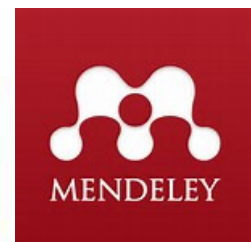


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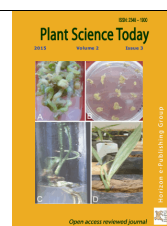
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## RESEARCH ARTICLE

# Agrobacterium-mediated genetic transformation of date palm (*Phoenix dactylifera* L.) cultivar “Khalasah” via somatic embryogenesis

Junaid Aslam<sup>1,2</sup>, Saeed Ahmed Khan<sup>1</sup> and Mustafa Abul Kalam Azad<sup>3</sup>

### Abstract

In present investigation, an efficient *Agrobacterium*-mediated genetic transformation was successfully carried out for a well known date palm (*Phoenix dactylifera* L.) cultivar “Khalasah” using matured somatic embryos. Somatic embryogenesis was initiated from offshoot's shoot tips of date palm cultivar. For genetic transformation, morphologically advanced matured somatic embryos developed on MS medium fortified with TDZ (1.0 mg/l) were co-cultured with *A. tumefaciens* strain LBA 4404 harboring binary vector pBI 121, containing *uidA* (*GUS*) and *npt II* genes and incubated for

4 days and later it was (somatic embryos) inoculated on germinating and plantlet conversion MS medium supplemented with BAP (0.75 mg/l) + kanamycin (100 mg/l). Prolific shoots developed from putatively transformed matured embryos showed 47.5 % transformation efficiency. A large number of transgenic plants were obtained and later established in black hard plastic bags. A strong *GUS* activity was detected in the putatively transformed plant leaves by histochemical assay and, the integration of *uidA* (*GUS*) and *npt II* genes into transgenic plants was confirmed by polymerase chain reaction (PCR) and Southern hybridization analysis. The established transformation protocol allows effective and quick regeneration via somatic embryogenesis, and it would be highly valuable for existing date palm orchards and improving their productivity.

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### Introduction

Date palm (*Phoenix dactylifera* L.) is the most important cash crop of Middle East and North Africa particularly in arid regions (Al-Khayri, 2001). It is a monocotyledonous and dioecious species belonging to the family Arecaceae. The Palmae is rich in diversity with 183 genera and more than 2,300 species with the greatest diversity in the Old World (Dransfield *et al.*, 2008). Economically, date palm provides a major source of income for farmers and

associated industries (Zohary and Hopf, 2000). Arab countries covered largest area for the cultivation of date palm which grow 62 million of the 105 million trees worldwide on an area of over one million ha., and occupy a total of 5% arable land (Anonymous, 1969). In Saudi Arabia, more than 90% of the land being used for the cultivation of date palm trees (Shaheen, 1990; Saker, 2011). The fruit of date palm is known as a date. According to FAOSTAT report, the world's largest date producing countries are Egypt followed by Iran, Saudi Arabia, United Arab Emirates, Pakistan, Algeria, Iraq, Sudan, Oman, Libya, Tunisia, Morocco, Yemen, Mauritania, Qatar, Chad, Israel, Bahrain and USA (Saker, 2011).

Date palm's fruit have a high tannin content and are used medicinally as a detersive (having cleansing power) and astringent in intestinal troubles. As an infusion, decoction, syrup, or paste, dates may be administered for sore throat, colds, bronchial catarrh, and taken to relieve fever and a number of other complaints (Vyawahare *et al.*, 2009). Its provides more calories than other fruits, cooked rice, bread or meat and is rich in sodium, iron, copper, calcium, zinc, magnesium, phosphorus and sulfur (Al-Shahib and Marshall, 2003). Fruits also contain water and fat soluble vitamins (Junaid *et al.*, 2011a,b,c), protein, fiber, lipids and are free of cholesterol (Toutain, 1967). The wood and leaves of the date palm are as important as the fruit itself. They provide fiber, fuel, clothing, furniture, hats, baskets and housing (Barreveld, 1993). The pit of the fruit is used to feed animals and the sap is used to prepare wine. Currently, date palm trees and leaves have become an important component in decoration. In addition to its nutritional value, the date palm tree tolerates adverse environmental conditions (abiotic stresses) and is important in reducing desertification and dune-encroachment (Saker, 2011).

It is propagated sexually through seeds and vegetatively by offshoot (Bonga, 1982). Although, date palm productivity can be achieved through increasing the productivity of the existing trees or expanding the palm cultivated areas. However, both approaches constitute a huge dilemma, because extensive breeding programs for the selection of superior date palm clones through traditional methods are tedious efforts due to the long life cycle and strongly heterozygous nature of the palm tree (Saker and Moursy, 2003). Nonetheless, insufficient and the expensive cost of offshoots is another serious limiting factor, which hinders expansion of palm cultivated area, renewing of existing palm orchards and improving their productivity (Anonymous, 1969).

In spite of that, date palm faces abiotic and biotic constraints; abiotic includes development under harsh desert conditions, where growth takes place without a regular supply of amendments. In biotic constraints, especially bayoud disease caused by *Fusarium oxysporum* f.

*sp. albedinis* (Malençon, 1934; Louvet and Toutain, 1973; Laville, 1973; Djerbi, 1988; Carpenter and Klotz, 1966). This disease is the most devastating infection of the date palm and was first described in southern Moroccan groves. Currently, it continues to spread across North African countries, especially in Morocco and Algeria where more than 12 million date palm trees have been destroyed so far (Sarkar, 2011). Therefore, serious efforts benefit from the application of plant tissue culture and gene transfer technology playing a potent role to overcome the date palm dilemma by developing high regeneration and disease free plantlets. Although notable progress had been made in date palm tissue culture (Tisserat, 1982; Bhansali *et al.*, 1988; Zaid and Hughes, 1995; Saker *et al.*, 2000; Bekheet *et al.*, 2001, 2002; Al Khayri, 2003; Saker *et al.*, 2006a; 2006b; Junaid *et al.*, 2009; 2011a). Genetic improvement of date palm through genetic engineering is the only realistic strategy to rapidly improve this tree, genetic transformation of date palm has not been reported or is still in its infancy (Saker and Ghareeb, 2007a; Saker *et al.*, 2007b; 2009). The tree is a target host for several pests and diseases, so it is necessary to focus on its *in vitro* propagation and genetic engineering to overcome some of these problems. Therefore, in present investigation we have been able to produce transgenic of "Khalash cv." through *Agrobacterium*-mediated genetic transformation technology and confirmation was made by the polymerase chain reaction and Southern hybridization analysis. To produce transgenic plants, regeneration protocol is an important step. In our previous studies, we have established high regeneration system *via* somatic embryogenesis (Junaid *et al.*, 2011a). To our knowledge, there is no report on the production of transgenic plant *via* *A. tumefaciens*-mediated transformation in date palm (*Phoenix dactylifera* L.) cultivar "Khalasah c.v." The established transgenic protocol would be helpful to produced plantlets with high quality fruits in future.

## Materials and methods

### Plant material

The offshoots of the date palm's "Khalash cv." was collected from the residential premises of the Chairman, Dubai Pharmacy College. Offshoots were 3-4 years old, each weighting approximately 30-40 kg (Junaid *et al.*, 2011a).

### Cleaning of explants

To remove the attached soil and other debris, the offshoots were washed with the tap water and the outer large leaves and fibres were carefully removed with the sharp knife until the shoot tip zone was exposed. Shoot tips were then trimmed to approximately 67 cm in length and 46 cm in width (Junaid and Khan 2009).

### **Disinfection and antioxidant treatment**

The excised shoot tips were washed three-four times with double distilled water. Thereafter, the cleaned shoot tips were subjected to two steps of disinfection: a) the washed shoot tips were dipped for 20 minutes in a fungicide (Benlate, 5 g/l) solution; b) the shoot tips were dipped in 33% commercial Clorox solution for 2530 minutes. The explants were then rinsed three times with autoclaved distilled water in a laminar flow hood. The disinfected explants were then soaked in an antioxidant solution to minimize oxidation of phenolic compounds (responsible for the browning of tissues), and to protect them from desiccation. The antioxidant solution consisted of 2 g/l polyvinylpyrrolidone (PVP, Mw = 40,000), 200 mg/l anhydrous caffeine and 100 mg/l sodium diethyldithio carbonate AR. The shoot tips were kept in this solution for 20 minutes and finally washed with double distilled water. (Junaid and Khan 2009).

### **Embryogenic callus induction and maintenance**

Embryogenic callus was induced from small pieces of shoot tips (Junaid *et al.*, 2011a). The cultures were maintained with periodic subculturing at an interval of four weeks.

### **Suspension culture**

Suspension culture was established according to the previous reports (Junaid *et al.*, 2011a).

### **Somatic embryo initiation and proliferation**

The suspended cells of the embryogenic calluses were implanted on MS medium supplemented with optimization concentration of NAA (Junaid *et al.*, 2011a), where undifferentiated heterogeneous masses of somatic embryos were produced.

### **Somatic embryo maturation**

Advanced globular embryos were separated out from the callus masses and placed on MS medium fortified with optimization concentrations of TDZ (Junaid *et al.*, 2011a). Matured somatic embryos were separated out from *in vitro* culture and finally used for transformation studies.

### **Kanamycin sensitivity test**

Matured somatic embryos were inoculated on germinating and plantlet conversion MS medium. Before inoculation, medium was supplemented with 10, 20, 30, 40, 50, 100 or 200 mg/l kanamycin (sterilized by Milipore membrane filter, 0.22 µm pore size).

### **Preparations of *A. tumefaciens* strain**

The fresh culture of *A. tumefaciens* was prepared by overnight growth of a single colony at 28°C in 10 ml liquid yeast extract mannitol bacterium (YEM) medium containing kanamycin (100 mg/l). The bacterial culture

was centrifuged at 5000 rpm for 10 min. The pellet was resuspended in 1-2 ml liquid MS basal medium to obtain a density of 10<sup>8</sup> cells per ml (OD<sub>540</sub> = 0.520).

### **Co-cultivation**

For the induction of infection, matured somatic embryos (25/conical flask) were dipped in freshly prepared *Agrobacterium* suspension for 10 min. In the similar manner, in order to study the effect of acetosyringone on transformation, different concentrations (0, 10, 20, 40, 80, 100 µM) of acetosyringone were treated during infection. After infection with *Agrobacterium* suspension, matured somatic embryos were blotted using the sterilized filter paper (Whatman No. 1) for 25 min. Blotting was carried out to away the excessive bacterial culture, the explants were incubated for 1, 2, 3 and 4 days in dark for co-cultivation.

### **Selection, germination and plantlet conversion**

The co-cultivated explants were washed with MS basal liquid medium, blotted dry on sterile filter paper and transferred to selection medium (MS basal medium with 3 % sucrose, 0.67 % Agar-Agar, 100 mg/l kanamycin (HiMedia, Bombay, India). Initially the cultures were subcultured for 2-3 times at weekly interval to avoid excessive bacterial growth. After four weeks, the matured somatic embryos were subculture to fresh medium for germination and plantlet conversion on MS medium supplemented with BAP (0.75 mg/l) + Kanamycin (100 mg/l). Data was scored in terms of somatic embryo germination and plantlet conversion percentage, number of shoots per explants and shoot length.

### **GUS activity**

Histochemical assay of *GUS* activity was carried out by the procedure of Xiao *et al.* (2005). Leaf of the somatic embryos derived transgenic plantlets were submerged in a substrate solution containing 100 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.3 % (m/v) *X-Gluc* and 0.1 % (v/v) *Triton X-100* and incubated overnight at 37°C. Expression of *GUS* gene was visualized after the tissues were destained by soaking and washing in 70 % ethanol for several times.

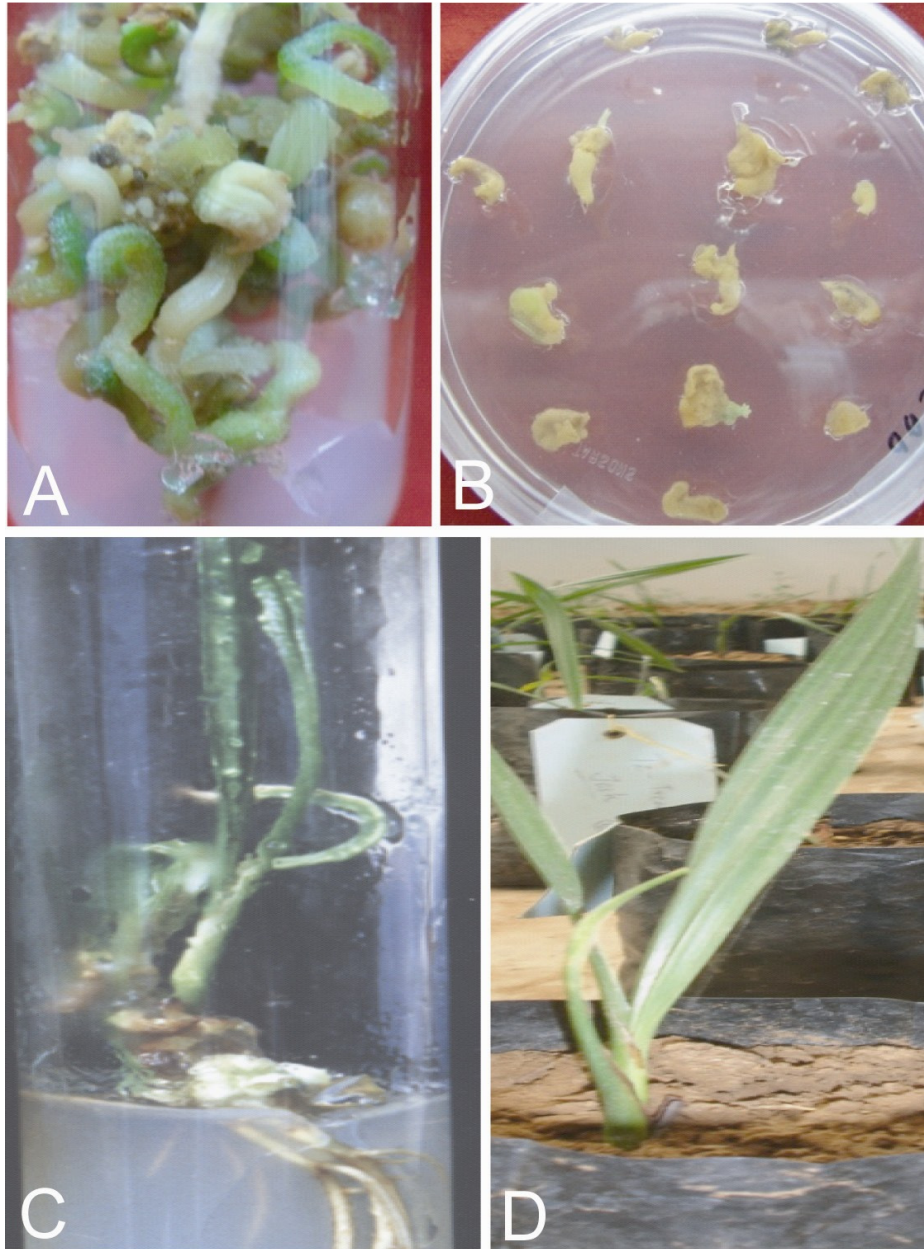
### **PCR analysis**

Total genomic DNA was extracted from fresh leaves of untransformed and putative transgenic (T<sub>0</sub>) plants (Doyle and Doyle, 1990). Integration of the transgene into the transgenic plants was checked through PCR analysis for the presence of the *uidA* (*GUS*) as well as *npt II* gene. The 514 bp coding region of *uidA* (*GUS*), and 750 bp coding region of *npt II* was amplified using sequence specific oligonucleotide primers (*uidA* (*GUS*): 5'-CTG TAG AAA CCC CAA CCC GTG- 3' (forward) and 5'-CAT TAC GCT GCG ATG

GAT CCC-3' (reverse), *npt II* : 5' CAA TCG GCT GCT CTG ATG CCG-3' (forward) and 5'- CAA TCG GCT GCT CTG ATG CCG-3'(reverse), 10 µg purified genomic DNA, and Taq polymerase (MBI Fermentas). PCR amplification was achieved by initial denaturation at 94°C for 4 minutes

### Southern analysis

For Southern analysis genomic DNA samples (10 µg) from putative transgenic and control *Phoenix dactylifera* cultivar "Khalasah" were restricted with *EcoRI* restriction enzyme



**Fig 1.** Transgenic plants development through mature somatic embryos of date palm (*Phoenix dactylifera* L.) cultivar "Khalasah". A) Matured somatic embryos on MS medium supplemented with TDZ (1.0 mg/l). B) Kanamycin (100mg/l) resistant somatic embryos on selection medium. C) Transgenic plantlet on germination and plantlet conversion medium (BAP (0.75 mg/l) + kanamycin (100 mg/l)). D) Transgenic plants growing in natural condition.

followed by 30 cycles of 94°C for 1 min, 58°C for 1 minute annealing, and 72°C for 1min and a final extension step at 72°C for 4 minutes hold was given to extend any premature synthesis of DNA. The PCR products were then separated on a 1 % agarose gel.

and fractionated by electrophoresis on a 1 % agarose gel, blotted on positively charged nylon membrane (Roche Diagnostics GmbH, Germany) and hybridized with non radioactive labeling dioxyguanine (DIG) method. The 750 bp product of *npt II* that was obtained from plasmid DNA

**Table 1.** Effect of acetosyringone (AS) and co-cultivation period on efficiency of transformation of matured somatic embryos of (*Phoenix dactylifera* L.) cultivar “Khalasah” after 4 weeks of culture on selection medium. Twenty five embryogenic calluses were cultured per Petri dish.

Acetosyringone ( $\mu\text{M}$ )	Number of GUS Positive Matured Somatic Embryos				Efficiency (%)
	2d	3d	4d	5d	
0	1.3 $\pm$ 0.23f	2.1 $\pm$ 0.32ef	3.3 $\pm$ 0.22e	1.4 $\pm$ 0.21d	7.5
10	2.6 $\pm$ 0.33c	4.1 $\pm$ 0.33cd	6.6 $\pm$ 0.32c	2.3 $\pm$ 0.12bc	14.0
20	3.4 $\pm$ 0.21ab	6.2 $\pm$ 0.51b	7.8 $\pm$ 0.33b	3.4 $\pm$ 0.43b	30.2
40	4.6 $\pm$ 0.32a	8.1 $\pm$ 0.12a	9.8 $\pm$ 0.14a	5.4 $\pm$ 0.33a	47.5
80	2.5 $\pm$ 0.62cd	5.7 $\pm$ 0.32bc	6.1 $\pm$ 0.43cd	0 $\pm$ 0.00e	10.5
100	1.1 $\pm$ 0.06e	2.5 $\pm$ 0.11e	0 $\pm$ 0.00f	0 $\pm$ 0.00e	8.7

Values are means of five replicates from two experiments. Means with common letters within each column are not significantly different at  $P \leq 0.05$ , according to DMRT.

**Table 2.** Morphological parameters (germination and plantlet conversion, number of shoots per explants and shoot length), when matured somatic embryos after successful selection were inoculated for germination and plantlet conversion on MS medium fortified with BAP (0.75 mg/l) + Kanamycin 100 mg/l). 25 explants/flask were tested.

Weeks	Germination and plantlet conversion (%)	Number of shoots per explants	Shoot length (cm)
2	25.64 $\pm$ 1.34d	3.0 $\pm$ 0.65d	0.5 $\pm$ 0.10d
4	62.34 $\pm$ 2.05ab	8.5 $\pm$ 1.23c	1.2 $\pm$ 0.62bc
8	75.64 $\pm$ 3.04a	12.0 $\pm$ 1.06ab	1.6 $\pm$ 0.43b
12	42.1 $\pm$ 2.03bc	15.5 $\pm$ 2.10a	1.8 $\pm$ 0.32a

Values are means of five replicates from two experiments. Data were scored after seven weeks of culture. Means with common letters within each column are not significantly different at  $P \leq 0.05$ , according to DMRT.

using random oligonucleotide primers was extracted from 1% low-melting-point agarose gel (Sigma-Aldrich) and used as the probe. Hybridization was carried out for 16 hrs at 65°C with gentle shaking.

#### Culture conditions

The pH of all the cultures was adjusted to 5.6 – 5.8 before autoclaving. The media were sterilized in an autoclave for 15 min at 121°C. Cultures were incubated at 25 $\pm$ 2°C under 16-h photoperiod with cool white fluorescent light (100 mol·m<sup>-2</sup>·s<sup>-1</sup> PFD).

#### Acclimatization

Transgenic plantlets were removed from culture vessels, transplanted in micro-plastic bags containing sterile soil rite, thoroughly covered with perforated polythene bags and grown for one month at 25 $\pm$ 2°C under a 16-h photoperiod (100 mol·m<sup>-2</sup>·s<sup>-1</sup> PFD). Plantlets were then transferred to black polythene plastic bags containing 1:1 soil rite and sand for another 2–3 weeks at room temperature, and finally planted in 100% soil under natural conditions.

#### Statistic Analysis

The data were statistically analyzed using Duncan’s multiple range test (DMRT) with the significance being determined at the  $P \leq 0.05$ .

#### Results

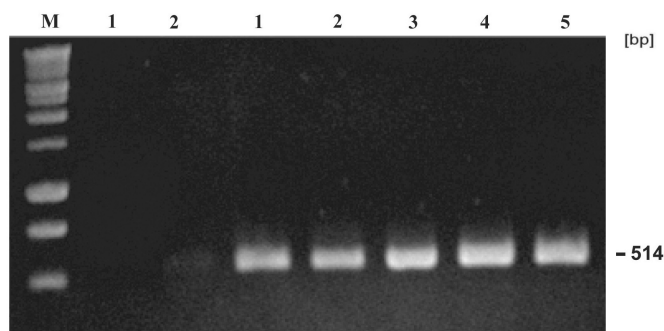
##### Somatic embryo initiation, proliferation and maturation

Embryogenic callus was induced from excised shoot tip on MS medium added with 2, 4-D (1.5 mg/l). It was maintained by continuous subculturing on fresh nutrient medium and the further rapid development was achieved on medium containing NAA (1.5 mg/l) following a suspension culture. Induced somatic embryos were in mixed population and could not be separated out easily. Advanced globular embryos were separated out from the callus mass and placed on MS medium fortified with TDZ (1.0 mg/l), where somatic embryos started to become greenish morphologically (matured somatic embryos)

after 2-3 weeks of inoculation (Junaid *et al.*, 2011a). Finally, matured somatic embryos (Fig. 1A) used for genetic transformation study.

#### Kanamycin sensitivity test

Amongst the tested different concentrations of Kanamycin, it was found that kanamycin at a concentration of 100 mg/l prove to be highly effective for the selection of matured somatic embryos which germinated and converted into plantlets directly. Kanamycin resistant matured somatic embryos were in bright yellow (Fig. 1B) colour morphologically (was obtained among the browned matured somatic embryos. In kanamycin free medium (control), the matured somatic embryos, after five weeks of inoculation started the germination and converted into plantlets. Concentration above the optimal level turned the material completely necrotic and retarded germination and plantlet conversion potentiality of the explants.



**Fig 2.** Confirmation of integration of *uidA (GUS)* gene (514 bp of amplified product) in the genome of date palm (*Phoenix dactylifera* L.) cultivar “Khalasah” plants by PCR. Lane M - 1 kb molecular size marker (*HindIII* digest of  $\lambda$  phage DNA), lanes 1,2 - non-transgenic plants, lanes 1 to 5 - transgenic plants.

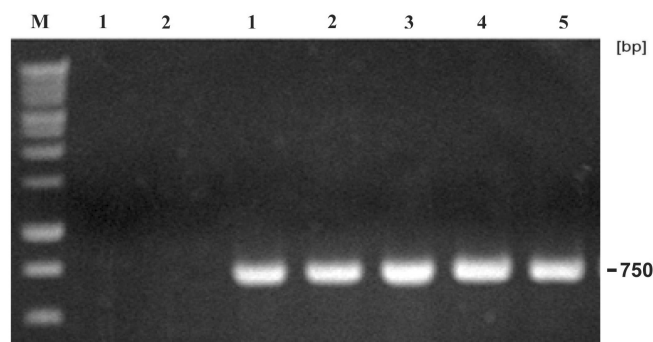
#### Co-cultivation and Selection and transgenic plant development

Matured somatic embryos were co-cultivated with *Agrobacterium* at different days interval, and thereafter, transferred to the selection medium (MS solid medium containing 100 mg/l kanamycin) containing various levels of acetosyringone. Of the tested different concentrations of acetosyringone, 40  $\mu$ M along with 4-d co-cultivation period showed maximum transgenic efficiency (Table 1). Co-cultivation of the matured somatic embryos for 4-d resulted in highest percentage of kanamycin resistant producing regenerating shoots buds directly (17.67%), whereas, 2-d followed by 3-d co-cultivation resulted in highest percentage of explants producing only roots (1.66%). The percentage of explants growing well on the selective medium was calculated as transformation

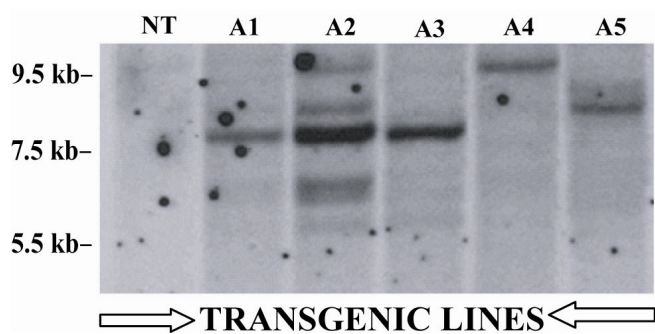
frequency. Table 2 shows growth performance of matured somatic embryos after successful selection on germination and plantlet conversion medium. A significant increase in somatic embryos germination and plantlet conversion percentage, number of shoots per explants and shoot length was noticed up to 8th weeks, thereafter, only germination and plantlet conversion started to decreased. After 4 weeks of culture, X-gluc reaction with somatic embryo derived transgenic leaf revealed that 4-d co-cultivation showed maximum efficiency of transformation. The X-Gluc reaction revealed that, transformed plantlets were all *GUS*-positive. Whereas, the non-transformed somatic embryo derived plantlets did not stain. Maximum plantlet conversion was reported after 8 weeks of somatic embryos germination medium with kanamycin. Transgenic plantlets with healthy roots (Fig.1 C) were tested for acclimatization, which showed a good survivability (Fig 1. D).

#### PCR and Southern analysis

PCR analysis showed amplification of integration of the *uidA (GUS)* as well as *npt II* gene. The 514 bp coding region of *uidA (GUS)* (Fig. 2) and 750 bp coding region of *npt II* was (Fig. 3) amplified using sequence specific oligonucleotide primers *npt II* gene indicating the presence of transgene in 5 out of 10 shoots analyzed. The transformation rate was 0.625 % (5/800). However, the transformation rate as determined by the number of PCR positive versus the number of somatic embryos mediated shoot was 2.52 % (5/198). Southern analysis of PCR positive plants of  $T_0$  generation revealed strong hybridization signal to the *npt II* gene probe indicating different *npt II* insertion sites and those had single copy to multiple copy of the *npt II* gene inserted. No hybridization could be detected for DNA samples from untransformed plants (Fig. 4).



**Fig 3.** Confirmation of integration of *npt II* gene (750 bp of amplified product) in the genome of date palm (*Phoenix dactylifera* L.) cultivar “Khalasah” transgenic plants by PCR analysis: Lane M - 1 kb molecular size marker (*HindIII* digest of  $\lambda$  phage DNA), lanes 1,2 - non-transgenic plants, lanes 1 to 5 - transgenic plants.



**Fig 4.** Confirmation of transgenic plants of date palm (*Phoenix dactylifera* L.) cultivar “Khalasah” by Southern blot analysis. Genomic DNA was digested with *EcoRI* and hybridized with the *npt II* probe: NT – non-transgenic plants, A1 to A5 – lines of transgenic plants. Molecular mass marker presented left side.

## Discussion

*Agrobacterium*-mediated genetic transformation based gene transformation technology for the development of particular trait, improvement of yield and to develop disease free transgenic has been almost exclusively focused on crops of high economic importance (Birch, 1997; Gheysen *et al.*, 1998; Li *et al.*, 1992; Cervera *et al.*, 1998; Saini and Jaiwal, 2007; Shrawat and Good, 2011; Abalaka and Mohammed, 2011; Ramamoorthy and Kumar, 2012; Li *et al.*, 2015). Unfortunately, other species native to developing countries has not attracted the interest of the well-known multinational seed and biotechnology companies due to their low socioeconomic status and minimal contribution to the national treasury. Date palm is one of these neglected crops on a global scale, and efforts at biotechnological improvement of this tree are limited to local researchers within the developing countries.

In present investigation we have been able to produced date palm “Khalasah” c.v. transgenic plantlets successfully. Co-cultivation had a marked effect on the production of kanamycin resistant material. We have observed that co-cultivation along with addition of 40  $\mu\text{M}$  acetosyringone in treatments upto 4-d cocultivation with *Agrobacterium*, further enhanced the recovery of more kanamycin resistant somatic embryos. The matured somatic embryos cell layer would have developed during the pre culture period and could have provided a place for *Agrobacterium* to penetrate during co-culture. Chemicals such as acetosyringone are recommended in most of the crops transformation protocols (Hiei *et al.*, 1994; Ishida *et al.*, 1996; Cheng *et al.*, 1997; Tingay *et al.*, 1997; Kumlehn *et al.*, 2006) for *vir* induction. Stachel *et al.* (1985) reported that, expression of virulence gene in *A.tumefaciens* was activated specifically by the acetosyringone and  $\alpha$ -hydroxyacetosyringone. Van Wordragen and Dons (1992) reported that the addition of acetosyringone is very

effective for transformation of recalcitrant crops. However, Godwin *et al.* (1991) stated that acetosyringone was not effective for transformation in some plant species.

Co-cultivation period longer than 4-d led to a reduction in transformation frequency in present study, where, explants become brown, it might be due to the bacterial overgrowth. The appropriate amount of kanamycin for the selection of callus or organs varied depending on the type of tissues, developmental stages and plant species (Han *et al.*, 2000; Franklin and Lakshmi Sita, 2003; Manickavasagam *et al.*, 2004). In the present study, 100 mg/l kanamycin was effective for the selection of transgenic somatic embryos. It has been reported that high concentrations of selection agent were effective for the selection of transformed callus and organs in other plant system (Li *et al.*, 1992; Cervera *et al.*, 1998; Saini and Jaiwal, 2007; Shrawat and Good, 2011, Junaid *et al.*, 2012a; Junaid *et al.*, 2012b; Liu *et al.*, 2014; Palla and Pijut, 2015).

The *GUS*-positive transgenic date palm “Khalasah” c.v. plants were analyzed by PCR reactions. The integration of the *uidA* (*GUS*) and *npt II* genes in the genome of date palm “Khalasah” c.v. was confirmed by the presence of an amplified fragment of 514 bp and 750 bp, respectively. Amplification of this fragment was not observed in non-transformed wild plants. Southern hybridization pattern of selected transgenic plants confirmed single as well as multiple gene insertion. Studies have shown that it is desirable to have single gene insertion in transgenic plants as multiple copies of T-DNA adversely influence the expression of the introduce gene (Stam *et al.*, 1997).

Plantlets with healthy roots were transplanted for acclimatization purpose, showed a good survivability percentage, and normal morphological appearance. Successful transplantation has been achieved in several other plants earlier (Dhar and Joshi, 2005; Junaid *et al.*, 2008; Ghanti *et al.*, 2010; Junaid *et al.*, 2011a; Shrawat and Good, 2011; Abalaka and Mohammed, 2011; Ramamoorthy and Kumar, 2012; Chafe *et al.*, 2015).

## Competing interests

The authors declare that they have no competing interests.

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