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Research Article

Embryogenic Callus Induction and Regeneration Efficiency in Date Palm

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ABSTRACT

The date palm (*Phoenix dactylifera*) is widely cultured for its medicinal and nutritional value as an edible fruit. Traditional vegetative propagation of date palm is time-consuming, requiring several years. However, modern biotechnological techniques, including plant tissue culture, have revolutionized mass production by significantly reducing the time required. Plant tissue culture involves using small tissues to grow and regenerate entire plants in an artificial (*In Vitro*) environment under sterile laboratory conditions. This study aimed to enhance the regeneration efficiency and induction of embryogenic callus in date palm. Callus induction was carried out using suckers of the date palm on Murashige and Skoog (MS) media with various concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D) in combination with different plant growth regulators (PGRs), including 2-isopentenyladenine (2iP), naphthalene acetic acid (NAA), benzylaminopurine (BAP), and indole acetic acid (IAA). Experiments were conducted in triplicate, with each hormonal treatment including three explants per replication. The best media combination for callus induction was MS + 2,4-D, 1.5 mgL^{-1} + 2ip 4 mgL^{-1} for variety Dakki and MS + 2,4-D 1.5mgL^{-1} + 2ip 5 mgL^{-1} for variety Hillavi in datepalm. Callus regeneration for Dakki was observed under treatment 3 containing MS + 3 mgL^{-1}IAA + 1.5mgL^{-1} BAP took minimum days for callus regeneration. Hillavi took minimum days for callus regeneration under treatment 9 contains MS + 2ip 6 mgL^{-1} + IAA 1mgL^{-1}. Hillavi took minimum days for shoot formation under treatment 2 contains MS + 1.5mg/L BAP + 1.5 mg/L IAA and Dakki took minimum days under treatment 8 containing different harmonal combination MS + 1mg/L IAA + 5 mg/L 2ip. Dakki took a minimum of days for new root formation under treatment 9 MS + 5 mgL^{-1} NAA + 4 mgL^{-1} IBA+4 mgL^{-1} IAA and Hillavi took minimum days under treatment 2 MS + 1 mgL^{-1} IAA + 1mgL^{-1} IBA. Data were analyzed using analysis of variance (ANOVA) under a completely randomized design, and means were compared using the least significant difference (LSD) test.

Keywords: Micropropagation, Embryogenic callus, Suckers, Date palm, In-vitro, Plant hormon**es**.

INTRODUCTION

The date palm's special qualities are appropriately referred to as the "Tree of Life. "It considered one of the oldest plants and is widespread throughout the Middle East, North Africa, the Southern Sahel, areas of East and South Africa and even certain parts of Europe and the USA. The date palm belongs to the family *Arecaceae*, which includes 183 genera and 2,600 species, including the bisexual (2n=36) and monocotyledon fruit plant (*Phoenix dactylifera* L.) [1]. In the past, date palms were cultivated throughout the prehistoric era . In date palm, it is easy to identify seedlings, plants produced by sexual reproduction, and offshoots taken from healthy female and male plants. The date palm, scientifically known as *Phoenix dactylifera* L., holds a significant position as one of the most important crop in horticulture globally and stands as one of the first cultivated tree crop [2].

The only tree that bears fruit is a female tree, and 50 female trees may pollinate one male tree. On the other hand, the effects of pollen on date quality through meta xenia have been extensively studied, and male genotypes with favorable features are commonly preserved in plantations and used for manual pollinating female plant [3]. Date palms were

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conventionally propagated through suckers although this method is slow and has a high risk of disease transmission with a poor survival probability for a single date palm tree [4]. The process of inducing callus in date palm offers multiple benefits, including the generation of pathogen-free plants, swift production of clones, and enhanced yield featuring nutritionally superior and advantageous variants [5]. Over the past few decades, traditional propagation techniques have been employed for standard varieties. However, these methods are proving inadequate to address the rising needs of a larger global population [6]. For the purpose of breeding and cultivating, growth regulators that are devoid of mutational effects are necessary. The outcomes demonstrated through the equilibrium between auxins and cytokines. Growth regulators, plant hormones, intracellular genes, and chemicals play a role in determining a plant's life because of inherent variables [7].

Nevertheless, the application of PGRs has required adjustment, taking into account the potential risk of inducing soma clonal variations [8]. In this study, different PGR concentrations were used to optimize a healthy callus from a date palm explant. Optimized growth medium can be utilized to generate new plants from a small section of plant tissue. The growth regulators have influenced the callogenesis and somatic embryo development processes in the date palm [9]. The use of growth regulators, the genotype, and the presence of an ex-plant all affect a cell's capacity to go through primary callogenesis. Unlike the tissues obtained from the base, the foliate sections collected from the ex-plant are optimal for callus formation. Primary callogenesis results in the formation of a globular compact callus. Plant tissue culture results in somaclonal variation, which is a variation in plants that arises from the proliferation of cells [10]. The inadequate effectiveness of organogenesis in date palms was primarily caused by tiny tissues of develop shoot lets. Growth of explants may depend on the genotype [11]. Meristematic tissues on the cut or wound surface of explants give rise to the callus, which can develop spontaneously. Incubation conditions, growth regulator addition, and medium type all affect callus induction [12]. By expanding propagation methods, it is also possible to increase the number of successful date palm offspring, which will increase the number of offspring produced by these procedures and will be essential for future cultivation efforts [13]. During *In vitro* micro-propagation shoot tips were treated more than the palm leaves. Juvenile leaves were used by embryogenic suspension cultures. Leaf segments from seedlings are used for callogenesis and rhizogenesis plantlet cultures [14]. During in vitro regeneration, 2,4-Dichlorophenoxyaceticacid has been applied to stimulate the most frequently utilized auxin in plants [15]. In this study, we have successfully optimized callus induction and regeneration for date palm varieties. It guarantees the quick development of disease-free plants. It also promotes sustainable agriculture practices by increasing yield potential and adaptability to environmental challenges.

Materials and Methods

Explants of two years old date palm varieties (Hillavi and Dakki) have been collected from Agriculture Mechanization Research Institute (AMRI), Multan. Collected suckers were rinsed under tap water for 10 to 15 minutes and then cut into small segments. Finally, sterile and disinfected explant segments were placed over the callus medium. Various concentrations of plant growth regulators were used. Data was examined based on characteristics such as the number of days taken for callus induction as well as callus shape. Three replications of each treatment and one control have been used for Days to Callus emergence (DCE), Days to Callus Regeneration (DCR), Days to new Shoots Emergence (DSE) and Days to new Roots Emergence (DRE). Complete randomized design was used in order to assess how different date palm varieties interact on various hormonal treatments. LSD was used to compare data means. Each replicate consisted of three explants. To check regeneration efficiency, callus formation and growth medium of a total of nine explant with different hormone levels were examined (Fig 1a and b).

Fig 1. (a) Sterilization of Ex-plant **(b)** Excision of Explant

Explant Inoculation

The laminar flow was treated with ethanol for disinfection. The disinfected forceps were placed in the laminar airflow. During this procedure, the small pieces of explants were placed vertically on the medium. After inoculation of ex plants, placed them into a growth room under controlled conditions in terms of temperature and light (Fig 2).

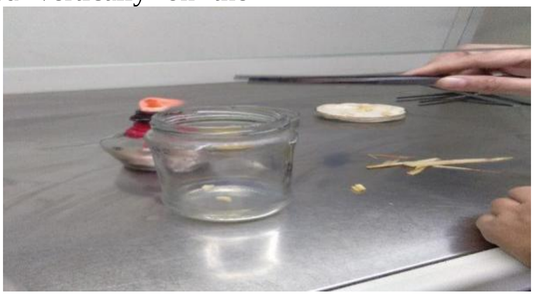

Fig 2. Culturing of Date Palm

Protocol Optimization for Sterilization of Explants

Sterilization of explants is a mandatory step to avoid contamination. Explants were cleansed with varying concentrations of mercuric chloride under different durations as shown in (Table.1).

Table 1. Different Concentration of Mercuric Chloride

Treatments	$HgCL_2$ Concentrations(%)	Time (Minutes)
T 1	Hg Cl_2 0.1%	5
T 2	Hg Cl_2 0.1%	10
T 3	Hg Cl_2 0.1%	15
T 4	Hg Cl_2 0.5%	5
T 5	Hg Cl_2 0.5%	10
T 6	Hg Cl_2 0.5%	15

Callus Induction

Two varieties of date palm (Hillavi and Dakki) were used for callus formation and regeneration. We used different treatments with varying doses of plant growth regulators. Nine different treatments were used for optimization of callus induction in two varieties of date palm. Details of the treatments were given in (table. 2).

Table 2. Protocol Optimization for Callus Induction

Treatment	Hormone Combination	Concentration mg/L
T0	MS	4.33 mg/L
T1	MS+ 2, 4-D + BAP	1 .5mgL + 0.6 mg L^{-1}
T2	MS+2, 4-D + BAP	1 .5mgL + 0.8 mg L^{-1}
T3	MS+ 2, 4-D + BAP	1 .5mgL + 1 mg L^{-1}
T4	MS+2, 4-D + NAA	1 .5mgL + 2 mg L^{-1}
T5	MS+2, 4-D + NAA	1 .5mgL+ 2.5 mg L^{-1}
T6	MS+2, 4-D + NAA	1 .5mgL + 3 mg L^{-1}
T7	MS+2, 4-D + 2ip	1 .5mgL + 3 mg L^{-1}
T8	MS+2, 4-D + 2ip	1 .5mgL + 4 mg L^{-1}
T9	MS+2, 4-D + 2ip	1 .5mgL + 5 mg L^{-1}

Under the T0 treatment, only Murashige and Skoog (MS) basal salts were used. For T1 to T3 a combination of 2, 4-D and Benzyl amino purine was used in concentration of 1.5mgL 2,4-D and BAP 0.6 mgL^{-1} to 1 mgL^{-1}. For the treatment T4 to T6 combination of 2,4-D and NAA used with concentration of 1.5mgL^{-1} 2,4-D and 2mgL^{-1} to 3mgL^{-1} NAA. For the treatment T7 to T9 combination of 2,4-D and 2ip used with concentration of 1.5mgL^{-1} 2,4-D and 3mgL^{-1} to 5mgL^{-1} 2ip. Data was collected using the following parameters

- Days to callus formation
- Shape of callus

Callus Regeneration

After induction of callus of date palm from sucker different growth hormone combinations were used for regeneration (Table 3).

Under the control treatment, only Murashige and Skoog (MS) basal salts were used. For the T1 to T3 a combination of indole acetic acid (IAA) was used in concentration 0.5mgL to 3mgL^{-1}and and benzyl amino purine (BAP) was used in concentration 0.5mgL^{-1} to 1.5mgL^{-1}. For the treatment T4 to T6 combination of benzyl amino purine and 2ip used with concentration of 1mgL^{-1} to 3 mgL^{-1} BAP and 1mgL^{-1} to 3mgL^{-1} 2iP. For the treatment T7 to T9 combination of 2ip and indole acetic acid used with concentration of 4mgL^{-1}to 6mgL 2ip and 0.5mgL^{-1} to 1mgL^{-1} indole acetic acid.

Table 3. Protocol for optimization of Callus Regeneration

Treatment	Hormone Combination	Concentration mg/L
T0	MS	4.33mg/L
T1	IAA + BAP	0.5mgL + 0.5mgL^{-1}
T2	IAA + BAP	2mgL + 1mgL^{-1}
T3	IAA + BAP	3mgL + 1.5mgL^{-1}
T4	BAP + 2ip	1mgL + 1mgL^{-1}
T5	BAP + 2ip	1.5mgL+ 2mgL^{-1}
T6	BAP + 2ip	3mgL + 3mgL^{-1}
T7	2ip + IAA	4mgL + 0.5mgL^{-1}
T8	2ip + IAA	5mgL + 0.8mgL^{-1}
T9	2ip + IAA	6mgL + 1mgL^{-1}

Shoot Formation in Date Palm

Under the control treatment, only Murashige and Skoog (MS) basal salts were used. For the T1 to T3, a combination of Benzyl Amino Purine was used in range from 1mgL to 2mgL^{-1} and (IAA) was used in concentration from 1mgL^{-1} to 2mgL^{-1}. For the treatment T4 to T6 combination of 2ip and Benzyl amino purine were used with concentration of 2mgL^{-1} to 3 mgL^{-1} 2iP and 2mgL^{-1} to 3mgL^{-1} BAP. For the treatment T7 to T9 combination of 2ip and Indole acetic acid used with concentration of 0.5mgL^{-1}to 1.5mgL 2ip and 1mgL^{-1} to 6mgL^{-1} IAA (Table 4).

Table 4. Protocol for optimization of Shoot Formation

Treatment	Hormone Combination	Concentration mg/L
T0	MS	4.33mg/L
T1	BAP + IAA	1mgL + 1mgL^{-1}
T2	BAP + IAA	1.5mgL + I.5mgL^{-1}
T3	BAP + IAA	2mgL + 2mgL^{-1}
T4	2ip + BAP	2mgL + 2mgL^{-1}
T5	2ip + BAP	2.5mgL+ 2.5mgL^{-1}
T6	2ip + BAP	3mgL + 3mgL^{-1}
T7	2ip + IAA	0.5mgL +1mgL^{-1}
T8	2ip + IAA	1mgL + 5mgL^{-1}
T9	2ip + IAA	1.5mgL + 6mgL^{-1}

Root Formation in Date Palm

Under the control treatment, only Murashige and Skoog (MS) basal salts were used. For the T1 to T3 a combination of indole Acetic acid and Indole Butyric Acid were used in concentration from 0.5 mgL^{-1} to 1.5mgL^{-1} . For the treatment T4 to T6 Naphthalene

Acetic Acid and IBA used with concentration of 2mgL^{-1} to 3 mgL^{-1} . For the treatment T7 to T9 combination of NAA, IBA, IAA used with concentration of 3mgL^{-1} to 5mgL^{-1} NAA, 2mgL^{-1} to 4 mgL^{-1} IBA and IAA (Table 5).

Table 5. Protocol Optimization for Root Formation

Treatment	Hormone Combination	Concentration mg/L
T0	MS	4.33mg/L
T1	IAA + IBA	0.5mgL + 0.5mgL^{-1}
T2	IAA + IBA	1mgL + ImgL^{-1}
T3	IAA + IBA	1.5mgL + 1.5mgL^{-1}
T4	NAA + IBA	2mgL + 2mgL^{-1}
T5	NAA + IBA	2.5mgL+ 2.5mgL^{-1}
T6	NAA + IBA	3mgL + 3mgL^{-1}
T7	NAA + IBA + IAA	3mgL + 2mgL^{-1} + 2mgL^{-1}
T8	NAA + IBA + IAA	4mgL + 3mgL^{-1} + 3mgL^{-1}
T9	NAA + IBA + IAA	5mgL + 4mgL^{-1}+ 4mgL^{-1}

The recorded data was observed during the Following observations:

- Number of days required for callus regeneration
- Number of days required for new shoots to form
- Days taken for new root formations

Statistical Analysis

To analyze the interaction of date palm explants in response to various hormone treatments, a CRD design with different factors was conducted in a controlled laboratory environment. To calculate the different observation data, three explant treatments and their data were studied using statistical methods. The calculated data were analyzed using ANOVA analysis of variance [16]. To test the relationship between the different hormone combinations on dates and compare the results with LSD.

RESULTS

ANOVA was performed on observation data which included the days required for induction of callus and callus regeneration, time taken for the development of new shoots, and the growth of new roots. This analysis was carried out using the Statistics 8.1 software program as mentioned in Table 6.

Table 6. Analysis of Variance for Different Parameters

Parameters	**MS(R)**	**MS(V)**	**MS(E)**	**Mean ± SE**	C.V (%)
Days to Callus Induction	23.8511	27.1567	15.1650	2.467	15.1650
Days to Callus Regeneration	39.5100	2.53160	1.69277	7.288	2.55
Days to New Shoots Emergence	61.3200	130.157	18.8150	1.613	9.21
Days to New Roots Emergence	49.2500	2.13260	13.3432	3.366	4.37

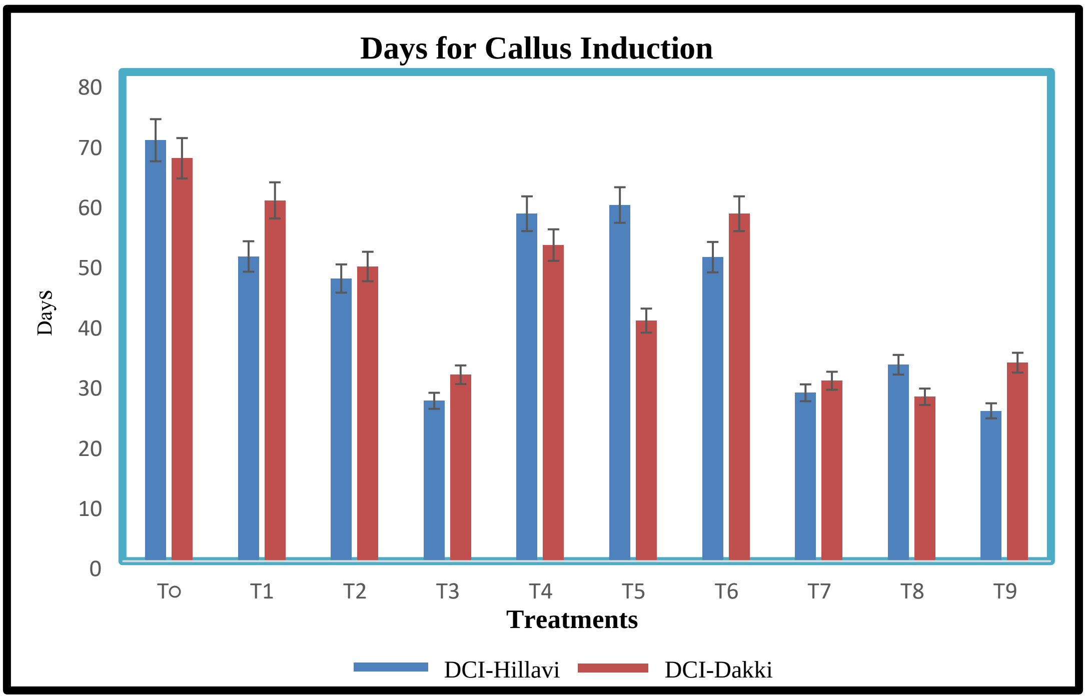

Figure 3. This graph illustrates days of callus induction under different treatments, including one control group comprising two different varieties. Hillavi is represented by the colour blue, whereas the Dakki variety is represented by the colour red. The above graphic representation results in the conclusion that treatment control takes more time but no callus was induced. Callus Induction was observed under treatments nine of the variety Hillavi and treatment eight of the variety Dakki take a shorter period of time for callus induction.

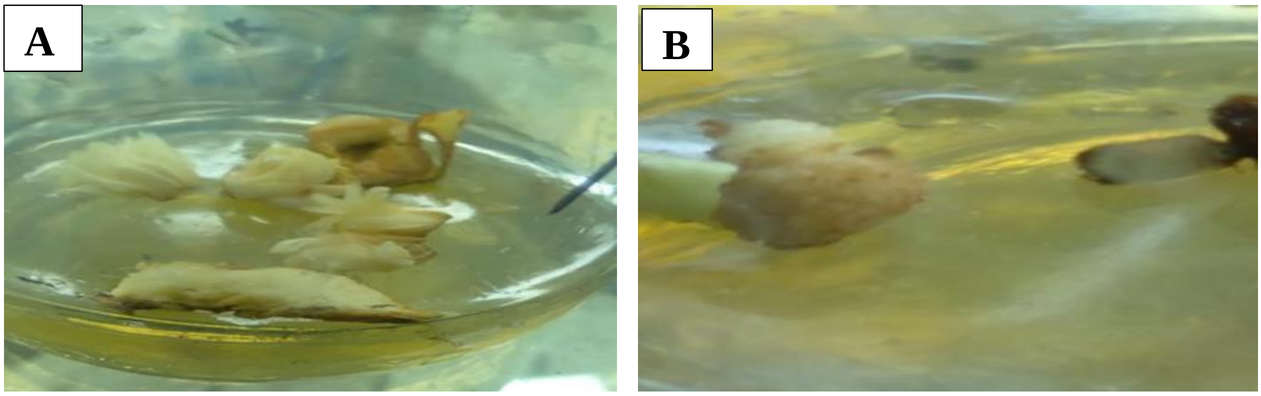

Figure 4. (A) Dakki under (T8) has taken 27days for callus induction (B) Hillavi under (T9) has taken 24 days.

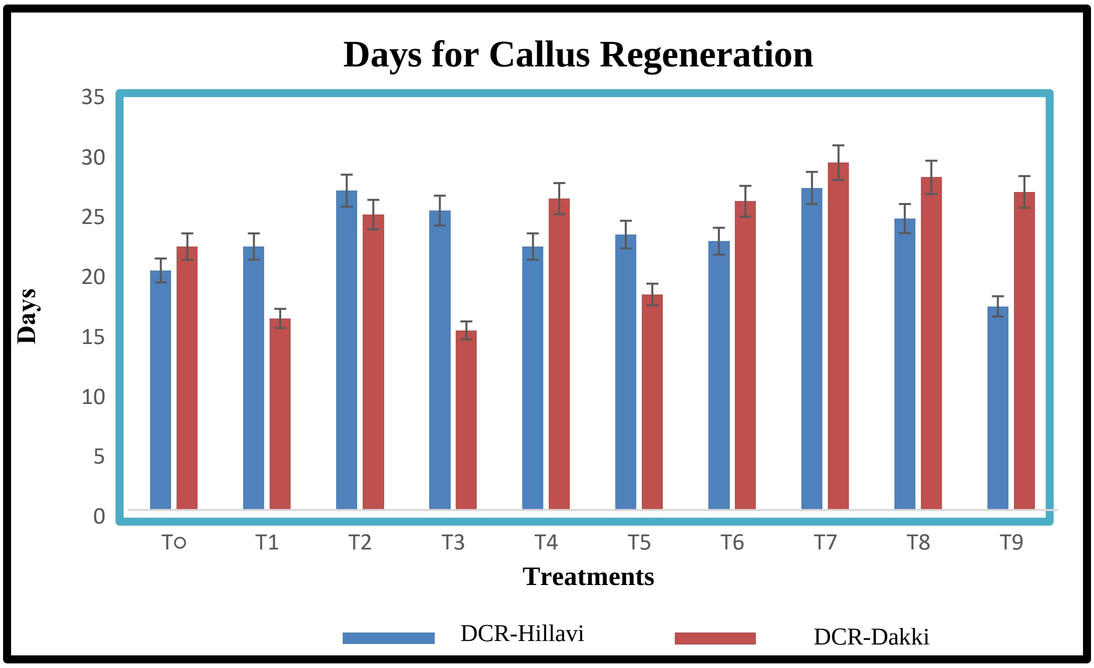

Figure 5. Minimum days for callus regeneration Under (Treatment 3) containing MS + 3mgL^{-1} IAA + 1.5mgL^{-1} BAP was observed for Dakki . Hillavi took minimum days for callus regeneration under (treatment 9) containing MS + 2ip 6 mgL^{-1} + IAA 1mgL^{-1}.

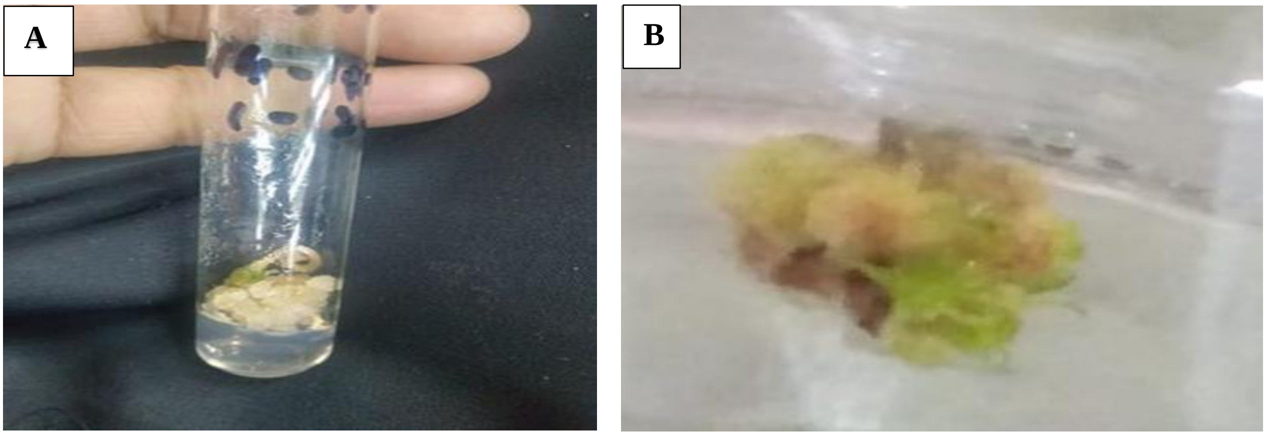

Figure 6. (A)Variety Dakki at (T3) took 15 days for callus regeneration. (B) Variety Hillavi at (T9) took 17 days for callus regeneration.

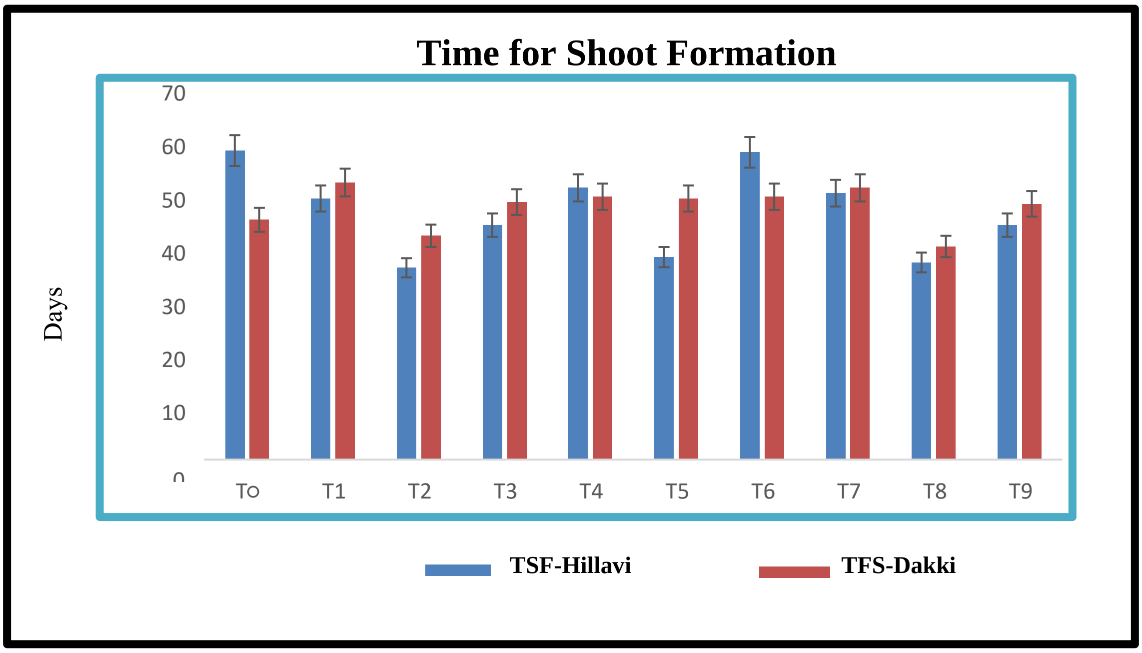

Figure 7. Hillavi took minimum days for shoot formation under (treatment 2) containing MS + 1.5mg/L BAP + 1.5 mg/L IAA and Dakki took minimum days under (treatment 8) containing MS + 1mg/L IAA + 5mg/L 2ip.

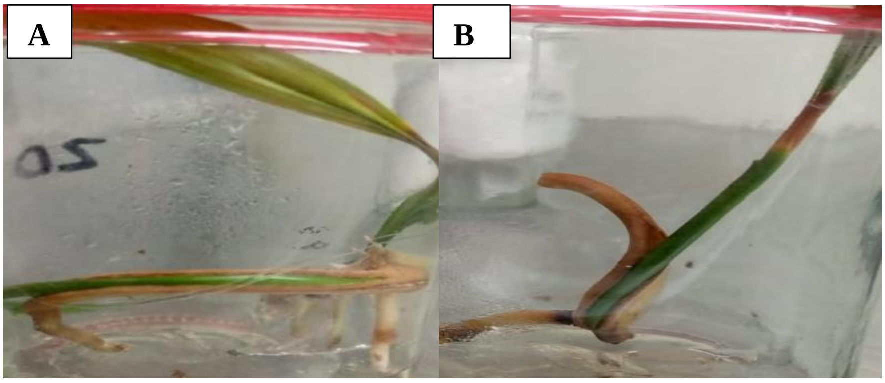

Figure 8. (A) *In- vitro* shoot formation of variety Hillavi took 38 days under (T2) . (B) *In- vitro* shoot formation of variety Dakki took 40 days at (T8).

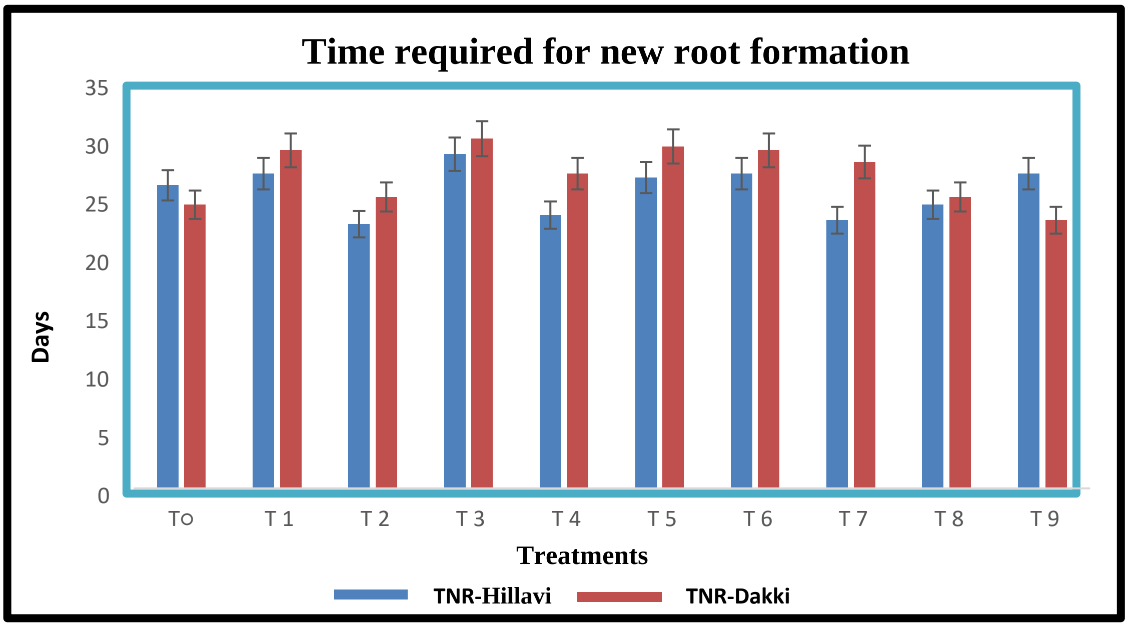

Figure 9. Dakki required the minimum days for new root formation under (Treatment 9), which consisted of MS medium supplemented with 5 mg/L NAA, 4 mg/L IBA, and 4 mg/L IAA. Similarly, Hillavi showed the shortest rooting time to emerge under (Treatment 2), containing MS medium with 1 mg/L IAA and 1 mg/L IBA.

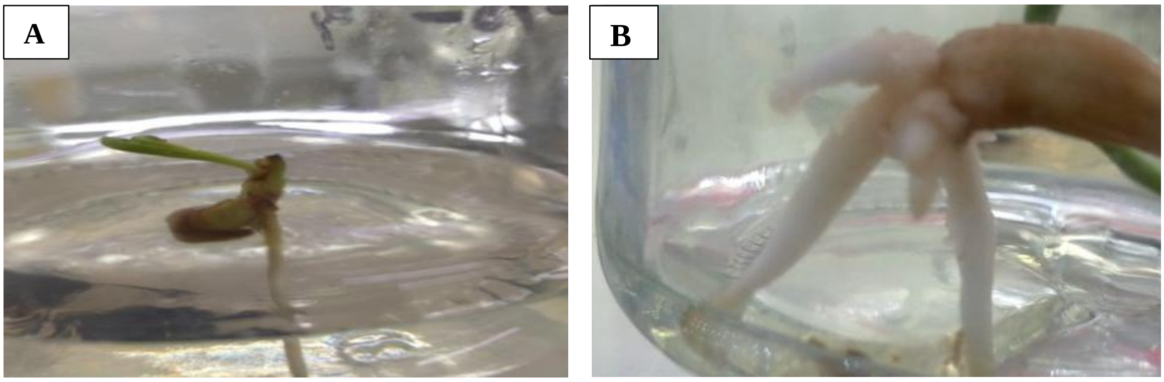

Figure 10. (A) Hillavi took 23 days under (T2). (B) Dakki took 24 days under (T9).

Callus Induction

Hillavi and Dakki varieties required a longer duration for callus induction under treatment (T1), but no callus was induced suggesting that this treatment was not very effective in enhancing callus formation. In Treatment (T2), the callus induction time remained relatively slow; however, a minor distinction was noted between the two varieties, with Dakki exhibiting a slightly quicker response compared to Hillavi. Treatment (T3) resulted in a marked decrease in callus induction time for both varieties, indicating that this treatment was more effective, with Hillavi showing a slightly better response than Dakki. Treatment (T4) produced moderate outcomes. In Treatment (T5), Dakki demonstrated a slower response. Treatment (T6) displayed consistent induction time, not presenting any significant advancement compared to T4. Treatment (T7) reflected another notable decrease in callus induction time. In Treatment (T8), particularly for Dakki, the induction time was shorter than all other treatments and white creamy callus were induced within short time. Under Treatment (T9) displayed a favorable response, for Hillavi variety demonstrating good callus induction as compared to other treatments. Treatment (8) is good for callus induction in Dakki and Treatment (9) is good for Hillavi.

Callus Regeneration

Nine different treatments mentioned in (Table 3) were used for callus regeneration. The maximum days for regeneration were taken under control treatment but no regeneration was observed for either-Hillavi or Dakki. Under (Treatment 3) minimum days for callus regeneration for Dakki was observed. Hillavi took minimum days for callus regeneration under (Treatment 9). Explants have taken maximum days for callus regeneration in other mentioned treatments, but no callus regeneration was observed for two varieties.

Time for Shoot Formation

Hillavi demonstrated the quickest shoot formation under Treatment 2, suggesting that this condition was optimal for its growth. Dakki showed the best response to Treatment 8, yielding shoots in the least amount of time. This difference in response indicates that the two varieties possess unique environmental or nutritional preferences that affect their growth rates. Importantly, in the control treatment, neither Hillavi nor Dakki produced any results, indicating that external factors or specific treatments were necessary for shoot initiation.

Time required to new root formation

Under the control treatment (T0), both varieties displayed no root development, indicating that external interventions were essential to stimulate root growth. The duration required for new root formation across various treatments for Hillavi and Dakki were observed under nine different treatments. The findings reveal that Dakki achieved the quickest root formation under (Treatment 9), demonstrating its efficacy in enhancing root growth for this variety. Conversely, Hillavi showed the quickest root development in response to (Treatment 2), implying that this particular treatment was most effective for its root growth.

DISCUSSION

The use of tissue culture techniques in date palm cultivation is an important approach to increase the yield and quality of this important crop with the help of biotechnology. Specifically, in vitro organogenesis was used in female date palm varieties to promote callus formation and shoot growth. Researchers such as Singh and Shekhawat [17] use this technology for good quality dates using shoot tips (typically 1.0 × 1.5 cm long). Scientists demonstrated the important role of the physical environment, especially the quality and intensity of light, on the organogenic response of the 'Medjool' date palm cultivar [18]. The results showed that light intensity in the range of 2000-3000 lux increased shoot length and chlorophyll synthesis, while higher light intensity had a negative effect on germination. However, a lower light intensity of 500 lux was favorable for root development, while a light intensity of 1000 lux was favorable for germination and vigorous growth [19]. The presence of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) in the culture medium was important for callus induction. The composition of the growth medium, especially the presence of the auxin 2,4-D concentrations required to induce cell formation in most plants within 8 to 12 weeks [20]. Their study showed that 2,4-D was important to other hormones in callus formation and that lower concentrations of 2,4-D did not produce significant results. Explant grown on Murashige and Skoog (MS) media were exposed to alternating light and dark. This process resulted in the formation of white nodule cells that served as markers of cell renewal leading to rooting and sprouting [21] suggest that combining root stage and seedling culture can save time, resources and energy and increase the yield of *in vitro* plants. Recent studies in this area have highlighted the importance of hormonal and environmental control in the regulation of cell structure and plant regeneration. For example, auxin 2,4-D is not only responsible for callus formation but also plays a key role in the initiation of somatic embryogenesis when combined with cytokinin such as BAP. In addition, IAA (natural auxin) and BAP have been shown to be effective in promoting cell regeneration in shoots and roots, thereby increasing the efficiency and economic viability of the entire culture process successful shoot regeneration has been reported on MS medium with 2,4-D and BA, with optimal concentrations leading to significant shoot formation [22]. Current studies in this area demonstrate the importance of hormonal and environmental controls necessary for callus formation and plant regeneration. The insights from these studies have led to significant advances in the field of tissue culture of date palm, which may have implications for other major commercial crops. This study showed great promise for the use of tissue culture in modern agricultural practices, which may increase the productivity and sustainability of date palm and other fields. The insights from these studies have led to significant advances in the field of tissue culture of date palm, which may have implications beyond other major commercial crops.

CONCLUSION

According to the study, the effects of various growth hormone treatments and their combinations, as well as the effect of a surface sterilant ($HgCl_2$) were examined. Temperature and light levels were maintained at 25°C with a cycle of 8 hours of darkness and 16 hours of light. It is suggested that date palm in-vitro micropropagation is the most effective way to address problems with hazardous diseases and physiological problems. In comparison to conventional methods, it might also be used to produce the maximum number of plantlets produced in a short time and confined space while maintaining controlled light and climatic conditions under optimized concentration of growth hormones.

AUTHOR CONTRIBUTIONS

SN and PK conceptualized the idea. SN did experimentation and wrote the manuscript. ZK, KR, MI, MR,SY and PK reviewed the draft for improvements.

DECLARATION OF POTENTIAL CONFLICTS OF INTEREST

The author states that there are no conflicts of interest.

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