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Effect of Coconut Milk, Casein Hydrolysate and Yeast Extract on the Proliferation of *in vitro* Barhi Date Palm (*Phoenix dactylifera L.*)

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Abstract: This research was aimed at the enhancement of shoot regeneration via the supplementation of natural compounds on Date Palm cv. Barhi. Small clusters consisting of (2-3 shoots) were used as explants and cultured on MS medium supplemented with three types of natural additives at three different concentrations: (10%, 20%, 30% for Coconut Milk and1.25g/l, 2.5g/l, 5.0g/l for Casein Hydrolysate and Yeast Extract), in addition to the control (0.05 BA mg/l, 0.1 NAA mg/l). Results showed the most efficient secondary somatic embryo formation in association with casein hydrolysate 5.00g/l and that for the growth vigor, coconut milk was the most effective component. The Yeast Extract produced the lowest readings in all assessed concentrations. Chemical analysis was also performed that tested Chlorophyll 'A' and 'B', total Carbohydrate, Protein, Amino Acid, Phenol and Indole. Coconut Milk 30% and Casein Hydrolysate 2.5g/l gave the best overall results, both in test responsiveness and regenerative abilities.

Key words: Phoenix dactylifera L · Coconut Milk · Casein Hydrolysate · Yeast extract · Proliferation · Somatic embryogenesis

INTRODUCTION

Date Palm (*Phoenix dactylifera L.*, 2n = 2x = 36) is a dioecious, perennial, monocotyledonous fruit tree belonging to Arecaceae family and it is one of the oldest fruit crops cultivated in North Africa and the Middle East [1]. Its growth is primarily exclusive to tropical and subtropical regions. Economically, date palm provides a major source of income for local farmers and associated industries in communities where it is produced [2]. The date palm tree plays an important role in the development of sustainable agriculture in several drought and salinity-affected regions globally [1]. The traditional method of date palm propagation is by offshoots. However, this method presents a multitude of disadvantages. Offshoots are produced in a limited number and the survival rate of offshoots is low, with a high chance of infection attributed to the abundance of pests; the technique is also considerably tedious and quite difficult [3]. In view of the inherent limitations associated with date palm propagation, tissue culturing has become an attractive alternative for the mass propagation of date palm [4]. It possesses advantages such as the propagation of healthy selected female cultivars, producing males with superior pollen and large-scale multiplication with no seasonal effect [3].

After opting for tissue culture, researchers have attempted to investigate the various means of enhancing the shoot and root proliferation. Foremost among these means was the supplementation of natural compounds, such as Yeast Extract, Coconut Milk, Casein Hydrolysate, Coffee and Pineapple [5].The effects of using Yeast and plant extracts *in vitro* have been investigated by a number of researchers. Undefined components such as Fruit Juices, Yeast Extracts and Protein Hydrolysate were frequently used in nutrient media as opposed to defined vitamins or amino acids, or even as a further supplementation [4]. The medium's uniformity and reproducibility is a significant aspect during its preparation and the employment of natural products has demonstrated improved results. For instance, Coconut Milk and Banana Homogenate are frequently used as a popular addition to the media of orchid cultures in the floral industry of tissue culturing [6]. The use of natural compounds instead of plant growth regulators in culture media may reduce or omit the possibility of genetic

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instability in plants. Organic additives such as Casein Hydrolysate and Coconut Water have been used to increase embryogenic callus growth and somatic embryogenesis in several plant species as well as date palm [1].

Adding complex organic additives (COA) to culture medium date palm *in vitro* was shown to enhance callus formation and accelerate growth and development of somatic embryos. Opposing, (COA) caused an increase in the diameter of shoots and the strongest plantlets raise was achieved when cultured on 50.0 or 100.0 mg/l malt extract [3]. In a study to determine the effects of date palm syrup on somatic embryogenesis induction, El-Khateeb [7] found that such a natural extract could be used at a 6% concentration as a replacement for sucrose. Date palm meristematic tissues extract also enhanced date palm somatic embryogenesis [8]. The objective of the present study was to examine the effect of varying concentrations of complex natural additives such as Coconut Milk, Casein Hydrolysate and Yeast Extract, with the purpose of enhancing the *in vitro* date palm cv. Barhi shoot proliferation.

MATERIALS AND METHODS

Plant Materials: All experiments were performed at the Central Laboratory of Date Palm Research and Development in Giza, Egypt. All materials were obtained through that lab; explants were retrieved through their cultivars. Multiplied shootlets were obtained, collected and used as explants in the form of small clusters consisting of (2-3 shoots), then were developed and cultured to their rooting stage to monitor growth efficiency.

The Assessment of Natural Compounds on Shoot Proliferation: There were three assessed natural components analyzed at three different concentrations: Casein Hydrolysate (CH) at 1.25, 2.5 and 5.0 g/l, Coconut Milk (CM) at 10%, 20% and 30%; and Yeast Extract (YE) at 1.25, 2.5 and 5.0 g/l. These concentrations were supplemented to a typical nutrient growth medium; controls were prepared via culturing the same explants on the same media under the same conditions without any supplements the treatment is denoted by (M0).

All culturing procedures were carried out under aseptic conditions. The media prepared was composed of MS according to Murashige and Skoog [9] basal nutrient medium and vitamins, 170 mg/l NaH_2PO_4, 0.4 mg/l Thiamine HCl, 100mg/l Myo-Inositol, 30 g/l Sucrose; pH was dispensed into small jars of 150 ml (40ml/jar) before autoclaving at 121°C and 15 Ibs/in ^{2} for 20 minutes for effective sterilization.

Culture jars of each treatment were divided into three replicates. Each replicate consisted of three culture jars. Every jar contained one shoot cluster explant. Culture jars of each treatment, as well as the control samples, were incubated under light conditions with 1500 lux for 16 hours. They were then subjected to 8-hr dark conditions at 27 ± 2°C for the shoot multiplication stage. Sub-culturing was performed twice on the control samples and three times for the natural additives with their three different concentrations. All procedures were carried out in a decontaminated horizontal laminar flow hood.

Data Collection: After the two subcultures, data was collected for shoot number per cluster, shoot length per cluster in cm, number of secondary embryo formed per cluster, growth vigor per cluster, number of roots formed and the length of roots per cluster in cm. Data for the secondary embryo formation per cluster and growth vigor per cluster were scored visually according to Pottino [10] and Mujib *et al*. [11].

Chemical Analysis: One gram of the fresh weight Barhi samples were used from each concentration of the investigated natural additives' samples as well as the control samples. The examined tests included: Chlorophyll A and B, which was according to the method described by Lichtentaler and Wellburn [12], the Total Protein content, as demonstrated by Bradford [13].

Total Carbohydrates were tested in agreement with Dubois *et al*. [14]. Total Amino Acids, Phenol and indole were analyzed in accordance with Bates *et al*. [15], A.O.A.C. [16] and Larsen *et al.* [17], respectively.

Chlorophyll A and B Testing: 1 gm. of leaf samples was collected from each concentration of the three additives and the control for this experiment. 25 ml of acetone was added on each sample and mixed gently. Spectrophotometric analysis was carried out for chlorophyll A and B at 440 nm and 640 nm, respectively. The results were then treated through a predetermined calculated equation.

Protein Testing: This entailed the use of 1 gm of fresh weight of the plant sample added to 1ml of Tris buffer in an autoclaved jar. The samples were incubated for 2 hours at room temperature. Bred Ford working buffer were added at a content of 3ml. The solution was analyzed in a UV spectrophotometer at 595 nm and sterilized water was used as a blank.

Total Carbohydrates Estimation: The sample preparation was carried out using the same method as the amino acid estimation. Following the same incubation method, the volume was at that stage equivalent to 60 ml. Samples were left to dry and 10 ml of Isopropanol 10% were added. 1ml of alcohol, 1 ml 5% phenol and 5ml of sulfuric acid were supplemented to the samples and left for a 15-minute incubation period. Spectrophotometric analysis and calculations were carried out in the same manner and at a wavelength of 490 nm.

Amino Acid Testing: When analyzing amino acids, similar assessments were carried out where the identical quantity and nature of a sample was used, 20 ml of ethanol 80%; the samples were incubated in a water bath 70 °C. This step was repeated 3 times for 24-hours until the samples dried. 10% Isopropanol was subsequently added on the dry samples. 1ml from the solution was then added to 3ml of distilled water and 1ml Acetate buffer. The mixture was then incubated for 15 minutes at 70°C in a water bath. 10 ml of ethanol 50% was added and the solution was analyzed in a similar manner.

Phenol and Indole Testing: 10ml of 80% methanol was added in a similar manner that occurred in the protein tests. Upon overnight incubation, 0.5 ml Phenol was added to the sample, as well as 1ml of sodium carbonate and 17.5 ml of distilled water. The samples were incubated for an hour and analyzed at a wavelength of 730nm. The Indole test was accomplished via the supplementation of 1ml of the sample solution to 4ml of PDAB (Para-Dimethyl-Amino-Benzaldehyde) followed by incubation for 1 hour at 40°C. Spectrophotometric analysis was carried out at 540 nm.

Statistical Analysis: A factorial design in completely randomized arrangement was used and data were subjected to analysis of variance. Difference of means among treatments was determined using L.S.D. test at the 5% significance level according to Smith *et al*. [18].

RESULTS AND DISCUSSION

The effects of natural compounds on the *in vitro propagation* of Barhi Date Palm were evaluated in terms of shoot and root proliferation. The results showed a significant difference amongst the three sources (Casein Hydrolysate, Coconut Milk and Yeast Extract) when compared to the control samples. In Table 1, regarding the number of shoots, Casein Hydrolysate 2.5g/l and Coconut Milk 30% showed the highest results both measuring at (8.667/cluster); the lowest results were attributed to the Yeast Extract 5g/l sample (3.333/cluster). When assessing the shoot lengths in cm, the best results were acquired with Casein Hydrolysate 2.5g/l (0.766/cluster) and Coconut Milk 30% (0.800/cluster) which were quite close; the lowest results were acquired in Yeast Extract 5g/l (0.200/cluster). Concerning the Growth vigor, the highest results were with Casein Hydrolysate 5g/l (3.667/cluster) and Coconut Milk 30% (4.0/cluster); and the lowest measurement was with Yeast Extract 5g/l (1.00/cluster). These results illustrated grave significance and demonstrate efficiency with both Casein Hydrolase and Coconut Milk. When examining other factors such as the secondary embryo, the highest results were observed in Casein Hydrolysate 5g/l (10.0/cluster) and Yeast Extract 2.5g/l (3.00/cluster); and the lowest results were viewed the Yeast Extract 5g/l (0.33/cluster).

Table 1: Effect of natural additives on number of shoots, shoot length, growth vigor, secondary embryos, number of roots and root length.

Treatments	Concentration	Number of shoots	Shoot length (cm)	Growth vigor	Secondary somatic embryos	Number of roots	Root length (cm)
Control	0.05 BA, 0.1 NAA mg/l	3.333	0.4333	2.33	5.00	2	2.00
Casein Hydrolysate	1.25 g/l	5.333	0.5667	2.667	2.33	2	1.66
	2.5 g/l	8.667	0.7667	3.333	3.66	1.66	1.46
	5 g/l	5.667	0.3000	3.667	10.0	1.33	1.40
Coconut Milk	10 %	6.667	0.5000	3.0	2.33	1.66	1.30
	20 %	7.667	0.6667	3.667	2.00	1.33	1.66
	30 %	8.667	0.8000	4.0	2.66	1	1.40
Yeast Extract	1.25 g/l	5.000	0.3333	2.333	2.667	1.3	0.96
	2.5 g/l	4.000	0.2333	1.333	3.00	0.6	0.93
	5 g/l	3.333	0.2000	1.00	0.33	0.3	0.86
	L S D at 0.05	3.19	0.3473	0.8679	2.315	0.05	0.06

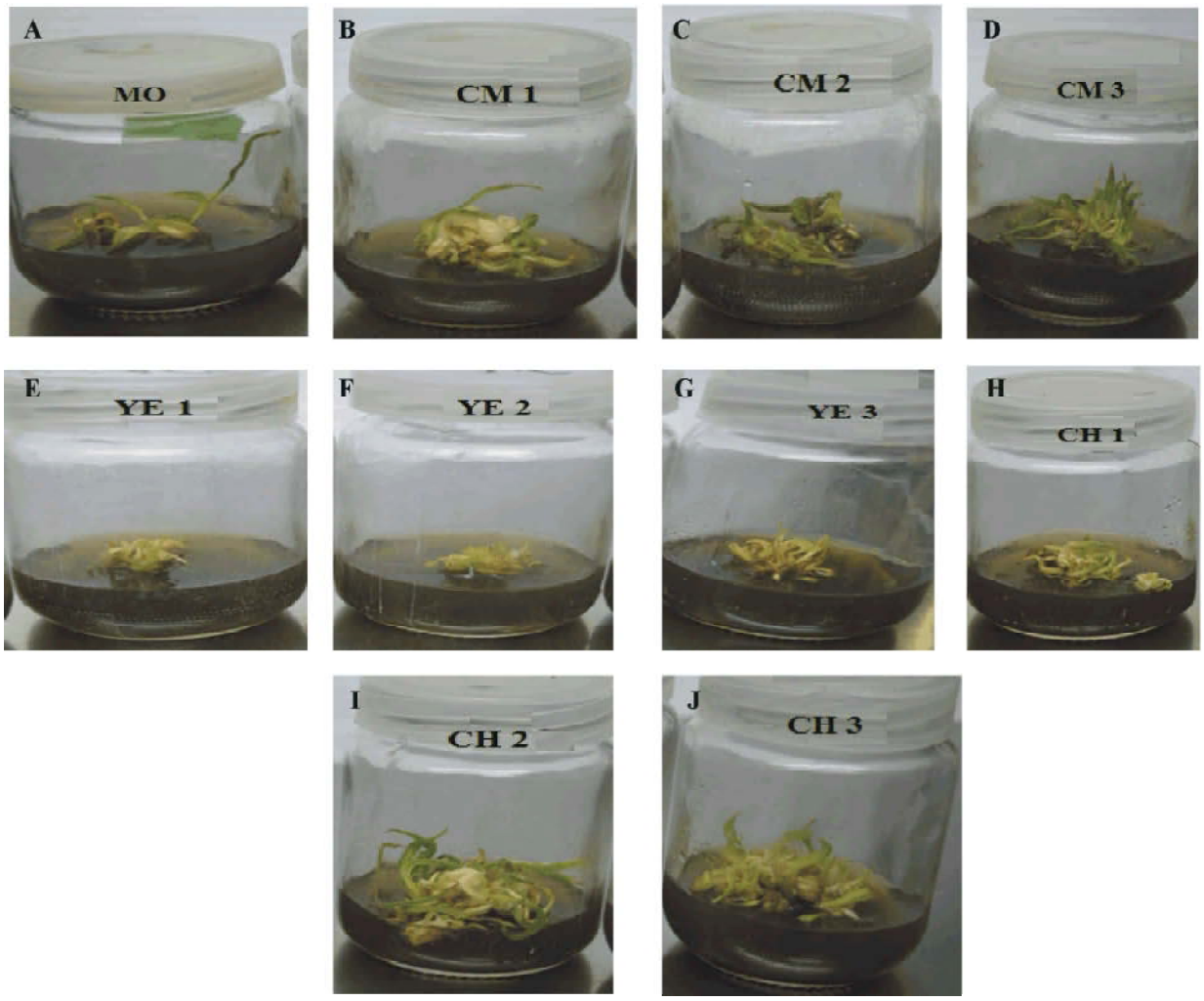

Fig. 1: Image representation of the results of natural compounds on the samples where: A MO signifies the Control, B CM1 is the Coconut Milk 10% sample, C CM2 is for Coconut Milk 20%, D shows Coconut Milk at 30%. E YE1 shows the Yeast Extract at 1.25g/l, F YE2 signifies Yeast Extract at 2.5g/l, G YE3 is Yeast Extract at 5g/l; H CH1 is Casein Hydrolysate 1.25%, I CH2 is for Casein Hydrolysate 2.5g/l and J CH3 is Casein Hydrolysate5g/l.

The number of roots showed ideal results in Casein Hydrolysate 1.25g/l (2/cluster) and the lowest result was accounted for in Yeast Extract 5g/l (0.3/cluster). When assessing root lengths in cm, the highest results were acquired and identical in casein Hydrolysate 1.25g/l and Coconut Milk 20% (1.66/cluster); the lowest results were in Yeast Extract 5g/l (0.86/cluster). Figure (1) demonstrates the results previously announced and determined where Casein Hydrolysate and Coconut Milk show the highest results in terms of growth, they are both quite evident as their proliferation is quite distinct when compared to both the controls, as well as the yeast extract samples.

Figure (2) is a collection of several graphic representations of the chemical analysis that was necessary in determining the overall health of the plantlets. A shows the results of Chlorophyll A and B Test, the highest results were observed in Casein Hydrolysate 2.5g/l and Yeast Extract 1.25g/l, while the lowest results were measured in Yeast Extract 5g/l. Data in Figure (2) B indicated that the highest results for the Total Carbohydrates were found in Coconut Milk 10%, 20% and Casein Hydrolysate 5g/l; the lowest results were found in Yeast Extract 5g/l.

Results presented in Figure (1) C for the Total Protein content showed that the highest figures were observed in Coconut Milk 10% and Casein Hydrolysate 5g/l. Figure (1) D were graphs where bars demonstrated the concentration of Amino Acids, which showed that

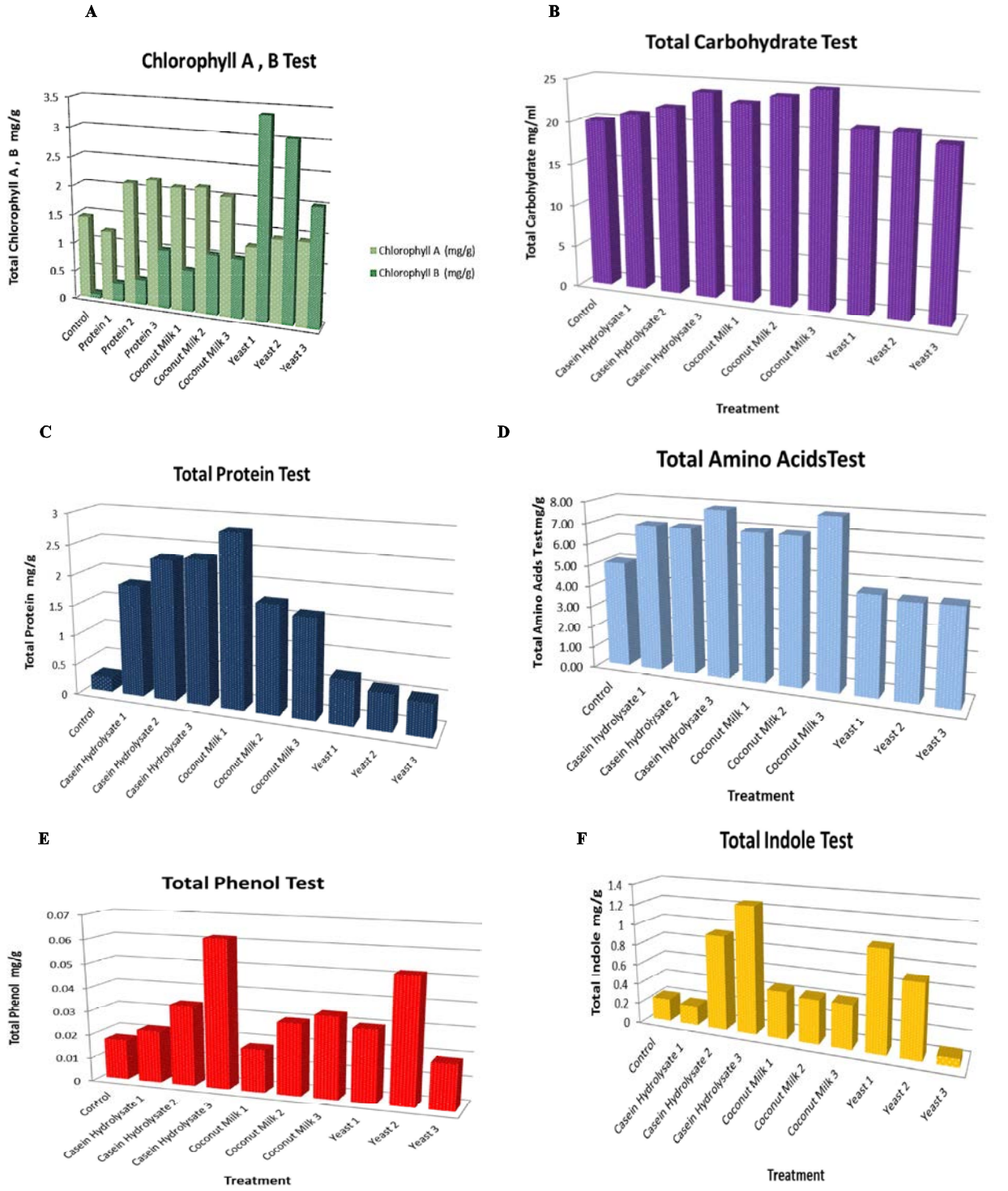

Fig. 2: Graphic representation of the results acquired from the chemical analysis tests, where (A) denotes chlorophyll A and B; (B) Denotes the total carbohydrate analysis; (C) represents the total proteins for the samples; (D) represents total Amino Acids; (E) Shows the total Phenol results and (F) Marks the total Indole tests. All measurements accounting for amounts were represented in mg/ml. The graphs include all investigated components and their subsequent concentrations.

Coconut Milk 30% and casein Hydrolysate 5g/l have given the highest results and Yeast Extract 2.5g/l showed the lowest content. Figure (1) E indicated that the most significant results in total Phenols, which was clearly shown in Casein Hydrolysate 5g/l and Figure (1) F confirmed the highest total Indole, which was found in Casein Hydrolysate 5g/l and Casein Hydrolysate 2.5g/l. while the lowest rank was denoted in Yeast Extract samples at 5g/l.

This investigation clearly established that natural compounds could stimulate proliferation. In vitro production of date palm via indirect organogenesis or somatic embryogenesis requires the application of relatively high concentrations of growth regulators such as 2,4-D or NAA for the initiation process [19, 20]. However, these auxins are known to be associated with genetic instability in plants [21, 22]. The use of natural compounds instead of plant growth regulators in culture medium may reduce or omit the possibility of genetic instability in plants. Organic additives such as Casein Hydrolysate and Coconut Water have been used to increase embryogenic callus growth and somatic embryogenesis in several plant species and date palm as well [4].

Since long-lived plants may develop mutants even in apical meristem and during the last few years, variations have been detected among in vitro date palm cultivars Barhi, Medjool and Khalas such as Dwarfism, delay in fruiting and fruiting set failure [23 - 25]. The effects of using Yeast and plant extracts in vitro culture have been investigated by a number of researchers. In media undefined components such as fruit juices, Yeast Extract and Casein Hydrolysate were frequently used in place of defined vitamins or Amino Acids, or even as further supplements. As it is important that a medium should be the same each time it is prepared, materials, which can vary in their composition, are best avoided if at all possible, although improved results are sometimes obtained by their addition [6, 26].

In many plants like *Cynbidium pendulum*31, *Phalaenopsis violacea*32 and *Paphiopedillum villosum.*33, other parameters like mean shoot length (4.72±0.06), Chlorophyll content (1.7±0.02), Protein content (22.4±0.08) and Carbohydrate content (22.5±0.01) were previously investigated by Sudipta *et al*, (2013), where superior results were acquired when 10% Coconut Water was supplemented in the multiplication media. Similarly, the use of Coconut Water in increasing the number of shoot, shoot length and number of nodes was also reported. Regarding the number of shoots and the shoot lengths, Casein Hydrolysate 2.5 g/l and Coconut Milk 30 % gave the highest results. Coconut Milk contains high protein content, while Casein Hydrolysate contains high Amino Acid and vitamin contents, so this proves that these natural additives increase cell division. In addition, both Coconut milk and Casein hydrolysate act as cytokinins, so they both affect the growth of shoots. These results are in accordance with Beshir *et al*.[5] where Coconut milk (5, 10, 20 cm) produced the highest number and length of shoots. Duhamet and Gautheret [26] stated that Coconut Milk are frequently used as a stimulator of cell division; this is due to the high Amino Acid content in Coconut Milk.

The results demonstrated that as the concentration of Coconut Milk increases, the number and lengths of shoot increase. This result is also in conformity with the work of Baque *et al*. [27], which shows that with the increase of Coconut Water concentration (10 to 50 ml/l) in the culture media, the shoot length, number of shoots and leaf area increase. The highest concentration, which was 50 ml/l of Coconut Water effectively, enhanced the plantlets growth when compared to the controls.

When assessing secondary embryo formation, Casein Hydrolysate 5g/l showed the most significant effects. This is due to the high Amino Acid content in Casein Hydrolysate, which leads to the increase in cell division. This concurs with Pelegrini [28], where the highest percentage of secondary somatic embryos induction (8.3 %) was observed in WPM culture medium containing 1g/l Casein Hydrolysate and the development of somatic embryos occurred.

Yeast Extract showed the lowest results regarding the number and length of both shoots and roots, compared with the control samples, Coconut Milk and Casein Hydrolysate samples as well. It showed also the lowest results in growth vigor. This proves that Yeast Extract does not have a great effect on the plantlets growth. According to Abraham *et al.* [29], Yeast Extract was used as a supplement in the proliferation medium, but it did not affect the shoot proliferation of *in vitro* plantlets of C.mangga.

These results agree with Zaid and De Wet [3] who reported that, all concentrations of Coconut Milk (50.0, 100.0 and 150.0 mg/l) and Malt Extract (100.0 mg/l) were effective for improvement and increase of secondary somatic embryos, while Casein Hydrolysate with various concentrations was ineffective. The data recorded the highest average number of shoots when Coconut Milk was added to the germination medium (3.77mg/l).

Supplementing various natural additives can increase the rate of *in vitro* multiplication. *In vitro* growth and regeneration of the plant can be improved by adding a small amount of organic nutrients to the medium [30]. Coconut Milk, the extract of white solid endosperm of mature coconut, is also used for inducing growth and morphogenesis without the need of a defined formulation [31].

The chemical analysis was used to investigate the effects of Natural Additives on the Metabolism inside the plant cells. Nasib *et al*. [32] reported superiority in 10% Coconut Water supplemented media. In the present work, the best chlorophyll A production was noticed in Casein Hydrolysate 2.5g/l due to its possessing the highest numbers and lengths or shoots. Yeast Extract 1.25g/l showed the highest results in Chlorophyll B, which was not expected. These results suggest a lack of correlation between shoot lengths and the percentage of chlorophyll in the leaves.

Carbohydrates are considered the fuel of metabolic and cellular respiratory processes. After analyzing their contents in the plantlets, it was found that the highest amounts were obtained in Coconut Milk 20% and 30 % and Casein Hydrolysate 5g/l. This could be attributed to the high protein and carbohydrates as the 240 gm include 13.3 gm. of it. Figure (2) B shows a correlation between the concentration of Coconut Milk and Casein Hydrolysate with the total carbohydrates; where both can be determined as directly proportional. Whilst understanding the prominence of carbohydrates for eukaryotic cells, these results demonstrate the role of Coconut Milk and Casein Hydrolysate in the enhancement of the growth of the plant. According to Sudipta *et al*. [33], the total Carbohydrate content (22.5±0.01) was found to be superior in 10% Coconut Water supplemented media, which positively corresponds to the study's findings. Regarding the presence of proteins, 10% Coconut Milk showed the most significant results which could be justified due to the presence of 5 gm of proteins in every 240 gm. Sudipta *et al.* [33] had similar results at (22.4±0.08) in 10% coconut water supplemented media which further justifies the findings. Due to the high Amino Acid content in Casein Hydrolysate, the highest result appeared in the 5g/l interval. Coconut Milk also showed high results, particularly in the 30% interval and that is attributed to the high protein content. Amino Acids play a significant role in the growth and development of living organisms including plants; a high amount is a demonstration of its health and readiness for further development.

Though Casein Hydrolysate 5g/l showed the highest result (0.06 mg/g), the value remained negligible. The low results observed from the Phenol Test showed that the used natural additives are beneficial to the *in vitro* Barhi samples and a continued growth is possible. Casein Hydrolysate 2.5g/l and 5g/l showed the highest results regarding total Indole content. Their amounts were found to be directly proportional, which suggests the presence of Indole in Casein Hydrolysate.

This test proved the reason why Casein Hydrolysate showed the highest number and length of roots, especially in Casein Hydrolysate 1.25g/l. Indoles are precursors for IAAs (Indole-3-Acetic acid), a type of auxin and auxins help in root formation and growth. Yeast Extract showed an inversely proportional relationship with indoles, which could be an indicator to least efficacy being attributed to them, where they acquired the lowest results in number and length of roots; McCubbin *et al.*[24] corroborates these findings.

Conclusions, Limitations and Further Work: Overall, the results demonstrate a conclusive significance in the supplementation of nutrient media with natural additives as growth regulators, capable of successful proliferation. It revealed Coconut Milk 30% and Casein Hydrolysate 2.5g/l as the most successful inducers and are recommended for the *in vitro* culturing of Barhi Date Palm (*Phoenix Dactylifera L.*). The markedly low figures of total phenols throughout the samples tend to usher a high probability of future continued growth of *in vitro* Barhi samples on cultured MS media supplemented especially with natural additives (Coconut Milk, Casein Hydrolysate and Yeast Extract). This study has provisioned successful results; however, the assessment of a larger interval of concentrations could have provided more significant results and enabled various statistical analysis techniques for the assessment of efficiency that was not possible with the present experiments. An acclimatization process and field analysis could also demonstrate more significant benefits.

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