 4-e: Effect of different concentrations of Lithovit (0.0, 0.25, 0.50, 0.75and 1.0 g/L) on the total phenols of date palm cv. Barhee after 8 weeks. LSD was calculated at 5% probability and Y-bars indicated the sample Variation.

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