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Research Article

Micropropagation of Date Palm (*Phoenix dactylifera* L.) Cultivar Gulistan Using Immature Inflorescence Explants

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Abstract: Current study described micropropagation of commercial date palm cultivar Gulistan through juvenile inflorescence explants. Immature spathes (20 cm in length) were excised before emergence from leaf axils in the crown at particular time i.e. early of February. For sterilization, the undissected spathes were dipped in NaOCl solution (40%) for 10 minutes on laminar air flow hood. Medium used for callus formation in the inflorescence explants was consisted of 0.1 mg/L 2,4-D, 0.1 mg/L IAA, 5.0 mg/L NAA induced significantly highest callus (87%) in primary explants. Highest somatic embryogenesis (84%) was obtained on the medium contained 0.1 mg/L NAA, 0.1 mg/L Kinetin. Shoot induction and multiplication (91%) was recorded on the medium consisted of 0.1 mg/L NAA, 0.05 mg/L BA. Leaves number (4.1), leaves length (17 cm), roots number (4.0), roots length (7 cm) were recorded as significantly highest on the medium consisted of 0.1 mg/L NAA. Trimming of embryonic root at 1-2 mm distance from plantlets' base produced 3-4 adventitious roots. *In vitro* hardening supported to choose healthy plantlets which survived well in greenhouse. Plantlets' survival percentage in greenhouse after 1M (88%) as significantly highest was recorded on the soil mixture contained peatmoss, river sand, perlite (1:1:¼ v/v). 46 cm long plantlets produced 4 small fronds/compound leaves, were cultivated in open field. Fruiting in trees started after three years of cultivation in open field. Introduction of elite date palm cultivars like Gulistan via micropropagation will be an addition to the existing cultivars in the area.

Keywords: Micropropagation, Somatic Embryogenesis, Plantlet Regeneration, *In vitro* Hardening, Acclimatization.

1. INTRODUCTION

Date palm (*Phoenix dactylifera* L.) a member of family Arecaceae is dioecious and diploid (2n = 36), contains vast number of varieties cultivated in arid and semi-arid regions in the world [1-3]. Date palm is titled as ''Tree of Life" due to its worldwide occurrence in Oasis and arid areas [4, 5]. Date palm yields up to 40-50 years; however, it may reach to 150 years age in certain conditions [6]. Dates contain vitamins, sugars, minerals, and various compounds used in the pharmaceutical industry for medicines' production [7-10]. Date palm generates considerable chances for the peoples living in rural areas, earning source for the date growers, increases income and is a good source of food [11]. Generally, the traditional propagation methods of date palm are by seeds and offshoots, but are labour intensive and time consuming. Commercially valued date palms are hindered via seed propagation due to heterozygous nature of the plants [12]. Generally, the date palm propagated via seeds either become male or female, and also produce inferior quality fruits, slow growth (8-10 years up to fruiting). Offshoot-grown date palm yields true-to-type fruits but show slow growth up to fruiting [13]. A date palm tree produces only 10-20 offshoots in whole life, its low survival in the field (50% mortality rate), and threat of Red Palm Weevil, Bayoud and Bayoud like diseases; such as, sudden decline disease [4, 14]. Keeping in view, the current scenario of date palm cultivation and production, the tissue culture is an additional possible way to multiply the selected local and exotic cultivars in a shortest

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time and space under controlled environmental conditions [2, 14, 15]. Simultaneously, the tissue culture-derived plants of date palm remain free from pests and diseases compared to the date palms propagated via traditional propagation methods. Micropropagation is suitable means of date palm propagation in contrast to seeds and offshoots, which can fulfil the requirement of date palm plants for large scale cultivation [16].

Additionally, the micropropagation is widely applied for the rapid production of the plant material [17]. Explants of the offshoots and inflorescences of the date palms have been used largely. Inflorescence based micropropagation proved as an efficient way for producing huge number of plants in a shortest time and space without sacrificing the whole tree; instead, a single inflorescence is excised from the tree without harm. Micropropagation is also a means for multiplication of the male or female recalcitrants of commercially important date palm cultivars [18]. A complete tree of date palm is utilized to obtain meristematic shoot tip explants, whereas, the tree remains un-damaged during excision of a spathe.

Several workers [19-26] utilized inflorescences of female date palms as an alternative to shoot tip explants; and found as a potential explant for *in vitro* culture compared to male inflorescence regarding induction of somatic embryos [22, 27]. Low somaclonal variation was observed in previous studies in the date palm plants derived from juvenile inflorescences [1, 28, 29]. Plant regeneration protocols of date palm through somatic embryogenesis were established through several studies [30-32]. Recently, inflorescence explants of date palm have been used widely in tissue culture [2, 15, 26, 33]. Commercial date palm cultivars in Pakistan are a few, simultaneously attacked by pests and diseases. Gulistan cultivar is a commercial semi-dry cultivar originally belongs to Dera Ismail Khan, Khyberpakhtunkhwa, Pakistan.

In this study micropropagation of commercial date palm cultivar Gulistan is an addition to existing cultivars in the area. Lot of work have been conducted on the micropropagation of several date palms in the world, but most of the studies were restricted to acclimatization stage, or if transferred in the open field produced fruits indistinguishable to the mother tree, or the trees were completely infertile due to irreversible genetic variations. On the contrary, this study described successful micropropagation of date palm produced fruits similar to mother palms; hence this study distinguishes to previously conducted studies on micropropagation of other date palm cultivars.

Present study designed to optimize stage-wise procedures for *in vitro* propagation of date palm via inflorescence explants, somatic embryogenesis, acclimatization of plantlets on various soil mixtures, transfer of plants in open field, and finally to check fruit quality are key objectives. Established tissue culture protocols of date palm by the current study will be applicable to micropropagate the other varieties and cultivars on commercial level growing in the world.

2. MATERIALS AND METHODS

2.1. Excision of Spathes and Explant Preparation

Spathes were collected from Orchard of Date Palm Research Institute, Shah Abdul Latif University, Khairpur located at latitude 27.490418˚ N, longitude 68.761593˚ E. Annual rainfall average of Khairpur is 87.6 mm and temperature (50 ˚C) in July. 20 cm long spathes were excised from the tree during first week of February in 2006; grown in the district Khairpur, Pakistan. Any residual particles on spathes' surface were removed with cotton. Later, spathes shifted to the laboratory, kept in closed ice box (4 ℃) to save from desiccation. Washing of spathes was carried out with tap water inside the laboratory. Further sterilization of spathes was carried out in culture room with NaOCl (40%) with 4-5 drops of tween-twenty for 10 min. After sterilization process, the spathe's cover was excised vertically with scalpel from both sides, keeping the inflorescence bunch intact.

After excision of both sides, the spathe was dissected horizontally to remove spathe cover entirely. Spikelets of 2-3 cm isolated from bunch and cultured directly on initiation media (kept vertically in culture tubes on the medium in a way the lower side of explant touched the medium); whereas, long spikelets were excised into 2-3 cm. Callus cultures were incubated under dark up to 7-9 months. Somatic embryos were induced under complete dark, and were multiplied under photoperiod 16/8 hours. Shoots (10 cm long) were shifted to elongation and rooting media for further

growth. After 1-2 subcultures, roots of the long plantlets were cut at 1-2 mm distance from plantlet's rooting area for production of multiple roots.

2.2. Media Preparation and Culture Conditions

Initiation medium was based on MS micronutrients, B5 macronutrients, sucrose 30 g/L, agar 2.2 g/L, gelrite 1.4 g/L, MS vitamins, KH_2PO_4 170 mg/L, glutamine 100 mg/L, adenine sulphate 40 mg/L, 2,4-Dichlorophenoxyacetic Acid (2,4-D) (0.1 mg/L), Indole-3-Acetic Acid (IAA) (0.1 mg/L), Naphthalene Acetic Acid (NAA) (5.0 mg/L) (M1). Initiation medium was based on 2,4-D 0.2 mg/L, IAA 0.1 mg/L, NAA 5.0 mg/L (M2). Maturation medium was based on Activated Charcoal (AC) 1.5 g/L, 2,4-D (5.0 mg/L), 2-Isopentenyladenine (2iP) (1.0 mg/L). Differentiation medium was consisted of full MS, NAA (0.1-0.2 mg/L) and Kinetin (0.1-0.2 mg/L). Multiplication medium was based on NAA (0.1-0.2 mg/L) and 6-Benzylaminopurine (BA) (0.05 mg/L). Rooting media were consisted of MS (¼), calcium pantothenate (0.1 mg/L), AC (3 g/L), NAA 0.1 mg/L (M1); Indole-3-Butyric Acid (IBA) 0.1 mg/L (M2), NAA 0.2 mg/L (M3), IBA 0.2 mg/L (M4). Initiation and differentiation were carried out under full dark (24 °C); while the rooted plantlets were grown under photoperiod (16/8 hours), temperature (27 °C). Multiplication and rooting were achieved under photoperiod 16/8 hours (fluorescent light intensity at 40-60 µmol m^{-2} s^{-1}). Light intensity during elongation and rooting stages was 160 µmol m^{-2} s^{-1}. At initiation stage, subculturing was carried out after every three weeks; while at multiplication and rooting stages; subculturing was done after every four weeks. pH of the media was fixed at 5.7 at all *in vitro* growth stages. Medium was dispensed either in 150 × 25 mm tubes or in 350 ml jars utilized for *in vitro* cultures. 20 ml medium dispensed in each culture tube or 25 ml medium dispensed per jar. Sterilization of the media was done by autoclave (Mitsubishi, Japan) for 15 min at 121 °C.

2.3. *In vitro* Hardening

In vitro hardening was performed inside laboratory during June, 2008 for 2-3 days before shifting the plantlets in the greenhouse. Plantlets used for *in vitro* hardening were consisted of 2-3 roots and 3-4 leaves. Liquid medium was used for *in vitro* hardening consisted of ½ MS, 15 g/L sucrose. Small holes were made in the cape of culture vessels for movement of gases in and out of the culture tubes 1-2 hours prior to transfer the plantlets in greenhouse.

2.4. Acclimatization

20 cm long plants with four simple leaves and adventive roots were transplanted in greenhouse during June, 2008. Plantlets directly exposed to greenhouse environment by removing the cap of the culture tubes for 1-2 hours prior to shift on soil mixture. Later, the plantlets taken out of vessels to remove gel from roots with sterilized water. Systemic fungicide (Carbendazim) solution (3 g/L) was used for washing the plantlets before shifting in 19 × 13 cm plastic bags on various mixtures of peatmoss, river sand, perlite (1:1:¼), (1:2:¼), (1:3:¼), (1:4:¼). Humidity was maintained to 85-90% inside the tunnel in the greenhouse for few weeks. Ventilation of the tunnel was carried out after a week for 10 min. After one month, the plantlets were exposed to the environment of greenhouse and the tunnel/polyethylene cover was removed completely. At this stage no any fertilizer was applied to the plants. One and half years old plants were shifted in 41.5 × 21 cm bags on soil mixtures used during acclimatization, and left for two and half years (up to end of 2010) until formation of compound leaves (3-4 per plant), and capable to grow well in open field environment. Plants' survival percentages were recorded subsequently at one and three months' interval.

2.5. Open Field Transfer

Plants about 46 cm long with maximum 4 fronds/compound leaves were cultivated in open field to evaluate true-to-typeness of tree and fruit. Fruiting in the plants started after two years of plantations in the field i.e. in the year 2012. NPK fertilizer was applied to the plants in the field as per need according to the age of the tree.

2.6. Statistical Analysis

Three spathes were obtained from cultivar Gulistan yielded total 150 explants. One explant per culture tube was cultured on initiation media. Completely Randomized Design was performed; whereas, the ANOVA and LSD tests were performed at $p < 0.05$ according to Steel and Torrie [34].

3. RESULTS AND DISCUSSION

3.1. Callus Formation in Primary Explants

2,4-D is one of the important plant growth regulators (PGRs) induces callus in immature inflorescence explants. In this study different auxins and their concentrations induced callus in the floral bud explants (Figure 1(a)) during third week of the initial culture (Figure 1(b)). About 120 out of 150 explants (on both treatments used during initiation stage) formed callus in the floral buds. Data in Table 1 show significantly highest callus formation (87%) in floral buds on the medium contained 2,4-D 0.1 mg/L, IAA 0.1 mg/L, NAA 5.0 mg/L. On the contrary, callus formation was decreased significantly (55%) on the medium contained 2,4-D 0.2 mg/L, IAA 0.1 mg/L, NAA 5.0 mg/L. Inflorescence explants can induce better callus using 2,4-D up to 0.5 mg/L [26]; however, in the current study three auxins were used in order to induce callus in the floral bud explants. Increase in 2,4-D (0.2 mg/L) decreased callus induction percentage in primary explants. 2,4-D concentrations i.e. 0.5 mg/L, 2iP 3.0 mg/L induced maximum callus; but further increase in 2,4-D to 1.0 mg/L showed vitrification [2]. Until maturation stage i.e. 7-9 months (induction of globular embryos) (Figure 1(c)); the cultures were retained on initiation media. Later, the callus cultures (7-9 months old) shifted to maturation medium contained 2,4-D (5.0 mg/L), 2iP (1.0 mg/L) for a single subculture. 2,4-D occurrence in calli initiation media exhibited positive effect on explants to induce highest callus, but higher 2,4-D levels may sometimes bring variations in plants; therefore 2,4-D in the media should be excluded once the calli is formed and matured. 2.4-D might bring permanent genetic variations at callus stage which may persist throughout the whole life making whole the cycle useless. Medium contained 2,4-D (100 mg/L), 2iP (3.0 mg/L) and AC (3 g/L) was used for propagating the date palm *in vitr*o from shoot tip [30]. Several workers [26, 35] reported the 2,4-D a major callus inducing PGR in primary explants. Al-Khayri [36] obtained callus in shoot tip explants on the medium comprised of 2,4-D (100 mg/L), 2iP (3.0 mg/L), AC (1.5 g/L). Badawy *et al*. [37] described the medium for callus formation in date palm cv. Sewi consisted of 2,4-D (100 mg/L), 2iP (3 mg/L). Auxins disrupt the normal development leading to callus induction in primary explants

Table 1. Influence of various combinations of PGRs on callus induction in spikelet explants of cv. Gulistan.

PGRs (mg/L)	Callus (%)
0.1 2,4-D + 0.1 IAA + 5.0 NAA	87±0.5[a]
0.2 2,4-D + 0.1 IAA + 5.0 NAA	55±0.2[b]
LSD (0.05)	**0.000*** **

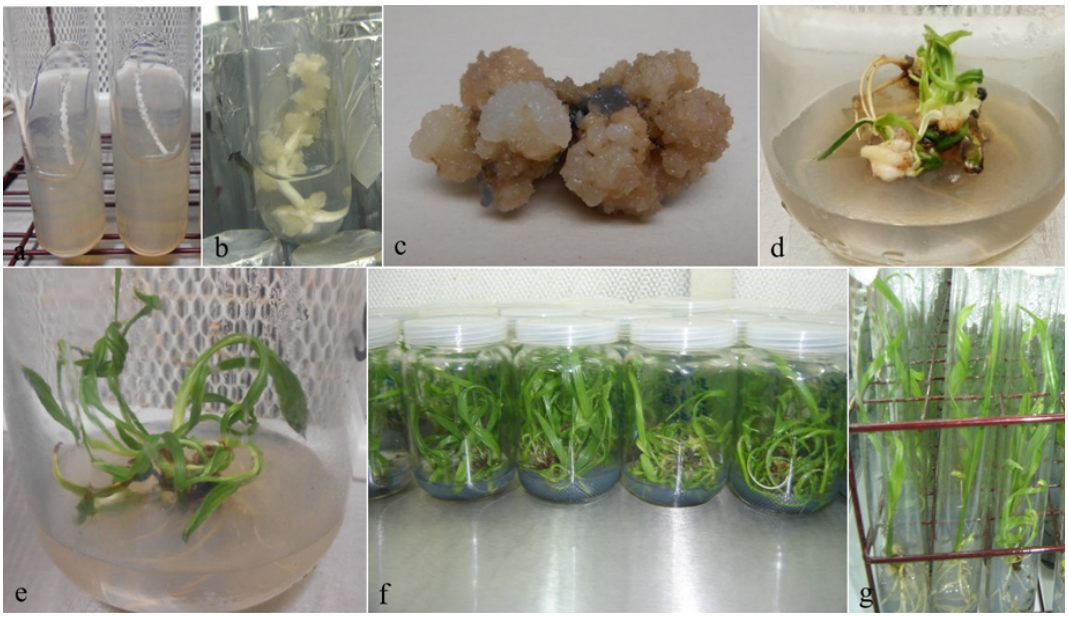

Fig. 1. (a) Inflorescence explants, (b) Callus formation in inflorescence explants, (c) Maturation stage of callus prior to differentiation, (d) Somatic embryogenesis and germination, (e) Small shoot clusters\, (f) Long plantlets in jars before separation, and (g) Isolated plantlets in culture vessels with healthy shoot and root ready for transfer in greenhouse.

[2, 38, 39]. Callus cultures should be retained on 2,4-D contained medium until appearance of small globular structures in the callus, indicates maturation stage of the callus. Several workers [15, 40, 41] studied callus induction behavior of soft, semi-dry or dry cultivars in inflorescence explants. Age of the spathe and explant size had the positive role in callus induction in the spikelet explants. 2-3 cm long explants can be easily submerged in the nutrient medium for callus induction. Any part of the explant away from the nutrient medium undergoes necrosis. Juvenile inflorescence explants used in the present study obtained from 20 cm long spathes excised during 1st week of February, 2006 induced significantly highest callus.

Previous studies used juvenile inflorescence explants obtained from spathes (15 cm long) obtained during early of February [42] formed calli in bulk in the primary explants. Several workers [43-45] observed that in date palm calli induction was supported by explants' age and PGRs' types. Most workers observed that the 2,4-D is an effective PGR among auxins regarding callus formation in initial explants [46-48].

3.2. Somatic Embryogenesis

Data in Table 2 exhibit significantly highest ($p < 0.000$) somatic embryogenesis (84% of the callus cultures obtained during initiation stage) on the medium containing NAA (0.1 mg/L), Kinetin (0.1 mg/L). Contrary, the lowest percentage of embryos (51%) was achieved on the medium containing NAA (0.2 mg/L), Kinetin (0.2 mg/L). Use of 2,4-D in the media was restricted up to maturation in callus, i.e., calli ready to produce embryos; however, the continued occurrence of 2,4-D in the media also retard somatic embryogenesis. Formation of somatic embryos require low 2,4-D concentration in the media or sometimes complete exclusion of 2,4-D. 2,4-D should be added up to the appearance of rounded structures in the callus cultures i.e. start of the differentiation stage [2, 26]. Embryogenic calli in inflorescence explants was also noted within six weeks on PGRs contained media [15]. Embryogenic callus cultures were declined gradually due to prolong subculture on the media consisted of 2,4-D under full dark. Shifting of callus on the media lacking 2,4-D retarded normal proliferation of callus under light. Furthermore, embryogenic clusters produced from friable callus under light developed into somatic embryos. Green photosynthetic leaves produced in plantlets upon germination (Figure 1(d)). 2,4-D concentrations i.e. 1.5, 5 and 10 (mg/L) also used for somatic embryogenesis in the callus [49-52]. Genotypic effects influence somatic embryogenesis which may take few to several months in different cultivars [46, 53].

3.3. Proliferation and Germination of Somatic Embryos

Data in Table 3 indicate that highest germination (91%) of somatic embryos achieved on medium consisted of NAA (0.1 mg/L), BA (0.05 mg/L) (Figure (1d)). Contrary, the lowest germination (57%) of somatic embryos was recorded on the medium contained NAA (0.2 mg/L), BA (0.05 mg/L). Increase in NAA concentration to 0.2 mg/L significantly decreased the multiplication of somatic embryos. Individual shoots formed after germination of somatic embryos were isolated and shifted on rooting media for elongation of shoot/root. Embryos exhibited highest multiplication and germination under light. Steward *et al*. [54] described the need of auxin in the medium until initiation of somatic embryos; whereas, complete removal of auxins from the media supports embryo maturation. Cytokinins bring maturation of somatic embryos [55], followed by cotyledon development [56]. Likewise, in this study the procedures were exploited for maturation of embryos at proper

Table 2. Influence of various combinations of PGRs on somatic embryogenesis in callus of cv. Gulistan.

PGRs (mg/L)	Embryogenesis (%)
0.1 NAA + 0.1 Kinetin	84±1.1[a]
0.2 NAA + 0.1 Kinetin	51±2.1[b]
LSD (0.05)	**0.000*** **

Table 3. Influence of various combinations of PGRs on somatic embryos germination in cv. Gulistan.

PGRs (mg/L)	Germination (%)
0.1 NAA + 0.05 BA	91±0.5[a]
0.2 NAA + 0.05 BA	57±2.2[b]
LSD (0.05)	**0.000*** **

growth stage. Two types of somatic embryos were recognized such as repeated i.e. cluster of embryos and non-repeated i.e. single embryos [2, 42]. Germination of somatic embryos (repeated and non-repeated) resulted in formation of green shoots with roots (Figure 1(d)). Up on germination, covering of embryos opens vertically, results in the emergence of shoot and root. After 1-2 subcultures on the multiplication medium, the plantlets were shifted on shoots and roots elongation medium (Figure 1(e-f)). Previous studies also observed germination of single and clustered embryos [15, 26, 42]. A comprehensive study conducted on micropropagation of date palm via floral bud explants [14]. However, in this study several combinations of PGRs used at each *in vitro* growth stages differ greatly to those of Solangi *et al*. [2].

3.4. Shoot Elongation and Rooting

Data in Table 4 describe highest response of NAA than IBA for shoot and root growth (Figure 1(g)). Total number of plantlets produced during elongation stage was 1100. About 100 plantlets were contaminated or died due to poor rooting, whereas remaining 1000 plantlets were survived after root trimming process. Results revealed that significantly highest ($p < 0.000$) leaves number (4.1), leaves length (17 cm), roots number (4.0) and roots length (7.2 cm) were achieved on the medium containing ¼ MS, calcium pantothenate (0.1 mg/L), AC (3 g/L), NAA (0.1 mg/L). Leaves number (3.2), leaves length (11 cm), roots number (3.1) and roots length (5.0 cm) were achieved on the medium contained ¼ MS, calcium pantothenate (0.1 mg/L), AC (3 g/L), IBA (0.1 mg/L). NAA and IBA (0.2 mg/L) induced lowest leaves and roots number. NAA and IBA (0.1 mg/L) have been widely exploited in the rooting media for date palm and other plants. 0.1 mg/L BA, 0.1 mg/L NAA significantly enhanced leaves and roots growth [14]. Several workers [15, 42] used BA 0.05 mg/L, NAA 0.1 mg/L for shoots and roots elongation in date palm. El-Sharabasy *et al*. [57] observed positive effect of 0.1 mg/L NAA on shoot length of cv. Zaghlool and cv. Sewi than IBA and IAA. Healthy root formation was acquired on the medium consisted of NAA (0.1 mg/L) [58]. Tisserat [59] also acquired adventive roots through re-culturing of isolated plantlets on the medium comprised of NAA (0.1 mg/L). Omar [60] obtained healthy plants of date palm by transferring small shoots on the medium comprised of NAA (0.1 mg/L) for improved rooting, and BA (0.01 mg/L) for improved shoot. NAA is widely used PGR for the rooting in date palm [35, 59, 61, 62], and several other plant species.

3.5. Root Trimming

Better survival of plants in greenhouse at acclimatization stage was based on the healthy roots developed during *in vitro* growth. In this way, the embryonic roots were cut at 1-2 mm distance to lower side of 10 cm plantlets to induce multiple roots in each plantlet (Figure 2(a)). Multiple healthy roots enhance survival percentage of plants during acclimatization. Plantlets acquired after germination of somatic embryos consisted of single embryonic root (Figure 2(b)). Several studies [14, 15, 62, 64] exploited root trimming for formation of healthy adventive roots in each plantlet. Root trimming is the means of getting higher survivability of plants in greenhouse. Solangi *et al*. [14, 26] achieved healthy growth and maximum survival of plantlets in greenhouse. Plantlets with untrimmed roots showed weak growth in the greenhouse compared to the plantlets with multiple roots developed via root trimming at proper growth stage.

3.6. *In vitro* Hardening

In vitro hardening was performed few days before to transfer the plantlets in greenhouse. Liquid nutrient medium consisted of ½ MS and 15 g/L sucrose was used for *in vitro* hardening. An opening was made in the cap of culture tube for gaseous movement just

Table 4. Influence of various combinations of NAA and IBA on shoot and root formation and elongation in cv. Gulistan.

PGRs (mg/L)	Cv. Gulistan			
	Leaves number	Leaves length (cm)	Roots number	Roots length (cm)
0.1 NAA	4.1±0.58[a]	17±3.10[a]	4.0±1.00[a]	7.2±2.00[a]
0.1 IBA	3.2±0.45[ab]	11±0.42[b]	3.1±0.58[ab]	5.0±1.14[b]
0.2 NAA	2.2±0.51[b]	9±1.12[c]	2.2±0.44[b]	2.3±0.46[b]
0.2 IBA	2.1±0.43[b]	8±1.16[c]	1.1±0.34[c]	2.1±0.58[b]
LSD (0.05)	0.03**	0.002**	0.04**	0.05**

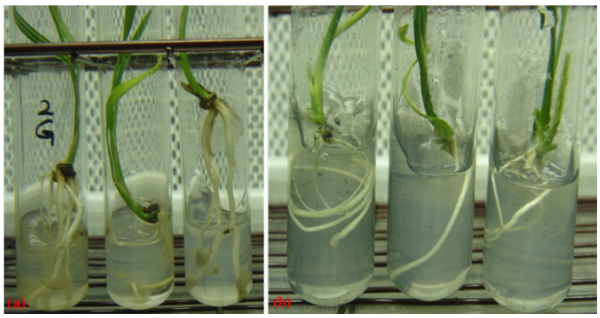

Fig. 2. (a) Multiple roots produced at the base of date palm plantlets after trimming the embryonic root, (b) Single root developed in plantlets with untrimmed embryonic roots.

one hour prior to shifting the plants in greenhouse. Number of *in vitro* hardened plants was 1000. *In vitro* hardening helps to bring changes in the physiological nature of plants from heterotrophic to autotrophic [14, 42]. *In vitro* hardening helped to isolate healthier plantlets able for acclimatization. More than 85% survivability of plantlets was acquired in greenhouse. Poorly developed plantlets turned into wilted leaves via *in vitro* hardening; such plantlets were recognized easily, and isolated before acclimatization. Hassan *et al*. [63] suggested hardening *in vitro* to choose healthy plantlets prior to acclimatization. *In vitro* hardening is an important final stage of *in vitro* growth [64]. *In vitro* hardening was also performed for many other crops [65]. This technique helps to adapt plantlets' physiology and anatomical features well suited to *ex vitro* environment to ensure maximum survival rate in greenhouse.

3.7. Plantlets' Survival in Greenhouse

Adaptation of *in vitro* grown plants in varied environmental conditions is called acclimatization [14, 42]. Plants develop varied physiology and anatomy grown under high humidity, low gaseous exchange, which needs to reverse to their normal structures by maintaining the humidity in the greenhouse during acclimatization [14, 42]. Number of plantlets acclimatized in the greenhouse was 980, which was 1000 before *in vitro* hardening. Only 20 plantlets showed wilted leaves during *in vitro* hardening and discarded before acclimatization. Table 5 shows highest survival (88% and 80%) of plantlets after 1M and 3M respectively on soil mixture contained peatmoss, river sand, perlite (1:1:¼). Gradual decrease was noted regarding survival of plantlets in greenhouse on soil mixture consisted of peatmoss, river sand, perlite (1:2:¼) (77% and 71% after 1M and 3M respectively). Results showed significantly highest values at $p < 0.000$ after 1M and 3M. Further decrease in the survival rate of the plants was noted on soil consisted of peatmoss, river sand, perlite (1:3:¼) and peatmoss, river sand, perlite (1:4:¼). Results showed that river sand is not rich in nutrients as peatmoss; however, river sand provides good aeration to the roots but deficient in nutrients compared to peatmoss. Peatmoss contains essential elements such as potassium, phosphorus, nitrogen, iron, magnesium, calcium etc. Peatmoss does not fulfil all nutrient requirements of the plants which always fulfilled with fertilizers when plants rooted in soil mixtures and start growing after acclimatization. Results revealed that total mortality rate of plants in greenhouse was 20% in 3 months on a soil mixture contained peatmoss, river sand, perlite (1:1:¼) and no more plants were died until shifting in the open field. On the contrary, significantly highest plant mortality rate (45%) was noted on a soil mixture contained peatmoss, river sand, perlite (1:4:¼) after 3 months. Phenomenon indicated that higher ratios of river sand were not suitable for acclimatization of date palm. Peatmoss should always be added with river sand, but the ratio of river sand should not increase to peatmoss ratio; however, peatmoss ratio can be increased; whereas, the perlite always should be in little amount. Perlite is a light weight porous volcanic glass used as a soil conditioner to improve drainage and aeration in the

Table 5. Influence of various mixtures of Peatmoss, River sand, Perlite on plantlets' survival in greenhouse cv. Gulistan.

Soil Mixtures			**Cv. Gulistan**	
Peatmoss	**River sand**	**Perlite**	**1 Month**	**3 Months**
1	1	½	88±1.3^{a}	80±2.2^{a}
1	2	½	77±0.5^{b}	71±0.2^{b}
1	3	½	65±0.4^{c}	60±1.2^{c}
1	4	½	60±2.1^{c}	54±2.2^{d}
LSD (0.05)			0.000***	0.000***

plants. It is created by heating volcanic glass, which causes it to expand. Perlite is pH neutral and helps to prevent soil compaction while creating air pockets for root growth. It also enhances water retention. In previous studies [63, 66, 67] low survival (25-30%) in date palm plants was obtained during acclimatization due to improper soil mixtures. Kurup *et al*. [68] noted 60% survival of plants on a soil mixture contained peatmoss and vermiculite (2:1) in cv. Kheneizi. Othmani *et al*. [51] obtained survival (60%) in date palm cultivar Boufeggous and 80% in cultivar Deglet Nour. 72-84% survival rate of date palm plants was obtained by Al-Khayri [48] in cultivars Nabout Saif and Khasab. Solangi *et al*. [14] recorded survival (92%) of plants on soil mixture contained peatmoss and river sand (3:1). Various studies [69, 70] used a soil mixture of peatmoss and vermiculite (1:1) for growing 12 cm plantlets in greenhouse.

Plantlets of date palm successfully acclimatized and established in the greenhouse (Figure (3a)). One and half year old plantlets in greenhouse with a compound leaf were transferred into 41.5 × 21 cm plastic bags (Figure (3b)). Two and half years old plantlets in greenhouse with four compound leaves (Figure (3c)) transferred in field showed 100% survival due to well established roots at base of plants grown in greenhouse. Fertilizer (NPK) was applied to plants in greenhouse and in open field according to the age of plants. Several studies [14, 15] applied 3 g/L NPK on six months old plants in greenhouse; whereas, 20 g/L NPK applied to two and half years old plants.

3.8. Vegetative Growth and true-to-type Fruiting of Date Palm in the Open Field Conditions

Plants grown in greenhouse up to two and half years produced 3-4 compound leaves and adventitious roots (Figure (3c)) were shifted in the open field for further vegetative growth and fruiting (Figure (3d)). Fruits of cv. Gulistan were similar morphologically to the fruits as in their native place (Dera Ismail Khan, Khyberpakhtunkhwa, Pakistan). Date palm cv. Gulistan started fruiting after three years of cultivation in the field (Figure 3(e-f)). Quality of ripened dates of the tissue cultured plants was also similar to the dates grown in their native place (Figure 3(g)). However, phenotypic abnormalities i.e. twisted inflorescence, fertilization failure, dwarf plants, bastard offshoots, ceased flowering, multiple carpels [71], were rarely noted after two years of cultivation in the open field, and were reversible. Mirani *et al*. [28, 29] observed epigenetic variations which were reversible to normal phenotypes after two years of cultivation in the field. Additionally, the variations like dwarf plants reverted to normal phenotypes were reported by Gurevich *et al*. [72]. Several workers [73-76] observed the epigenetic variations in tissue cultured plants of date palm reverted to normal phenotypes. Climate and soil conditions of Khairpur, Pakistan were suitable for growing date palm cultivars grown in other provinces of Pakistan. Mirani *et al*. [28, 29] conducted detailed study on somaclonal variations in tissue cultured date palm cvs. Gulistan, Dedhi, Kashuwari. Mirani *et al*. [28] conducted study on reversion of phenotypic variations in tissue cultured

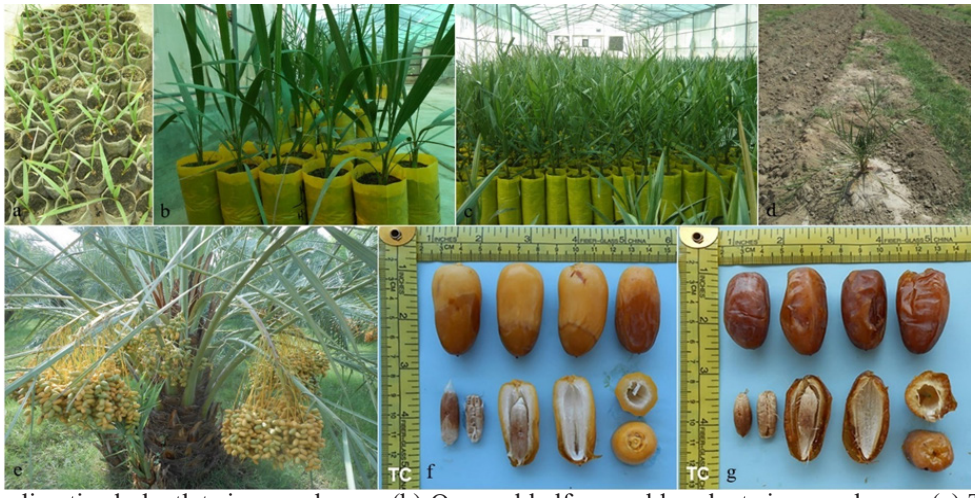

Fig. 3. (a) Acclimatized plantlets in greenhouse, (b) One and half year older plants in greenhouse, (c) Two and half years older plants in greenhouse, (d) Plants growing in the open field, (e) Date palm cv. Gulistan with true-to-type fruits, (f) Rutab stage dates of TC derived cv. Gulistan, (g) Tamar stage dates of TC derived cv. Gulistan.

date palm, and observed that phenotypic variations occurred in tissue cultured plants were epigenetic variations (reversible variations). Solangi *et al*. [77] conducted comparative study on field performance of tissue cultured and offshoot-grown date palm cvs. Gulistan, Kashuwari, Dedhi, and observed that tissue cultured plants produced true-to-type fruits. Date Palm Research Institute, Shah Abdul Latif University, Khairpur witnessed to micropropagate around 2400 plants of date palm cvs. Gulistan, Kashuwari, Dedhi, Samany and Bertamoda, which were distributed among the active growers, belong to different areas of Pakistan produced true-to-type fruits. However, still there is need to have a tissue culture laboratory for production of huge number of plants for commercialization. Tissue culture is expensive; consume high cost; but simultaneously huge number of elite and rare plant species can be propagated within shortest time and space. In this study true-to-type fruits were obtained, which were also confirmed by genetic studies conducted by Mirani *et al*. [29].

4. CONCLUSIONS

Commercially valued date palm cultivar Gulistan was successfully micropropagated through immature inflorescence explants. PGRs exhibited significant role in promoting the growth in each *in vitro* growth stage. Improved germination and multiplication of somatic embryos were obtained, led to shoot formation and rooting. Growth of shoots and roots improved on the media comprised of NAA or IBA. 3-4 adventive roots produced per plantlet by trimming the embryonic root. *In vitro* hardening helped to change slowly from heterotrophic mode of nutrition to autotrophic (to obtain food by photosynthesis). In greenhouse plantlets showed better survival rate on the soil mixtures contained equal ratios of peatmoss and river sand. 100% plants survived in open field due to well-formed roots. Normal tree growth (except reversible changes) and true-to-type fruits were noted showed similar colour, size, shape and taste. Results obtained in this study can be applied to micropropagate elite date palm cultivars grown in Pakistan and around the world.

5. CONFLICT OF INTEREST

The authors declare no conflict of interest.

6. ACKNOWLEDGMENT

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