

Effect of Natural Additives as Coconut Milk on the Shooting and Rooting Media of *in vitro* Barhi Date Palm (*Phoenix dactylifera* L.)

H.S. Ghazzawy^{1,2,a*}, S.F. El-Sharabasy¹

¹Date palm Research Center of Excellence, King Faisal University, Saudi Arabia

²Central Laboratory for Date palm Research and Development, Agriculture Research Center, Giza, Egypt

^ahishamdates@hotmail.com

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Abstract. The objective of the research study was to determine the effect of addition of different concentrations of three types of natural additives on Date Palm cv. Barhi: (1.25g/l, 2.5g/l, 5.0g/l for Casein Hydrolysate and 10%, 20%, 30% for (Coconut Milk and Yeast Extract), in addition to the control (0.05 BA mg/l) for shooting stage and (0.1 NAA mg/l, 3 g/l AC) for rooting stage. The results show that the use of 30% Coconut Milk achieved a high number of shoots and the highest shoot length was recorded with 10% Coconut Milk. In the date palm rooting stage, the results show that the use of 30% Coconut Milk increased the number of roots, shoot thickness and rooting percentage. However, root length was increased with 10% Coconut Milk. The lowest values were recorded with using Yeast Extract in this stage.

Introduction

Date palm (*Phoenix dactylifera* L.) has a great economical importance and agricultural uses throughout human's history. Also, it is one of the oldest cultivated fruit trees in the world. Date palm is a very important crop in the Middle East, since it can grow well in both semi-dry desert areas and the newly cultivated land. The production of Arab world of dates is about 80% of the total production of the world. Egypt is the world largest date producing country i.e. more fruitful female palms (1.5M tonnes/annum) produce 1.694.813 tons of dates [9], [7]. In Egypt, date palm trees distribution covers a large area extends from Aswan to north Delta, beside the Oasis of Siwa, Bahriya, Farafra, Kharga, Dakhla. Egypt is one of the most productive countries of dates in the world, the number of fruitful female palms in Egypt is about 15 million produce 1.694.813 tons of dates [9]. Date palm is commonly propagated by ground offshoots; however, a female date palm produces only 10-20 offshoots in its entire life [20], which is a limiting factor for the propagation of commercial cultivars. A non-conventional technique of *in vitro* culture is widely used in many species including date palm [14]. The production of plants through *in vitro* culture is successfully introduced in many species [23]. The technique of tissue culture for propagation date palm, also called *in vitro* propagation, has many advantages as large scale multiplication throughout the year, production healthy female cultivars, (disease and pest-free), or males having superior pollen; production of genetically uniform plants [19]. Recently, the natural products is using Yeast and plant extracts *in vitro* which have been discovered. Some undefined components such as Yeast Extracts, Fruit Juices and Protein Hydrolysate were frequently used in nutrient media as opposed to defined amino acids or vitamins as a further supplementation [4]. In addition, some other natural additives as Coconut Milk is frequently used as a popular addition to the media of orchid cultures in the floral industry of tissue culturing [5]. Natural extract could be



used at a 6% concentration as a replacement for sucrose [7] the utilization of natural additives compounds instead of hormones in culture media may decrease the possibility of genetic instability in plants. Organic additives such as Coconut Water and Casein Hydrolysate have been used to rise embryogenic callus growth and somatic embryogenesis in several plant species as well as date palm [6].

The aim of the research study was to determine the effect of different concentrations of combination natural additives such as Coconut Milk, Casein Hydrolysate and Yeast Extract, with the goal of enhancing the in vitro date palm cv. Barhi shoot and root proliferation.

Materials and methods

Explant and sterilization: The experiments were carried out in the Tissue Culture Laboratory for Date Palm Research and Development, Agriculture Research Center, Giza, Egypt. Four-year-old female offshoots of date palms cv. Barhi were collected and used as explants. Preparation of explants was done by removing the roots and outer green mature leaves from the offshoots, then reducing the size to less than 25 cm. remaining mature leaves were removed gradually from the bottom offshoot to the top in the laboratory [14]. The gradual removal of white young leaves and surrounding white fibrous leaf sheath resulted in 5 cm shoot tips, which were further trimmed to 2 cm for explant use. All excised shoot apexes were stored temporarily in an anti-oxidant solution (150 mg/l ascorbic acid and 100 mg/l citric acid) prior to surface-sterilization. Under aseptic conditions, shoot apexes were soaked in 70% ethanol alcohol solution for 30 seconds, followed by immersion in (1.0 g/l) of mercuric chloride for 5 min and thoroughly washed with sterilized distilled water for one-time. After that additional leaf primordial were removed from sterilized explants and then these explants were sterilized in 50%(v/v) commercial bleach (Clorox) 5.25% w/v, sodium hypochlorite NaOCl plus 1 drop Tween 20 for 15 min with rotary agitation, rinsed three times with sterilized distilled water.

Effect of different natural additives on shooting and rooting stages:

Shoots clusters which have been received from indirect somatic embryogenesis as recommended by (El-Dawayati et al, 2018) were used as explants in this experiment.

Different concentrations of three natural additives as follows: (1.25g/l, 2.5g/l, 5.0g/l for Casein Hydrolysate and 10%, 20%, 30% for Coconut Milk and Yeast Extract), were supplemented to a standard nutrient growth medium (control treatment without natural additives) for shooting and rooting. Control (treatments) were prepared by culturing the same explants on the same media under the same conditions without any supplements to study their effects on shoots development during shooting and rooting stages. All refined techniques were completed under aseptic conditions. Standard growth media preparation for shooting stage was composed of $\frac{3}{4}$ MS basal nutrient medium according to Murashige and Skoog with vitamins [16, 22], with addition of 100 mg/l Myo-Inositol; 80 Adenine Sulfate; 170 mg/l $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$; 0.3 mg/l Ca pantothenic acid; 0.4 mg/l thiamine- HCl; 2 glycine; 0.5 mg/l nicotinic acid; 0.5 mg/l pyridoxin-HCl; 100 myo-inositol; 30g/l Sucrose; 0.05 mg/l (BA) and 0.1 NAA mg/l growth regulators and 6 g/l Agar; 7000 [Agar-agar/Gum agar] (Sigma Chem. Co., St. Louis, MO) (in mg l^{-1}) [1]. Standard growth media preparation for rooting stage Also the same different three natural additives at different concentrations were added to rooting media which consist of the same components of previous standard growth media of shooting but supplemented only with 0.1 NAA mg/l growth regulator, with the addition of 1.5 g/l activated charcoal and 0.3 mg/l Ca pantothenic acid; 0.4 mg/l thiamine- HCl; 2 glycine; 0.5 mg/l nicotinic acid; 0.5 mg/l pyridoxin-HCl; 100 myo-inositol; 200 glutamine; 1g; 30000 sucrose; and 6000 agar.

The pH value was adjusted at 5.7 before adding agar gerlite and autoclaving the medium at 1.2 Kg.cm⁻² equivalent to 121°C for 20 min. The nutrient media was dispensed into small jars twenty-five ml of media for shooting stage. The plantlets were cultured in tube size (25 x 250 mm) each tube contained 25 ml for rooting stage. Explants of each treatment and control treatments were transferred and repeatedly recultured for 2 recultures every 8 weeks into fresh medium of the same composition. [10]. all samples were incubated for 16 hours under 1500 lux light conditions shooting stage and 3000 lux light conditions for rooting stage. They were then subjected to 8-hr dark conditions at 27 ± 2°C for the shoot multiplication stage. Subculturing was performed twice on the control samples and three times for the natural additives with their three different concentrations [4]. All procedures were carried out in a decontaminated horizontal laminar flow hood. The experimental design was completely randomized with three replicates in each treatment. Data recorded of 10 treatments were first analyzed as a whole using the aforementioned statistical design and then it was divided into groups as follows [14]:

Table 1, Different concentrations of three types of natural additives (1.25, 2.5, 5.0 mg.L⁻¹) for Casein Hydrolysate and (10%, 20%, 30% for Coconut Milk and Yeast Extract)

- T₁ Control 0.5 BA mg.L⁻¹ (Shooting stage).
- T₂ Control 0.1 NAA mg.L⁻¹ + 3AC g.L⁻¹ (Rooting stage).
- T₃ Casein Hydrolysate 1.25 mg.L⁻¹.
- T₄ Casein Hydrolysate 2.5 mg.L⁻¹.
- T₅ Casein Hydrolysate 5 mg.L⁻¹.
- T₆ Coconut Milk 10%.
- T₇ Coconut Milk 20%.
- T₈ Coconut Milk 30%.
- T₉ Yeast Extract 1.25 mg.L⁻¹.
- T₁₀ Yeast Extract 2.5 mg.L⁻¹.
- T₁₁ Yeast Extract 5 mg.L⁻¹.

Collected data for shooting were calculated by estimated the number of shooting, shoot length per cluster in cm and shoot thickens per cluster, number of roots formed rooting % and the length of roots per cluster in cm.

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. [11].

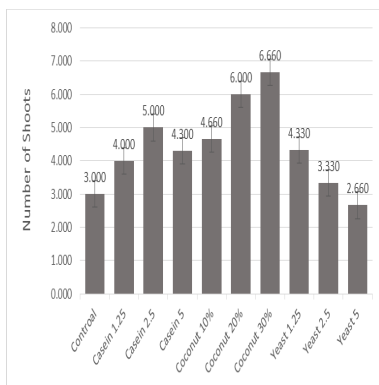


Fig (1): Effect of Coconut milk, Casein Hydrolysate and Yeast extract on No. of shoots of in vitro Barhi CV. (*Phoenix dactylifera* L.).

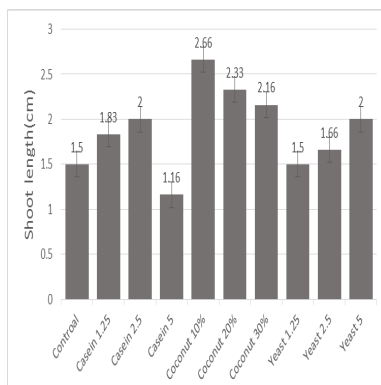


Fig (2): Effect of Coconut milk, Casein Hydrolysate and Yeast extract on shoot length (cm) of in vitro Barhi CV. (*Phoenix dactylifera* L.).

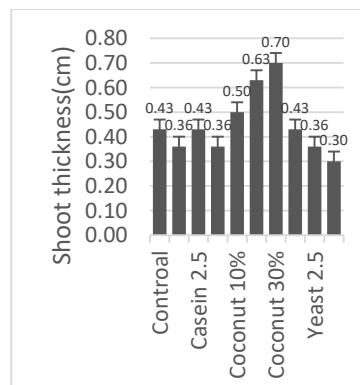


Fig (3): Effect of Coconut milk, Casein Hydrolysate and Yeast extract on (shoot thickness cm) of in vitro Barhi CV. (*Phoenix dactylifera* L.).

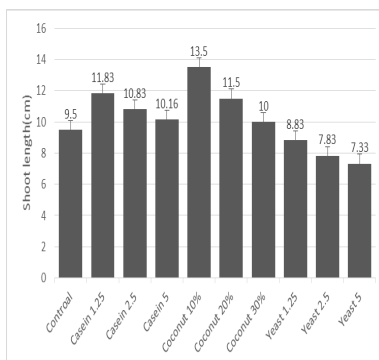


Fig (4): Effect of Coconut milk, Casein Hydrolysate and Yeast extract on shoot length (cm) of in vitro Barhi CV. (*Phoenix dactylifera* L.).

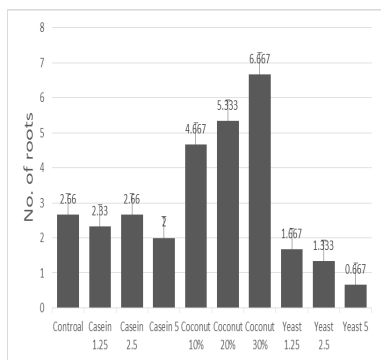


Fig (5): Effect of Coconut milk, Casein Hydrolysate and Yeast extract No. of roots on of in vitro Barhi CV. (*Phoenix dactylifera* L.).

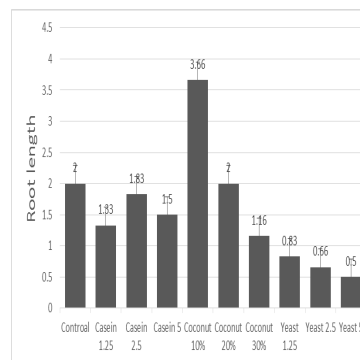


Fig (6): Effect of Coconut milk, Casein Hydrolysate and Yeast extract on root length (cm) of in vitro Barhi CV. (*Phoenix dactylifera* L.).

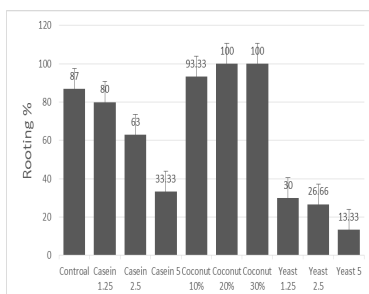


Fig (7): Effect of Coconut milk, Casein Hydrolysate and Yeast extract on rooting (%) of in vitro Barhi CV. (*Phoenix dactylifera* L.).

Results and discussion

Data in Fig (1) show the effect of addition of different concentrations of three types of natural additives on Date Palm cv. Barhi: (1.25g/l, 2.5g/l, 5.0g/l for Casein Hydrolysate and 10%, 20%, 30% for Coconut Milk and Yeast Extract), in addition to the control (0.05 BA mg/l mg/l) among different treatments regarding the number of shoots, Maximum increase in number of shoots with (6.66) was observed when Coconut Milk 30% (T7) added as addition to the control (0.05 BA mg/l) for shooting stage, followed by the same materials (T6) of Coconut Milk 20% and (T3) Casein Hydrolysate 2.5 g/l (5.00) *i.e.* T5, T4, T8, T2, T9, T1 and T10 (2.66). Data regarding *in vitro* shoot length (cm) showed that T5 (Coconut Milk 10%) had highest value (2.66) followed by T6, T7 were quite close (Coconut Milk 20% & 30%) among various means of different concentrations of three types of natural additives on Date Palm cv. Barhi the three sources (Casein Hydrolysate, Coconut Milk and Yeast Extract) when compared to the control samples. The lowest values were attributed to T4 (Casein Hydrolysate 5g/l) (1.16) then followed by T1, T8 (1.50) (Fig 2). Concerning the shoot thickness (cm) the highest results were with (T7) of Coconut Milk 30% (0.70) and (T6) of Coconut Milk 20%, then (T5) of Coconut Milk 10% (0.63 & 0.50), respectively as shown in Fig (3).

Shoot length (cm) (Fig 4) clarified gave demonstrate efficiency with T5 (Coconut Milk 10%) which recorded (13.50) followed by T2 (Casein Hydrolysate 1.25 g/l) and T6 (Coconut Milk 20%) (11.83 & 11.50) and the lowest results were viewed the Yeast Extract 5g/l (7.33). Higher value recorded for number of roots (6.67) was founded in T7 (Coconut Milk 30%) followed by T6 and T5 (5.33&4.66), respectively as shown in Fig (5). Results presented in Figure (6) when assessing root lengths in (cm), the highest results were acquired and identical in Coconut Milk 10% (T 5) and Coconut Milk 20%, control were quite close (2 cm), on the hand, lowest results were in Yeast Extract 5g/l (0.50). Regarding to Fig (7), the highest same values were cleared observed with three treatments (T1, T6 and T7) which recorded (100%), the lowest results were in Yeast Extract 5g/l (13.33). using of natural additives instead of plant growth regulators when added to culture medium may be gave minimum or reduce the possibility of genetic instability in plants [4]. Our results showed the potential use of natural additives to stimulate proliferation. Medium composition, genotype and plant hormones some factors, which affected on multiplication. [12] date palm cv. Maktoom showed higher shoot-bud multiplication in MS medium with a hormone combination of 1 mg L⁻¹ NOA, 1 mg L⁻¹ NAA, 4 mg L⁻¹ 2iP and 2 mg L⁻¹ BAP. Half-strength MS medium improved with 0.5 mg L⁻¹ NOA and 0.5 mg L⁻¹ Kin produced (23.5) shoot buds per explant after 3 months of multiplication in cv. Najda [13]. Average of production an of 18.2 buds per culture in cv. Hillawi, in the MS medium containing 1 mg L⁻¹ BAP and 0.5 mg L⁻¹ TDZ [2]. Many researchers [20] studied the effects of using plant extracts and Yeast *in vitro* culture. 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 essential that a medium should be the same each time it is prepared, materials, which can differ in their composition, are best avoided if at all possible, although improved results are sometimes obtained by their addition [4, 5, 15]. High protein content was founded in Coconut Milk, while high Amino Acid and vitamin were in Casein Hydrolysate, so this confirms that these natural additives increase cell division. Additionally, both Casein hydrolysate and Coconut milk act as cytokine, so they both affect the growth of shoots. These results are in accordance with [8]. Duhamet and Gautheret [26] declared that Coconut Milk are frequently used as a stimulator of cell division; this is due to the high Amino Acid content in Coconut Milk as mentioned with [17]. [18] Suggested 1 mg L⁻¹ NAA

induces optimum and better rooting at the same concentration IBA or IAA. Mejhoul cv. [3] reported that the shoots grew an average of (13.4 cm) with an average 4.6 roots number per shoot with wide and green leaves from (3 months) old hormone-free 1/2 MS medium strength. 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; [19] corroborates these findings. [17] declared that the most efficient secondary somatic embryo formation in association with coconut milk was the most effective component and 5.00g/l casein hydrolysate that for the growth vigor,. The Yeast Extract [2] produced the lowest readings in all assessed concentrations. In addition, chemical analysis was performed that tested Chlorophyll' A' & 'B', Amino Acid, total Carbohydrate, Protein, Indole and Phenol. In addition, the results showed that Coconut Milk 30% and Casein Hydrolysate 2.5g/l gaved the best results, both in test responsiveness and regenerative abilities.

Summary

Our findings validate the results in the supplementation of nutrient media with natural additives as growth regulators. It revealed Coconut Milk 30%, 20%, 10% then 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.).

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