

DIRECT SHOOT REGENERATION SYSTEM FOR DATE PALM (*PHOENIX DACTYLIFERA* L.) CV. DHAKKI AS A MEANS OF MICROPROPAGATION

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Abstract

This study aimed *in vitro* multiplication of date palm (cv. Dhakki) via direct shoot regeneration. Initially, screening of media with various supplements (activated charcoal, citric acid and ascorbic acid) was carried out to evaluate the maximum survival of cultures, followed by the analysis of different explants for their competence for direct shoot regeneration. The explants were cultured on media supplemented with 1 mg/l naphthalene acetic acid (NAA), 3 mg/l 2-isopentenyladenin (2-iP) and 3 mg/l 6-benzyl amino purine (BAP). Shoot tips revealed the highest capacity for direct shoot regeneration among all the explants tested. Following the direct emergence of 2-3 shoots, explants were shifted to various shoot proliferation media. The medium supplemented with 0.5 mg/l BAP, 0.5 mg/l Kinetin and 1 mg/l NAA proved effective for achieving optimum number of shoots with appropriate length. The rooting medium supplemented with 1.5 mg/l NAA resulted in maximum number of roots per shoot. However, date palm plantlets rooted on medium containing 0.5 mg/l NAA observed with highest survival efficiency after eight weeks of *ex vitro* transplantation.

Introduction

Date palm (*Phoenix dactylifera* L.) the dioecious, monocotyledon species belonging to the family Arecaceae is a multipurpose tree having food, medicinal and ornamental importance. With the present uncertainty in the world food supply and the expected increase in demand, the date palm could be a good source of food of high nutritional value. Pakistan is one of the leading dates' producers in the world. The dates are the third most important fruit in Pakistan and grown on an area of 93336 hectares, with a total production of 680107 tons per annum (Anon., 2010) which is far below its actual potential. The most serious hurdle in the expansion of this crop is unavailability of good quality and disease free planting materials to increase area under crop. The vegetative multiplication of date palm is traditionally achieved by offshoots. This offshoot propagation has limitations such as slow propagation rate, transmission of disease-causing pathogens and insects and production of offshoots in a limited number for a certain period in the life time of a young palm tree (Gueye *et al.*, 2009). Date palm readily grows from seeds but half of the seedlings may turn out to be males and high proportion of inferior quality segregates (Al-Khalifa, 2000). Furthermore, seedlings take 6 to 10 years to fruit so male and female trees are not identifiable until flowering (Othmani *et al.*, 2009). Hence, *in vitro* propagation is the only available alternative to produce disease free, uniform and good quality planting material to establish large scale cultivation within a short period of time.

Date palm is mainly *in vitro* multiplied through somatic embryogenesis (Rashid & Quraishi, 1994; Fki *et al.*, 2003; Al-Khateeb, 2008a; Othmani *et al.*, 2009). Most of the protocols for somatic embryogenesis of date palm made the use of high concentrations of 2, 4-dichlorophenoxyacetic acid (2, 4-D) in media which is known to be associated with genetic instability in regenerated plants. Furthermore, callogenesis is

prerequisite for somatic embryogenesis in date palm (Gueye *et al.*, 2009) which enhances the possibility of producing off type plants (Saker *et al.*, 2006). Conversely, micropropagation through direct organogenesis lacking callus phase, has the advantage of producing highly identical plants in their vegetative characteristics, with the mother plant. Although there are few reports on direct organogenesis (Hussain *et al.*, 1995; Bekheet & Saker, 1998; Al-Khateeb, 2008b) of date palm, however these are confined to specific genotypes. Because of plant genotype specificity even closely related cultivars of date palm observed with variable growth behavior to the same culture conditions (Al-Khayri & Al-Bahrany, 2004). This distinct influence of genotypic specificity puts emphasis on the optimization of direct shoot proliferation protocol for elite cultivars of date palm.

Among the elite cultivars of Pakistan, Dhakki is marked with high economic importance for its distinctive characteristics such as large fruit (4-5 cm long and 2-3 cm thick) with a small pit, fine texture, high flesh, relish taste and reasonably longer shelf life. Average monetary return from Dhakki date palm is also highest among other cultivars. Moreover, Dhakki is the highest yielding date palm cv. of Pakistan with more than 120 kg per tree and is also found as high total sugar containing variety with low moisture content (Alizai *et al.*, 2005). Due to its demand in local, domestic and international markets large quantity of Dhakki dates are planted every year in Dera Ismail Khan. Total area under dates cultivation is approximately 256,000 sq. acres, with approximate total production of dates in Dera Ismail Khan reaches 11000 tons. In recent years 80 % of total dates production is contributed by Dhakki dates.

Although, there are few reports published on tissue culturing of cv, Dhakki, via somatic embryogenesis and callus cultures (Rashid & Quraishi, 1994; Quraishi *et al.*, 1997) but this study describes a comprehensive protocol on micropropagation of same cv. through direct shoot regeneration system, strengthening the possibility of producing genetically stable plants.

Materials and Methods

Plant material and its primary preparation: Female date palms cv. Dhakki were obtained in the form of 3-4 years old offshoot from the fields of Dera Ismail Khan, Pakistan. The primary preparation of explants was done outside the laboratory by removing the roots, brown fibrous leaf sheaths and outer green mature leaves from the offshoots reducing the size to 30 cm. In the laboratory, remaining mature leaves were removed gradually from the bottom to the top, exposing the white young leaves. The gradual removal of white young leaves and surrounding white fibrous leaf sheath resulted in 8 cm shoot tips.

Surface sterilization and dissection of explants: Shoot tips of date palm were treated with 50% (v/v) commercial bleach solution (5% w/v sodium hypochlorite), containing

1 drop of Tween-20 per 100 ml as a surfactant and stirred gently for 30 minutes. These shoot tips were rinsed in sterile distilled water three times for five minutes each and then disinfested with 0.2% (w/v) mercuric chloride solution for 10 minutes and finally rinsed thoroughly with sterile distilled water three times. The upper and lower most regions of shoot tips exposed to disinfectants were removed. To scrutinize the best explant for direct shoot regeneration, explants of variable sizes were excised. The shoot tips of 6 cm long were shortened by removing the leaf primordia surrounding the meristematic region acropetally. These detached leaf primordia were used as explants (Fig. 1a). Further trimming of shoot tips was done to get 2 cm explants keeping 2-4 primordial leaves intact (Fig. 1b). A certain number of shoot tips with 2-4 primordial leaves were used as explants while remaining were further sectioned longitudinally into 3-5 small pieces to employ as explants (Fig. 1c).

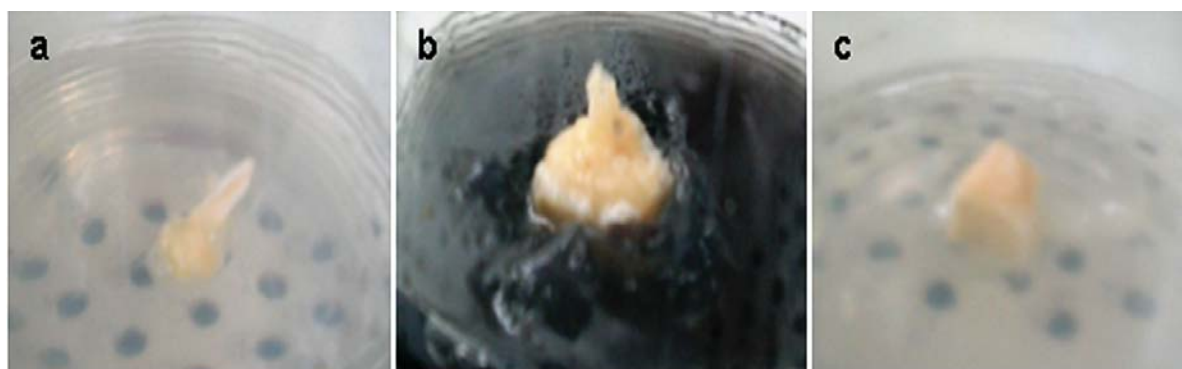


Fig. 1. Various explants inoculated on initiation media for direct shoot regeneration a. leaf primordia b. shoot tip with primordal leaves c. section of apical meristem.

Preliminary screening of media for maximum survival of explants at initiation stage: To reduce explants' browning and their subsequent death at initiation stage, media with various supplements were screened. As a preliminary step, explants were incubated on basal MS media (Murashige & Skoog, 1962) and other modified

MS media containing different combinations of ascorbic acid, citric acid and activated charcoal (Table 1). The survival efficiencies of different explants were recorded after 4 weeks of incubation. The experiment was repeated three times with 4 replications of each explant for the media used.

Table 1. Media containing various supplements evaluated for explants' survival efficiencies at initiation stage.

Media	Media composition
M-0	Basal MS
M-1	Basal MS + 0.3 g/l Activated charcoal
M-2	Basal MS + 0.75 g /l Citric acid + 0.75 g/l Ascorbic acid
M-3	Basal MS + 0.3 g/l Activated charcoal + 0.75 g/l Citric acid + 0.75 g/l Ascorbic acid

Culture initiation and maintenance: Explants were inoculated on initiation media containing MS salts and vitamins supplemented with 75 mg/l ascorbic acid, 75 mg/l citric acid, 0.3 g/l activated charcoal, 40 g/l sugar, 1mg/l naphthalene acetic acid (NAA), 3 mg/l 2-isopentenyladenin (2-iP) and 3 mg/l 6-benzyl amino purine (BAP). The explants were kept in dark at 25 ± 2 °C. The cultures were transferred to fresh media of same composition after every two weeks interval during the initiation period for sustained growth. Cultures were

exposed to photoperiod of 16/8 h after five weeks. To evaluate the morphogenic competence of various explants (Fig.1) five replications of each explant were used for initiation media.

All media were autoclaved at 121° C, at 15 psi for 15 minutes and adjusted to pH 5.75 using 0.1N NaOH or 0.1N HCl and solidified with 2.5 g/l phytigel.

Shoot proliferation and elongation: After direct regeneration of 2-3 shoots, cultures were shifted to MS

media containing 30 g/l sugar, 2.5 g/l phytagel and additionally supplemented with different concentrations and combinations of BAP, Kinetin and NAA (Table 2). Cultures were maintained at 16/8 h

photoperiod, $25 \pm 2^\circ\text{C}$ and subcultured after every 6 weeks. Data recorded in terms of number of shoots per explant and length of shoots after six week on various media (Table 2).

Table 2. Effect of BAP in combination with NAA and Kinetin on shoot proliferation and elongation of date palm after 6 weeks.

Media	Growth regulators (mg/l)	Shoot number ^a / Explan	Shoot length ^b (cm)
SM-0	0	2.90 ± 0.28	7.5 ± 0.99
SM-1	BAP (0.5)	4.80 ± 0.55	5.0 ± 0.69
SM-2	BAP (0.5)+NAA (1)	5.80 ± 0.60	8.2 ± 0.55
SM-3	BAP (0.5)+Kinetin (0.5)	8.10 ± 0.43	4.5 ± 0.31
SM-4	BAP (0.5)+Kinetin (0.5)+NAA(1)	7.95 ± 0.86	8.0 ± 0.40

^{ab}Values represent means \pm SE, where $n = 10$

Development of roots and acclimatization of date palm plantlets: When date palm plantlets reached approximately 6-8 cm in length, they were shifted to rooting media consisting of MS salts and vitamins, 30 g/l

sugar, 1 mg/l biotin, 6 g/l agar provided with different concentrations of NAA (0.1 to 0.5 mg/l). Data recorded for root length and number of roots per shoot after eight weeks (Table 3).

Table 3. Effect of NAA on root development and ex-vitro survival efficiency of date palm plantlets.

NAA (mg/l)	Root length ^a (cm)	Root number ^b / Shoot	Ex vitro survival (%) ^c
0.0	7.23 ± 0.10	0.91 ± 0.36	ND*
0.1	6.51 ± 0.21	4.13 ± 0.83	58 ± 3.57
0.5	5.23 ± 0.15	6.11 ± 0.35	82 ± 4.38
1.0	3.98 ± 0.21	9.50 ± 0.78	45 ± 3.70
1.5	3.01 ± 0.11	11.82 ± 0.67	26 ± 3.9

^{abc}Values represent means \pm SE, where $n = 10$

*ND: Not Determined

Date palm plantlets of suitable height (10-14 cm) having 2-3 leaves and roots not less than three in number selected for *ex vitro* transplantation in green house (Fig. 6a, b). The plantlets were taken out from the test tubes and adhering agar was removed in running tap water. Then plants were shifted to 18 cm plastic pots filled with soil medium consisted of sand and peat moss mixture in 2:1 ratio (v/v) (Fig. 6c). Plants with plastic pots covered with polyethylene bags for one week to maintain high humidity. After one week bags were removed. The plants were irrigated with water every day and half strength MS salt solution was given after two weeks. The survival efficiency of plantlets was determined after five weeks.

Results and Discussion

Influence of various media supplements on survival efficiency of explants: Explants' blackening and release of growth inhibitory exudates into medium, is the most frequently occurring hindrance in date palm initiation and establishment *In vitro*. Browning of the tissue and the adjacent medium is assumed to be due to the oxidation of polyphenols and formation of quinines (Zaid, 1987) which are lethal to the tissues resulting in subsequent death of high proportion of explants at the initiation stage. To minimize the death rate of explants as a consequence

of browning, as a preliminary step the explants incubated on various MS media (Table 1) revealed differences in survival efficiencies (Fig. 2) recorded after four weeks.

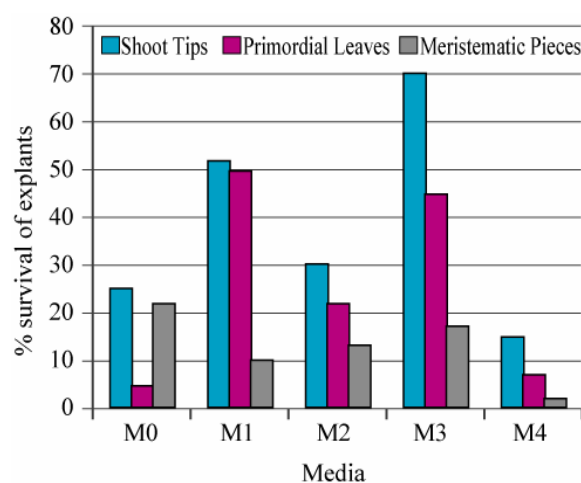


Fig. 2. Effect of different media (M0 – M4) on percent explants' survival where M0 = Basal MS, M2 = Basal MS + 0.3 g/l activated charcoal, M2 = Basal MS + 0.75 g/l citric acid + 0.75 g/l ascorbic acid, M3 = Basal MS + 0.3 g/l activated charcoal + 0.75 g/l citric acid + 0.75 g/l ascorbic acid.

The shoot tips with few intact leaf primordia survived best on M3 comprising combination of citric acid, activated charcoal and ascorbic acid (Table 1) followed by moderate survival on M1 with ascorbic acid as a single supplement (Fig. 2). These findings exposed the synergistic interactions of citric acid, ascorbic acid and activated charcoal in establishing the newly initiated shoot tips. This positive effect on survival of explants might be correlated to the possible influences of supplements used for alleviation of browning in media. Ascorbic acid might act by preventing the oxidation of phenols and rapidly removing any quinone formed. Citric acid presumably acts as a chelating agent and may therefore delay the breakdown of ascorbic acid (George, 1993). The beneficial effects of activated charcoal can be attributed to the removal of inhibitory substances from the media released by the tissue and drastic decrease in the phenolic oxidation or brown exudates accumulation.

These results are in partial accordance with Tisserat (1984b), who incorporated 3 g/l activated charcoal for establishment of date palm shoot tips. However, the present research made use of 0.3 mg/l activated charcoal in media due to the fact that high concentration demands 10-100 times more addition of auxin to media and alters the properties of the medium by adsorbing growth regulators and other components Khan *et al* 2011. The primordial (immature) leaves showed maximum survival on M1 followed by survival on M3 (Fig. 2). These findings confirmed the positive effect of 0.3 mg/l activated charcoal on survival of immature leaf explants of date palm as observed by Othmani *et al.*, (2009) at initiation stage. None of the media proved effective for enhancement of survival efficiency of small sections of apical meristem but M0 to some extent which was devoid of supplements. In the present study, it was noticed that the positive effects of activated charcoal, citric acid and ascorbic acid on survival efficiencies of explants at the given concentrations alone and in combinations decreased as the size of explants got smaller. Probably it points towards the use of low concentrations of these supplements in media when employing the small sized explants for initiation of culture. Therefore there is a need to further test the various concentrations of activated charcoal, citric acid and

ascorbic acid when using explants of different types and sizes to achieve maximum survival.

The findings (Fig. 2) clearly indicate that shoot tips with intact leaf primordia had higher survival efficiency than other explants on all the media tested. Therefore, it can be assumed from the results that large sized explants have more potential to cope up with the problems at initiation stage so as a consequence survive more frequently than very small ones Khan. *et al* 2010 This might be due to the loss of some essential substances released from the excised plant tissues. The large plant tissues can withstand these losses whereas small sized explants can't compensate these by the medium provided Saifullah & Khan, 2011. This suggests the use of appropriate sized explants for successful establishment of date palm explants.

Screening of explants for morphogenic competence:

To probe the explant with best possible morphogenic potential for direct shoot regeneration, shoot tips with 2-4 primordial leaves, small sections of apical meristem and detached primordial leaves (Fig. 1) were incubated on initiation media as described in methods and materials. These explants demonstrated variations in morphogenic response (Figs. 3 and 4).

The swelling of explants started after one week of incubation but considerable increment in size of explants was noticed after two weeks. Induction of calli from primordial leaves was observed at the start of third week of incubation on initiation media. The most of primordial leaves (47%) completely transformed into calli within the five weeks of incubation (Fig. 3b, Fig. 4). Fki *et al.*, (2003) also reported the callus formation from juvenile leaves of date palm cv. Deglet Bey. Similarly, Gueye *et al.*, (2009) found the highest callogenic capacity in the elongation zone of young leaf of date palm. In this study none of the primordial leaves gave rise to direct shoot regeneration. Whereas, Othmani *et al.*, (2009) reported direct shoot regeneration from the base of the few leaves in addition to callus formation using 10 mg/l 2, 4-D in media. A small proportion of tiny pieces of meristematic tissues showed the emergence of 1-2 shoot primordia from the base of explants after 8 weeks of incubation. Remaining tissues transformed into calli after 5 weeks on the initiation media (Fig. 3c).

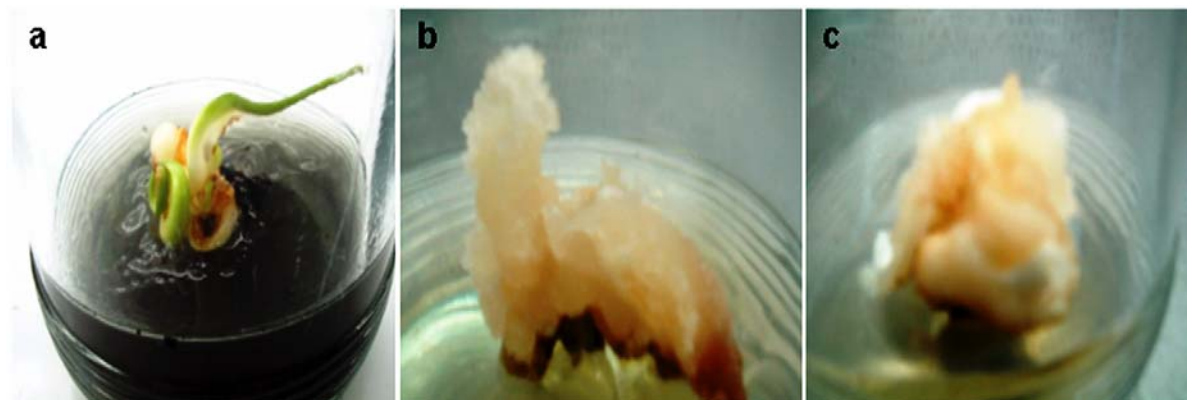


Fig. 3. Diverse morphogenic responses of different types of explants on initiation media containing 1 mg/l NAA, 3 mg/l BAP, and 3 mg/l 2-iP **a.** direct shoot regeneration from shoot tip after 10 weeks of incubation **b.** callus formation from leaf primordia and **c.** section of apical meristem transformed into callus after 5 weeks of incubation.

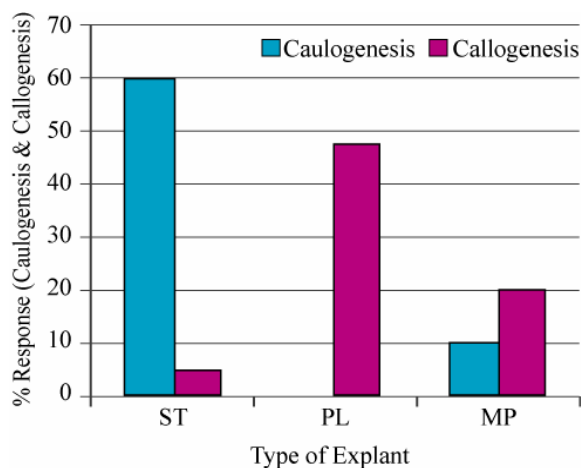


Fig. 4. Percent response of shoot tips (ST), Primordial leaves (PL) and Meristematic pieces (MP) to caulogenesis (direct shoot regeneration) and callogenesis on media supplemented with 1 mg/l NAA, 3 mg/l BAP, and 3 mg/l 2-iP.

The shoot tips with 2-4 leaf primordia resulted in 2-3 shoots by direct organogenesis (Fig. 3a) after incubation of 10 weeks. More than half number of shoot tips responded to direct shoot regeneration (Fig. 4). These results are in accordance with the results obtained by Bekheet & Saker (1998) where they got direct shoot proliferation from shoot tips of date palm on MS media supplemented with the same growth regulators' combination as used in the initiation media of the present study but relatively at higher concentrations of cytokinins. Similarly, Tissert (1984) got the same results on LS media provided with 10 mg/l NAA and 3 g/l activated charcoal. On the other hand, Taha *et al.*, (2001) reported the callus formation from shoot tips by using 2 mg/l 2-iP and 1 mg/l NAA.

The diverse morphogenic response of explants excised from the shoot apex of date palm, on the same initiation media might be the result of differences in the differentiation state of tissues. This is also reported by Gueye *et al.*, (2009) that different segments of 5 cm leaf of date palm exhibited marked variation in callogenic capacity on the same media. They directly correlated this callogenic capacity with the gradient of tissue differentiation in leaf

segments. Moreover, Othmani *et al.*, (2009) got callus as well as direct shoot regeneration from immature leaves of date palm on the same media supplemented with 10 mg/l 2, 4-D. Besides the different state of tissue differentiation, the levels of endogenous hormones have significant influence on determination of morphogenic response of excised tissue (Gueye *et al.*, 2009).

Influence of plant growth regulators' interaction on shoot proliferation and elongation:

After direct emergence of 2-3 shoots, cultures were transferred to shoot proliferation media supplemented with various combinations and concentrations of BAP, NAA, and Kinetin. It is evident from the results (Table 2, Fig 5a) that medium supplemented with 0.5 mg/l BAP and 0.5 mg/l Kinetin revealed as the most effective one for maximum shoot bud proliferation. These cytokinins enhanced shoot proliferation due to their possible role in cell division and inhibition of apical dominance in multiplication media. The combined use of BAP and Kinetin in the culture medium had a significant synergistic effect on the number of shoots (8.10). These results are in agreement to Kathal *et al.*, (1988). They reported more effective shoot proliferation in the presence of more than one type of cytokinin. However shoot elongation on this medium was the least (4.5 cm) among all the media tested. It supports the assumption that concentrations of cytokinins, effective for shoot bud induction and proliferation are suppressive to shoot elongation. Of all the media evaluated, the longest shoots (8.2 cm) were obtained on the medium supplemented with 0.5 mg/l BAP in combination with 1 mg/l NAA. It is also worth mentioning in this study that NAA in combination with BAP and Kinetin (Table 2) exhibited pronounce effect on shoot elongation. In this study the medium responsible for maximum shoot elongation also resulted in minor signs of callus formation at the base of shoots. The results (Table 2, Fig. 5b) illustrated that use of medium containing combination of two cytokinins (BAP and Kinetin) and one auxin (NAA) proved more promising for attaining cultures with adequate number (7.95) of shoots with sufficient length (8.0 cm).



Fig. 5. Shoot proliferation and elongation after six weeks **a.** profuse shoot proliferation on media supplemented with 0.5 mg/l BAP and 0.5 mg/l Kinetin **b.** Optimum shoot multiplication and elongation on media supplemented with 0.5 mg/l BAP, 0.5 mg/l Kinetin and 1 mg/l NAA **c. d.** Rooting of date palm shoots after eight weeks of culturing on MS media containing 0.5 mg/l NAA and 1 mg/l NAA respectively.



Fig. 6. *Ex vitro* transplantation and acclimatization of date palm plantlets **a.** **b.** date palm plantlets ready for *ex-vitro* transplantation after removal of agar in running tap water **c.** date palm plantlets transplanted in plastic pots filled with mixture of sand and peat moss in 2:1 ratio (v / v).

Effect of NAA on rooting and *ex vitro* survival efficiency of plantlets: For initiation of roots, date palm shoots measuring approximately 6-8 cm long separated and transferred on MS media consisting of supplements described as in materials and methods. Among the auxins, NAA is the most frequently exploited exogenous hormone for roots' development of *in vitro* date palm's shoots. However genotypic specificity of a particular cultivar resulted in variations for optimum concentrations of NAA. Therefore media with different concentrations of NAA were evaluated for attaining the best quality roots to achieve maximum *ex vitro* establishment of date palm (cv. Dhakki) plantlets. The results (Table 3) showed that number of roots increased with the ascending concentrations of NAA. 1.5 mg/l NAA favored the profuse rooting (Fig. 5d) of date palm shoots followed by 1 mg/l NAA. However, these roots were very thin. These results are in agreement with those obtained by Bekheet & Saker (1998). They found efficient rooting effect of 1 mg/l NAA rather than IBA and IAA at the same concentrations. On the other hand, Tissert (1984) reported profuse rooting of date palm plantlets on medium supplemented with 0.1 mg/l NAA. Table 3 shows that rising levels of NAA negatively affected the root length of date palm plantlets.

When the rooted plantlets transplanted to *ex vitro* conditions, the marked variation in survival efficiency (Table 3) was observed among the plantlets rooted on MS medium provided with various concentrations of NAA. Of all plantlets transplanted *ex vitro*, the plantlets harvested from medium consisting of 0.5 mg/l NAA found to have highest survival proportion (83 %). The roots of these plantlets were thicker than the others (Fig. 5c). The plantlets obtained on the media supplemented with 0.1 mg/l NAA had greater survival efficiency (58 %) than plantlets harvested from 1 mg/l NAA and 1.5 mg/l NAA containing media (Table 3). These results are also reported by Johnson & Burchett (1991). They found highest survival rate in plantlets supplemented with lower concentrations of IBA and IAA despite of the fact that these plantlets had comparatively less number of roots

than those obtained at higher concentrations of auxins. Thus it can be depicted from the results of this study that selection of appropriate concentration of NAA should be made to get plantlets with adequate number and length of roots to achieve maximum *ex-vitro* establishment.

Conclusion

This report uncovered the morphogenic potential of different types of explants suggesting the exploitation of shoot tips with 2-4 intact leaf primordia as the most appropriate explant source for *in vitro* multiplication of date palm (cv. Dhakki) through direct shoot regeneration. It can also be inferred that combination of citric acid, ascorbic acid and activated charcoal might have pronounced positive effects on shoot tip establishment by mitigating explant blackening. The results of this study further illustrate that the combined use of BAP and Kinetin had a considerable synergistic effect on shoot proliferation whereas interaction of these cytokinins with NAA exerted significant effect on shoot elongation of date palm (cv. Dhakki).

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