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Enhancing the Acclimatization of In Vitro-Propagated Date Palm (*Phoenix dactylifera* L.) cv. Barhi Plantlets Using Coumarin

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Abstract

The current study was carried out in the Plant Tissue Culture Laboratory at the Date Palm Research Center, University of Basra from 2024 to 2025 to investigate the effect of different concentrations of coumarin (0, 15, 30 and 45 mgL^{-1}) on the improvement of acclimatization of Barhi date palm *Phoenix dactylifera* L. plantlets. The results revealed a significant effect of coumarin on the success rate of acclimatization, with the highest success soaring to 90% when treated with 45 mgL^{-1} of cou. Furthermore, the study revealed that the concentration of carbohydrates, proline and chlorophyll of leaves increased significantly in the 45 mgL^{-1} coumarin treatment as compared to the control. Incredibly, this concentration also resulted in the highest number of roots and zero percent contamination, better than 0 mgL^{-1} coumarin treatment.

Key words: In vitro ; Coumarin ; Acclimatization ; Contamination ; Date palm

Introduction

Phoenix dactylifera is a male and female (unisexual) dioecious tree. It is peculiar for palm species because it can give birth to offshoots. It is common throughout most of the world, ranging from 10° – 30° North and as far South as 20°. Its capacity to withstand extreme environmental conditions like high temperatures, dry winds and salinity of soil makes it remarkable (Ibrahim, 2013).

Sexual reproduction has not been deemed an appropriate way of commercial reproduction of date palms. Its use is largely limited to breeding initiatives, mainly because it cannot ensure the genetic fidelity of offspring to the parent plant. Moreover, in sexual propagation, about half of the descendants are male. On the other hand, offshoot propagation has been internationally acclaimed as the best option for vegetative reproduction of date palms, enabling the procurement of desirable cultivars (Al-Bakr, 1972). A notable decline in date palm populations has been observed in Iraq, especially in the city of Basrah. This degradation can be attributed to a combination of factors, especially prolonged wars, agricultural neglect, and harsh environmental stressors,

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which have collectively had a deleterious effect on the national economy (Al-Yasiri, 2018). In addition, several obstacles and challenges are associated with offshoot propagation, including the limited number of offshoots the mother palm produces during its initial stages of development (Al-Qatrani *et al*., 2023).

As a result, scientific research has focused on the use of tissue culture technology to produce numerous plantlets, including date palm apical meristem culture on synthetic nutrient media, which has been successful (Muhsen, 2004). Plant cell and tissue culture is a science that focuses on the In vitro culture of plant cells, tissues, and organs using specific techniques. These methods include the acquisition of plant parts to be used as culture (explants), their proper handling and sterilization, and the selection of appropriate culturing methods, nutrient media, and environments (Al-Miyahi, 2023). The use of substances that induce stress in the culture media of tissue-cultured plantlets, such as coumarin, polyethylene glycol, sucrose, and mannitol, plays a pivotal role in enhancing the acclimatization of these tissue cultures to external environmental conditions. Lowering the internal moisture content within these media is crucial, as it enables the plantlets to thrive under low-humidity conditions, thereby accelerating their adaptation to the outside world (Mohsen, 2013). Coumarin, a naturally-occurring phenolic compound in numerous plants, is especially known for its antioxidant activity, and is one of the major stress-inducing factors in the growth environment of plants (Stringlis *et al*., 2019).

Due to the absence of any research on the impact of coumarin in tissue culture and acclimatization of date palms, this pioneering study sets out to explore how this growth inhibitor can enhance the acclimatization of date palm plantlets.

Materials and Methods

Experiment Location

This study was conducted in the Tissue Culture Laboratory at the Date Palm Research Center, University of Basra, during the period 2024-2025. The objective was to improve the acclimatization of plantlets of date palms grown in vitro.

Medium Preparation

To improve the acclimatization of date palm plantlets, four concentrations of the growth inhibitor, coumarin (cou) (0, 15, 30 and 45 mgL^{-1}) were studied. The treatments were added to the nutrient medium which contained full strength MS salts, sucrose, selected amino acids, vitamins, activated charcoal, agar solidifier, Isopentenyl adenine (2ip) and Naphthalene acetic acid (NAA). The pH of the medium was carefully adjusted to 5.7 by measuring it with the digital pH meter. Following this, agar was added as the gelling agent. The medium was

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then heated to 95°C before being dispensed into glass culture jars, each holding 50 ml. The jars were sealed and sterilized in an autoclave at 121°C under a pressure of 1.05 $kgcm^{-2}$ for 20 minutes. After sterilization, the culture vessels were gently shaken to ensure uniform mixing of the medium's components and left to solidify until planting. Uniform plantlets, matched by leaf number and root and shoot length, were planted, with 10 replicates per treatment. The cultures were maintained in the nutrient media for one month to monitor the experiment's progress.

Planting or Transferring Cultures

The planting and transfer plantlets inside a Laminar Air Flow cabinet, where, before starting the process, the cabinet was sterilized by running ultraviolet (UV) light for 10 minutes to ensure a pristine, aseptic conditions. Following this, the cabinet was also wiped down with 70% ethyl alcohol, pre-sterilized with formaldehyde. To sterilize scalpels and forceps, a benzene burner and concentrated alcohol were used for flame sterilization, after which the instruments were cooled with sterile distilled water. All instruments, including culture tubes and Petri dishes containing the cultures to be transferred, were carefully sterilized before being introduced into the Laminar Air Flow Cabinet. The openings of the culture tubes were sealed and handled close to the flame to prevent contamination. Furthermore, the rims of Petri dishes and culture tubes were briefly passed through the flame to maintain aseptic conditions and ensure the prevention of contamination. Prior to culture establishment, dead roots and leaves were carefully excised, and plantlets exhibiting uniform growth characteristics, including length, leaf number, and root biomass, were selected. The plantlets were then inoculated onto the prepared culture medium at a rate of ten replicates per treatment and incubated under controlled environmental conditions for further growth and development.

Incubating the plantlets

After completing the planting process, the plantlets were incubated in the growth room at 27°C ± 2°C for 16 hours of light followed by 8 hours of darkness, with a light intensity of 4000 lux. The plantlets were kept in the growth room for 30 days, as represented in Plate (1) and were frequently observed and notes taken during this period.

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Picture (1): The plantlets Within the Growth Chamber

Acclimatization and Its Specific Growth Medium

Following one month of culture on the designated media, the plantlets were carefully excised from the culture vessels and thoroughly rinsed under running water to remove residual culture medium and nutrient deposits adhering to the root system. This cleaning step was considered crucial for reducing the incidence of bacterial and fungal contamination during the subsequent growth and acclimatization phases. Wilted leaves and damaged roots were gently removed before the plantlet were immersed in a sterilizing solution containing 1 gram per liter of Cuprosate for 10 to 15 minutes, a vital shield against fungal threats (see Picture 2) . Afterward, the plantlets were carefully extracted from the sterilization medium and planted into small pots (Torpedo) filled with a growth medium, a precise blend of peat moss and perlite mixed in a 1:3 ratio. In this medium a steam autoclave had been used to pre-sterilize it for 20 minutes at 121°C and 1.05 Bar of steam, after planting of the plantlets the growth medium was gently pressed around the roots to avoid creating air pockets. After completing the planting, the plantlets were watered with a half-strength nutrient solution derived from the sterilization salts, containing 0.5 grams per liter of Cuprosate. Finally, the pots were placed in the acclimatization chamber, ready for the next stage of growth. Wrapped snugly in agricultural polyethylene film, shaped into an arch like the frame in Picture (3), the torpedo-shaped seedlings were cradled in a nurturing germination chamber set at a cozy 28 ± 2°C. Bathed in a gentle glow of 1000 lux for 16 hours, followed by 8 hours of restful darkness, the plantlets thrived, drawing moisture from the humid embrace inside the plastic wrap. The cover was slowly removed after 10 days, which gradually decreased the humidity. Each week, these young plants were fed with a nutrient solution, and with distilled water when necessary, and they were safeguarded from contamination whenever it began by a

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gentle spray of sterilizing solution. After 45 days of this tender care the covers were completely lifted and the plantlets were carefully observed and notes were made about the traits to be studied.

Picture (2) plantlets immersed in the sterilizing solution (Cuprosate)

Picture (3) plantlets inside the acclimatization room, wrapped snugly within the agricultural plastic film

The success rate of acclimatization =Number of acclimatized plantlets/ Total number of plantlets x100

Estimating Leaf Tissue Content from Total Chlorophyll

The chlorophyll content in leaf tissue was measured at the Palm Research Center laboratories following the method described by Porra (2002).

Total Carbohydrate Content in the Leaf

The total soluble carbohydrates in the leaves, resulting from the effects of hardening and acclimatization processes, were estimated in the Palm Research Center laboratories. The following procedures were carried out based on fresh weight, according to the method of Watanabe et al. (2000).

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Total Proline Estimation in Leaves

The proline content in plant tissue was measured at the Palm Research Center laboratories, following the method of Bates et al. (1973).

Statistical Analysis:

The experiment was crafted as a straightforward trial following the Completely Randomized Design (C.R.D). Significance between means was tested using the Least Significant Differences test (L.S.D) at a 0.05 probability level, with 10 replicates per treatment (Al-Rawi and Khalaf Allah, 2000). The ready-to-use statistical software Genstat v. (2007) was employed to unravel the results.

Results and Discussion:

The Impact of Coumarin on Contamination Rates, Root Count, and Acclimatization Success

The results presented in Fig (1) show that the level of contamination was significantly affected by the addition of cou. As the concentration of cou increased, contamination rates dropped markedly. Treatments with 30 and 45 mgL^{-1} recorded the lowest contamination rate—an impressive 0% for both, compared to the control treatment, which suffered a 20% contamination rate. The treatment with 15 mgL^{-1} of cou had 5% contamination, which was not significant compared to the control.

As shown in the study results in Fig (1) the effect of the addition of cou on the count rate of the root was detected. As the concentration of cou increased, the number of roots also increased with 45 mgL^{-1} having an impressive 3.67 roots compared with an average of 2.33 roots for the control treatment. There were no significant differences between the means of the 30 mgL^{-1} treatment with an average of 3.00 roots.

The results in Fig (1) is quite interesting as it shows that grafting was more or less successful when conducted under different concentration of cou. The highest concentration of 45 mgL^{-1}, with a fantastic success rate of 90%, stood out as the crown jewel among all the treatments, even in the control treatment. In stark contrast, the comparison treatment barely reached a 20% success rate. Meanwhile, the 15 mgL^{-1} and 30 mgL^{-1} cou treatments achieved respectable success rates of 70% and 80%, respectively, each standing apart with their own meaningful impact.

Microbial contamination is one of the biggest challenges encountered in the tissue culture propagation of date palms and is the main barrier to successful and long-term propagation of date palms in tissue culture. (Ahmed,

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2023). Phenolic compounds play a heroic role in defending plants against pathogens, soaking up harmful UV rays and curbing the growth of rival plants (Taiz and Zeiger, 1998). Cou, in particular, reshapes root architecture by interacting with the polar transport of auxin, proven to suppress the primary root growth while sparking the development of lateral roots (Liu *et al*., 2025). Low and high concentrations of phenolic compounds were found to display the best efficacy in inhibiting the growth of several plant and pathogen species than the control (cultures without the phenolic compounds) (Bhattacharya *et al*., 2010). The results obtained in the present study are in agreement with those reported by Jassim *et al*., (2024), who harnessed cou to curb fungal pathogens lurking around the date palm, resulting in a remarkable boost in survival rates. It also correlates with Waikhom & Louis (2014) who investigated the rooting and acclimatization potential of two bamboo species Bambusa tulda and Melocanna baccifera under the conditions of acclimatization room with survival rate of 81.81% and 70.31% respectively. These results echo the insights of Li & Gao (2011) demonstrating that low concentrations of cou enhance the number of lateral roots and the length of root hairs. Furthermore, this study agrees with that of Bruno *et al*., (2021) with *Arabidopsis thaliana* (L.), showing that desetters may be the most effective method of controlling aphid populations. Heynh revealed the ability of the cou treatment to inhibit the growth of the primary root, but stimulate the growth of lateral roots, suggesting a mode of action similar to auxin.

L . S . D	Pollution Rate 6.66	Acclimatization Success 9.41	Number of Roots 0.941

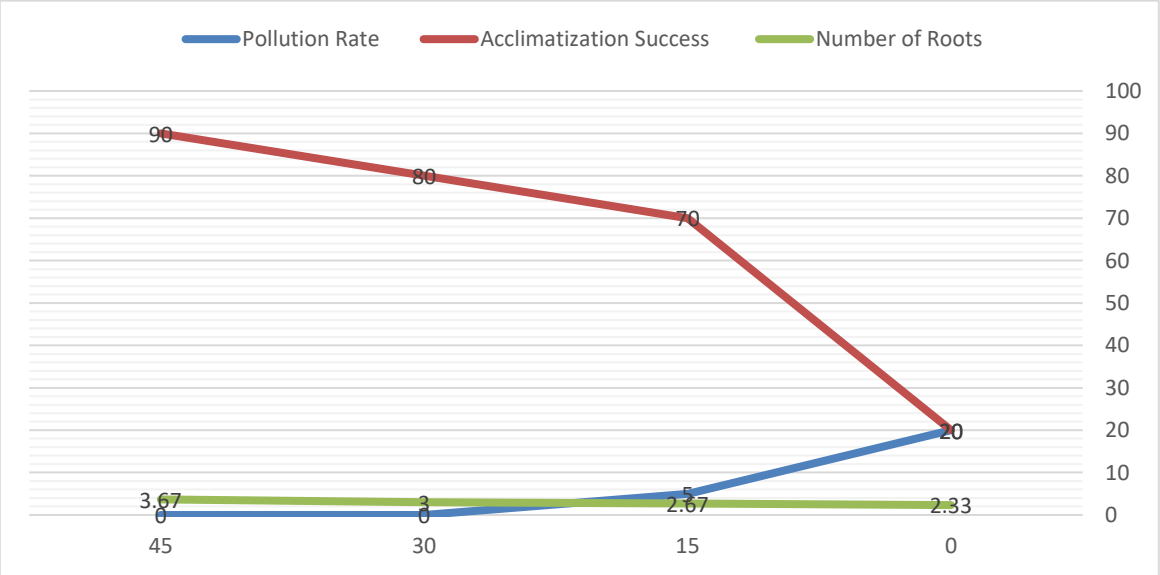

Fig (1) The Impact of Using Cou on Pollution Rate, Number of Roots, and Acclimatization Success

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The impact of using cou on the content of total soluble carbohydrates in leaves, as well as the amounts of free and bound proline, and chlorophyll levels before and after treatment.

Remarkable differences between the total carbohydrate contents of plantlets leaves was obtained from the statistical analysis results presented in Fig (2) as a result of the treatment with cou. The brightest treatment was the one with the highest concentration of the cou (45 mgL^{-1}), which outperformed all other treatments with the highest leaf total carbohydrate content of 20.783 $mg100g^{-1}$. The control treatment, however, was the last to make progress with a carbohydrate level of only 9.950 mg per 100g.

Fig (2) shows that the application of cou caused an immense increase in the content of proline in leaves. Treatment with 45 mgL^{-1} of cou was found to be significantly high with leaf proline value of 29.983 micrograms per gram. However, the leaves of the control treatment had the least amount of proline with 11.197 micrograms per gram.

Fig (2) reveals that the use of cou had a significant impact on the chlorophyll content in leaves before germination. The treatment with cou at a concentration of 45 mgL^{-1}stood out, significantly surpassing the others and delivering the highest boost in chlorophyll content, reaching 1.083 mg per gram of fresh weight. The same trait registered significant values for the same treatments (15 and 30 mgL^{-1} of cou) with values of 0.807 and 0.950 mgg^{-1} of fresh weight, respectively. The control treatment, however, was the last to catch up with the others, with the lowest chlorophyll concentration of 0.783 mgg^{-1} FW.

Fig (2) illustrates the impact of cou on the chlorophyll content in leaves after rooting. The treatment with 45 mgL^{-1} of cou was significantly different from all the other treatments and even higher than the control with an average of chlorophyll content 3.830 mgg^{-1} fresh weight. The control treatment, on the other hand, showed a significantly lower level of chlorophyll 1.900mgg^{-1} fresh weight. Notably, the cou concentrations of 15 and 30 mgL^{-1} also demonstrated a remarkable edge over the control, boasting chlorophyll levels of 2.273 and 3.060 mgg^{-1} fresh weight, respectively.

Under stress, the phenolic compounds are important because the phenolic acids and cou are involved in plant stress resistance processes through growth processes developmental aspects and other mechanisms (Lattanzio, 2013). A mechanism to achieve this is osmotic regulation, which is done by accumulating the dissolved substances (Rhodes *et al.,* 2002).

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These are molecules which are low in molecular weight and they concentrate and become high in cytosol without any damages to cell. They contain sugars, sugar alcohols and proline (Shao *et al.,* 2009). These substances not only boost the cell's osmotic pressure and help maintain its turgor, but they also shield the cell's compartments from oxidative damage (Sekmen *et al.,* 2014). The rise in chlorophyll levels likely highlights the crucial role these compounds play in enhancing the plant's physiological resilience and reducing oxidative harm to the chloroplasts. Cou, at low to moderate concentrations, stimulates antioxidant activity, which in turn curbs the buildup of free radicals and preserves the integrity of chloroplast membranes, thereby preventing the breakdown of chlorophyll pigments. This leads to the continuous increase in the photosynthetic efficiency and chlorophyll content in the leaves. At high concentrations, however, it may have inhibitory effects (Sultana *et al.,* 2022).

The results agree with Li *et al*., (2013) who emphasized the effect of some phenolics on the accumulation of carbohydrates in leaves of cucumber under stress conditions. In the same way, the findings of this study also corroborate the findings of Sultana *et al*., (2022) who found that cou increased the amount of chlorophyll pigment in the leaves of maize. The findings of this study are in line with those of Araniti *et al.,* (2017) who investigated the effect of cou on A. thaliana, which also showed enhanced proline and carbohydrate levels in leaves. Furthermore, the findings were comparable with those of Tao *et al*., (2019) who reported a reduction in the carbohydrate level with increasing coumarin level. Last Saleh & Madany (2015) showed the effects of cou treatment on wheat plants on increasing the amount of carbohydrates and proline in the leaves, which was a perfect match with the current results

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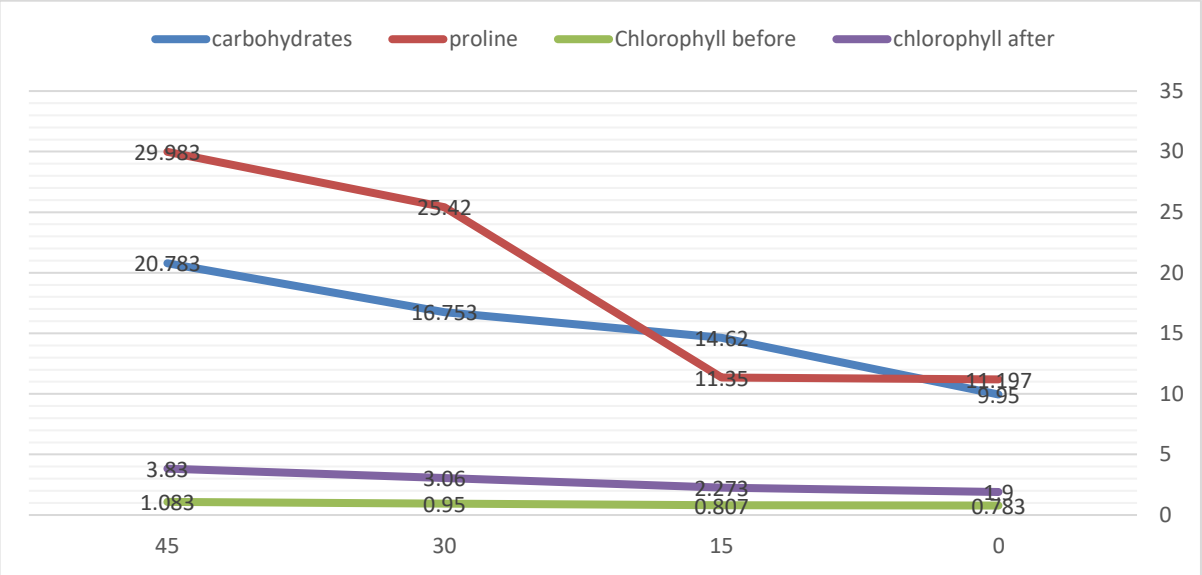

L . S . D	0.5525Carbohydrates	proline 0.1230	Chlorophyll before 0.0918	0.1481 Chlorophyll after

Fig (2) The impact of cou use on the total soluble carbohydrate content in leaves (mgg^{-1} fresh weight) and the average amount of free and bound proline in leaves (µgg^{-1} fresh weight), along with the average chlorophyll content in plantlets leaves (mgg^{-1} fresh weight) before and after acclimatization.

Conclusions

From the current study, we conclude that adding cou to the culture medium for growing date palm tissue cultures before acclimatization significantly enhanced acclimatization rates, carbohydrate and proline content, as well as chlorophyll levels both before and after acclimatization, alongside improved rooting percentages. It also reduced contamination rates in plantlets. Therefore, this study recommends conducting further research on other date palm varieties and different plant species using varied concentrations of this compound.

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