RESEARCH ARTICLE

AN IMPROVED AND EFFICIENT ORGANOGENIC REGENERATION PROTOCOL USING EPICOTYL SEGMENT OF *IN VITRO* GROWN KAGZILIME (*CITRUS AURANTIFOLIA*) SEEDLING

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Abstract: In the present study, an improved and efficient plant regeneration protocol of Kagzilime (*Citrus aurantifolia*) using epicotyl segment of *in vitro* grown seedlings was developed. Kagzilime seed sterilized with Bavistin @ 0.1% for 30 min followed by Mercuric chloride @ 0.1% for 15 min was found to be optimum to reduce the contamination and efficient seed germination. About 0.75-1.0 cm long epicotyl segments of *in vitro* grown 21 days old seedlings were found suitable explants for efficient plant regeneration. The best regeneration efficiency of 84% with 5 shoots/explant was obtained at BAP @ 2.0 mg/l. The higher efficiency of root induction of 60.60% with 4.40 roots/shoot was observed at lower concentration of NAA@ 0.5 mg/l. Over 90% of plantlets were acclimatized and grown at pot mixture of soil, sand and vermiculite @ 1:2:1 in greenhouse. The efficient regeneration protocol developed in this study will be useful for mass propagation of root stock, biological indexing of virus diseases, production of disease free elite planting material, plant transformation and *in vivo* expression of desired viral gene.

Keywords: Age of explants, Epicotyl segment, Kagzilime, Multiple shoots, Regeneration

INTRODUCTION

Kagzilime/Acid lime (*Citrus aurantifolia*) is an important citrus fruit grown in the tropical and sub-tropical regions of the world. It is the third most important commercial citrus fruit after mandarin (*C. reticulata*) and sweet orange (*C. sinensis*) in India. This fruit is used to make lemonades and pickles, and its juice is added to various food preparations to enhance flavor. Improvement of commercial citrus cultivars through conventional breeding is not effective, because of its complex reproduction biology like polyembryony, self- and cross-incompatibility and long juvenile periods (Singh and Rajam, 2009).Micropropagation is a powerful tool for mass multiplication of horticultural crops in relatively short time with high fidelity index. It is needed for plant improvement to achieve the objectives those are not obtained by conventional methods. It is also an ideal system for production of disease-free planting materials for horticultural crops like citrus. Many citrus cultivars have been improved through somatic hybridization to circumvent the problems of sexual and graft incompatibility (Gloria *et al.*, 2000; Khan, 2007).

The various method of tissue culture for citrus plant regeneration includes: callus culture (Chaturvedi and Mitra, 1975); nucellar embryogenesis (Button and Kochba, 1977); pistil and ovule culture (Mitra and Chaturvedi, 1972); androgenic plants from anther culture (Chaturvedi and Sharma, 1985) and epicotyl segment culture (Sim *et al.*, 1989). However, epicotyl segments have been proved to be favorite explants for plant regeneration because of its *in vitro* morphogenic response (Costa *et al.*, 2004). *In vitro* plant regeneration from epicotyl segments has been achieved in many citrus species using appropriate concentration of growth regulators (Cervera *et al*., 1998; Bond and Roose, 1998; Luth and Moore, 1999). Tissue culture based regeneration has been reported in many commercial citrus; however efficiencies are generally very low, and the protocols available are moreover species or cultivar dependent (Cervera *et al*., 1998; Moore *et al*., 1992; Kaneyoshi *et al*., 1994; Khawale *et al*., 2006). It is also known that regeneration of citrus is a slow process (Singh and Rajam, 2009). *In vitro* regeneration protocol of Kagzilime was not reported so far from India.

Kagzilime is the indicator plant of many virus and virus like diseases. Thus this plant is required for biological indexing of citrus viruses, for identification of citrus virus cross protection mild strains; thus production of quality seedlings is essential. Further, with the advancement of molecular biology and biotechnology and modern applied science, organogenic regeneration of citrus and its efficient protocol using epicotyl explants of *in vitro* raised Kagzilime is obvious for various purposes like citrus transformation and genetic improvement of plant and *in vivo* expression of cloned virus genes. Therefore, in the present study, effort has been made to develop an improved and efficient plant regeneration protocol of Kagzilime using epicotyl segment of *in vitro* grown seedlings.

MATERIALS AND METHODS

Plant material and culture conditions

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Matured seeds collected from healthy bold Kagzilime fruits were rubbed with muslin clothe and washed thoroughly in running tap water to remove mucus and sugars from the seed coat and then air dried for 8-10 hr at room temperature. The seeds were treated with Bavistin at 1.0 g/kg, packed in polythene bags and stored at 4^0C. The seeds were taken from store and the seeds integument was peeled off carefully using pointed forceps. Care was also taken so that the cotyledons are not damaged because nucellar embryos are closely embedded with cotyledons. Surface sterilization of seeds was carried out by single and combined treatment of 0.1% Bavistin (w/v) solution, 0.1% Mercuric chloride ($HgCl_2$) and sterile distilled water. The surface disinfected seeds were placed on the surface of MS basal medium (Murashige and Skoog, 1962) solidified with 7 % (w/v) plant tissue culture agar (HIMEDIA, India) in test tubes (25×150 mm), and then incubated in the dark at 27^0C for 2-3 week.

Explant preparation and regeneration

About 0.75-1.0 cm long epicotyl segments from *in vitro* grown 14-56 days old Kagzilime seedling were used as explants for regeneration. To know the effect of BAP (6-benzylaminopurine) on shoot induction of Kagzilime, different concentrations of BAP was used; 0, 1.0, 2.0, 3.0 and 4.0 mg/l. To get higher regeneration, the explants were sliced at an angle increasing surface and placed the explant horizontally dipping the basal end on medium. Explants were cultured petriplates (90 mm) containing 25 ml MS medium supplemented with BAP and incubated at 26 ± 2^0C in the dark for 2 weeks. Explants were sub-cultured every 2 weeks in a fresh medium with same composition and kept under a 16-h photoperiod with cool-white florescent light (30-55 µmol m^{-2} s^{-1}). When explants were started to regenerate transferred singly in cultured tube. Multiple shoots were regenerated from single explants. Multiple shoots were separated from shoot cluster carefully and individual shoots were cultured in MS medium supplemented with BAP.

Rooting and hardening

For root induction, about 2.0-2.5 cm long regenerated shoots were transferred to MS medium supplemented with different concentrations of NAA (Napthalene acetic acid) at 0, 0.5, 1.0 and 1.5 mg/l. The plantlet with appropriate root system was removed from the culture tube and the residual agar at the root system was washed, and transferred into liquid Hoagland media containing minimal salt and kept for 2-3 weeks covering the plant with polythene sheet to avoid desiccation. After complete maturation, plantlets were transferred into 4-inch plastic pots filled with autoclaved sand, and watered with diluted Hoagland and liquid MS medium without sucrose (1/100 dilution). Pots covered by clear plastic sheet with 100 pinholes for aeration and kept in culture room for one month. The pots were watered at regular interval with the same gradient until the plant was finally transferred to 6-inch mud pots containing autoclaved pot mixture prepared by soil, sand and vermiculite @ 1:2:1 ratio in green house, and covered with polythene sheet for 2 weeks in greenhouse. Then the polythene cover was gradually removed and observations were recorded periodically for those plants which survived in greenhouse.

Statistical analysis

For each set of experiments, 5 replicates were used per treatment, and the experiment was repeated twice. One way analysis of variance and comparisons between means were made following the Fisher's least significant difference test (LSD) at $P < 0.05$ by using the SPSS 19.0 (IBM Corp., Armonk, New York, USA).

RESULTS AND DISCUSSION

Effect of surface disinfectant for seed germination

In vitro germination of Kagzilime seed in MS medium is reduced because of high contamination of seeds with bacteria and fungi. Therefore, a protocol for surface sterilization was standardized using sterile distilled water and different concentration of surface disinfectants (Bavistin and Mercuric chloride). About 80% of seeds were germinated after washing seeds with sterile distilled water only, but it showed very high contamination of bacteria and fungi. About 70% seeds were germinated after treatment with 0.1% Bavistin and 67% with 0.1% Mercuric chloride alone. However, combined treatment of Bavistin @ 0.1% and Mercuric chloride @ 0.1% showed similar of about 76% seed germination, but no contamination of bacteria and fungi was observed when seeds were treated with combination of both the surface disinfectants. The duration of treatment of these disinfectants was also found to be a critical factor for removing contamination. Seeds treated with 0.1% Bavistin for 30 min and then with 0.1% Mercuric chloride for 15 min showed no contamination even after 20 days (Fig. 1) It was reported earlier that the seeds sterilized with 0.75% hypochlorite (NaOCl) solution with 0.1% Tween-20 for 10 min gave maximum germination of Nagpur mandarin (*C. reticulata*) (Khawale *et al*., 2006). Seeds of Carrizo citrange (*C. sinensis* x *P. trifoliata*) disinfected with 0.5% NaOCl containing 0.1% Tween 20 for the duration of 10 min and rinsed thrice with sterile water and the surface disinfected seeds were used for *in vitro* seedling production (Cervera *et al*., 1998).

Effect of the age of explants for efficient regeneration

Regeneration efficiencies are reported to higher using epicotyl segments of many citrus hosts, Tryor orange (*C. sinensis* x *P. trifoliata*) (95%), Carrizo citrange (*C. sinensis* x *P. trifoliata*) (95%), grapefruit (*C. paradisi*) (90%) and sweet orange (*C. sinensis*) (70%) (Costa *et al.*, 2004). As 1 cm long epicotyl

segments of Kagzilime seedling has shown effective regeneration efficiency compared to 0.5 cm long segment (Moore *et al*., 1992), 0.75-1.0 cm long epicotyl segments of *in vitro* grown Kagzilime seedling was used in this report (Fig. 1). Age of explants was found to be important factor for efficient regeneration of Kagzilime. In this experiment, two concentration of BAP of 1.0 mg/l and 2mg/l supplemented with MS medium were used. At 2 mg/l of BAP, 60.20-80.40% of explants were regenerated, whereas at 1mg/l of BAP it was of about 4.60-21.80%, irrespective of age of the seedlings (Table 1). As maximum plant regeneration was observed at 2mg/l of BAP, this concentration was used to test efficiency of regeneration of explants of different ages. Explants of 21days old showed maximum regeneration efficiency of 80.40% with 5 shoots/explant. Regeneration rates were significantly reduced using older and too young (14 days) explants; 69.40, 64.60, 60.20 and 65.00% regeneration efficiency using 28, 35, 56, and 14 days old explants. However, maximum regeneration efficiency 44.6% has been reported earlier using 20 days old Trifoliate orange (*Poncirus trifoliata*) explants (Kaneyoshi *et al*., 1994). Earlier, reduced regeneration efficiency (70-77%) has been shown using older explants (35-56 days) of Washington navel orange (*C. sinensis*) compared to young explants of 21 days old (87-95%) (Bond and Roose, 1998). The present result in agreement with result of Bond and Rose (1998), where in the best results of regeneration was obtained using epicotyl segments of 3 weeks old seedlings of citrus. The present and previous data (Bond and Roose, 1998) reveals that older explants have poor regeneration performance; these findings are attributed to the few cells of older epicotyl explants actively dividing during regeneration. The present and previous (Kaneyoshi *et al*., 1994; Bond and Roose, 1998) data showed that regeneration efficiency of citrus varied in citrus species to species in MS medium. Further, in the present study, increased rate of regeneration was observed, the epicotyl explants was sliced at an angle and placed into medium horizontally dipping the basal end in the medium in order to increase nutrition uptake (data not shown).

Optimization of BAP concentration for efficient shoot regeneration

Response of epicotyl segments of Kagzilime cultured on MS medium supplemented with different concentrations of BAP (0, 1, 2, 3, 4 mg/l) was studied in order to identify the most suitable concentration of BAP. Data was recorded on regeneration frequency and number of shoot per explant from 3-6 week after culture (Table 2). Initiation of adventitious shoots was observed at the cut end of the segments at all the different BAP concentration used. Although there is a significant effect of BAP at different concentrations on regeneration of explants, the highest frequency of 84.00% was observed in the medium containing 2 mg/l BAP after 6 week of culture (Table 2 and Fig. 1). The regeneration frequency was found to be decreased (50.00 to 68.60%) with a progressive increase of the concentration of BAP (3-4 mg/l). Similarly, lower level of BAP (1mg/l) also reduced regeneration efficiency (24.80%). Further, number of shoots induced per explant varied with the varying concentration of BAP. The maximum (5.0) number of shoots per explant was recorded in the medium supplemented with 2 mg/l of BAP (Table 2 and Fig. 1). At higher (3-4mg/l) and lower (1mg/l) levels of BAP, a decline was recorded with respect to the number of shoots. Epicotyls segments did not show regeneration in the culture till to six week of inoculation when BAP was not supplemented with MS medium (Table.2).

Shoot multiplications in regenerated citrus plants are affected mostly by concentration of BAP (Al-Khayri and Al-Bahrany, 2001; Pena *et al*., 1997). Al-Khayri and Al-Bahrany, (2001) reported that most of the explants showed a single shoot when BAP was not supplemented with the medium, but shoot multiplication was shown to be stimulated significantly when BAP @ 0.25mg/l was supplemented and also showed that high level of BAP reduced shoot elongation. Earlier, BAP@1 mg/l in medium gave good responses for shoot induction from epicotyl explants of lime (*C. aurantifolia*) (Pena *et al*., 1997). In the present study, different concentration of BAP of 1mg/l to 4 mg/l was used and evaluated for regeneration of epicotyl segments of kagzilime, and shoot induction was observed with all the concentration with varied frequency. However, BAP concentration of 2.0 mg/l showed the best result of regeneration of epicotyl segment of kagzilime with maximum number of shoot formation.

Rooting and hardening of regenerated plant

Earlier, it is reported that NAA @ 0.1 and 0.5 mg/l gave good result for root induction in Mexican lime (*C. aurintifolia*) (Moore *et al*., 1992) and Trifoliate orange (Kaneyoshi *et al*., 1994). Thus, in the present study, different concentrations of NAA were evaluated for efficient root induction. The four-leaf stage proliferated long shoots of 2.0-2.5 cm were cultured in MS medium supplemented with different concentrations of NAA (0, 0.5, 1.0, 1.5 mg/l). Efficiency of root induction was higher of 60.60% at 0.5 mg/l NAA (Fig. 2), but lower of 40.00 and 30.40% at higher concentration of NAA @1.0 and 1.5 mg/l respectively (Table 3). Number of roots/shoot was found to be higher of 4.40 at 0.5 mg/l NAA (Fig. 2) and lower of 3.40 and 2.80 at 1.0 and 1.5 mg/l NAA respectively (Table 3). Earlier, NAA alone (1 mg/l) or in combination (0.5 mg/l NAA + 2.0 mg/l IBA) with IBA induced high frequency of roots from the *in vitro* grown shoot of lime (*C. aurantifolia*) (Al-Khayri and Al-Bahrany, 2001). However, contrary to the present findings,

Goswami *et al*. (2013) reported that NAA supplemented media did not induce root of *in vitro* raised shoot of seedless lime Kaghzi Kalan (*C. limon*). In this study 0.5 mg/l NAA was found to be optimum for high frequency root induction from kagzi lime explants. These results indicate that root induction was varied with the type and concentration of auxin in different citrus species. In the present study, initiation of root induction was observed 3 weeks after transfer of shoot on rooting medium and for development of adventitious root system the shoots took another 2 weeks (Fig. 2).

To encourage *ex vitro* root development the explants were removed from the culture tube and the residual agar at the root system was washed, and transferred into liquid Hoagland media (Fig. 2). After 3 weeks of growth in the media the plantlets with appropriate root system were transferred to plastic pot containing sand, and kept in culture room for 1 month and then transferred to mud pots containing autoclaved pot mixture prepared by soil, sand and vermiculite @ 1:2:1 ratio in greenhouse. Over 90 percent of plantlets were acclimatized and grow normally in greenhouse with development of new leaves (Fig. 2). Earlier, soil mixture consisting of soil, sand and organic material/FYM (1:1:1) (Normah *et al*., 1997; Shah *et al*., 1999) have been shown to be suitable for survival and growth of citrus plantlets under greenhouse condition. The present study reports that potting mixture containing soil, sand and vermiculite is very effective for survival and healthy growth of Kagzilime plant.

In conclusion, a simple and efficient regeneration protocol using 0.75-1.0 cm long epicotyl segment of 21 days old *in vitro* grown Kagzilime seedling was developed in the present study. BAP @ 2 mg/l is optimum concentration for efficient regeneration and NAA @ 0.5 mg/l for high frequency root induction. The efficient regeneration protocol developed in this study will be useful for mass propagation of root stock, biological indexing of plant virus, and production of disease free elite planting material, plant transformation and *in vivo* expression of desired viral gene

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Table 1. Effect of age of the epicotyl explants of Kagzilime seedling for *in vitro* regeneration

Organogenic shoot Regeneration	Treatments (mg/l)	Age (days) of explant used				
		14	21	28	35	56
Percent of Regeneration	B_1	9.60±0.22 a[x]	21.80±0.44 b	14.60±0.46 c	14.60±0.22 c	4.60±0.22 d
	B_2	65.00±0.63 a	80.40±1.04 b	69.40±0.36 c	64.60±0.22 a	60.20±0.59 d
No. of shoots/explants	B_1	1.20±0.18 a	1.40±0.22 a	1.20±0.18 a	1.00±0.18 a	1.00±0.18 a
	B_2	2.00±0.28 a	5.00±0.28 b	3.00±0.28 c	1.80±0.18 a	1.80±0.40 a

B: 6-Benzylaminopurine (BAP), subscripts 1 and 2 denote concentration of BAP at 1 and 2 mg/l in MS medium. The observations were taken after 5th week of inoculation. Each point corresponds to the average value of 5 replicates ± SE. [x]Different lowercase alphabets within the same row indicates that values are significantly different at $p < 0.05$ (Fisher's least significant difference, LSD)

Table 2. Response of 6-benzylaminopurine (BAP) on *in vitro* regeneration of epicotyl explants of Kagzilime

Treatments (mg/l)	Percent of organogenic shoot regeneration after week				Average no. of shoots/explant after 6th week
	3 week	4 week	5 week	6 week	
B_0	-	-	-	-	-
B_1	14.00±0.28 a[x]	19.00±0.28 e	24.40±0.46 b	24.80±0.66 b	1.20±0.18 g
B_2	65.20±0.33 b	73.00±0.28 f	82.00±0.63 c	84.00±0.49 e	5.00±0.40 h
B_3	52.60±0.36 c	58.40±0.36 g	67.00±0.28 d	68.60±0.61 f	3.20±0.18 a
B_4	42.00±0.28 d	46.20±0.33 h	49.20±0.44 a	50.00±0.49 a	2.40±0.22 b

B: 6-Benzylaminopurine (BAP), subscripts 0, 1, 2, 3 and 4 denote concentration of BAP at 0, 1, 2, 3 and 4 mg/l in MS medium, -: no response. The epicotyl explants of 0.75 to 1.0 cm in length from 21days old *in vitro* raised Kagzilime seedling were used. The first observation was taken after the 3rd week of inoculation and data were recorded at weekly interval upto 6 week. Each point corresponds to the average value of 5 replicates ± SE. [x]Different lowercase alphabets within the same row and same column indicates that values are significantly different at $p < 0.05$ (Fisher's least significant difference, LSD)

Table 3. Effect of Napthalene acetic acid (NAA) on rooting of Kagzilime shoots

Treatments (mg/l)	Percent of rooted shoots	Average no. of roots/shoot
N_0	10.20±0.44 a[x]	1.60±0.22 a
$N_{0.5}$	60.60±0.96 b	4.40±0.22 b
$N_{1.0}$	40.00±0.80 c	3.40±0.22 c
$N_{1.5}$	30.40±0.83 d	2.80±0.18 c

N: Napthalene acetic acid, subscripts 0, 0.5, 1.0 and 1.5 denote concentration of NAA at 0, 0.5, 1.0 and 1.5 mg/l in MS medium. Proliferated shoot of 2.0-2.5 cm in length with four-leaf stage was cultured in rooting medium. The observation was taken after the 3rd week of inoculation in rooting medium. Each point corresponds to the average value of 5 replicates ± SE. [x]Different lowercase alphabets within the each column indicates that values are significantly different at $p < 0.05$ (Fisher's least significant difference, LSD)

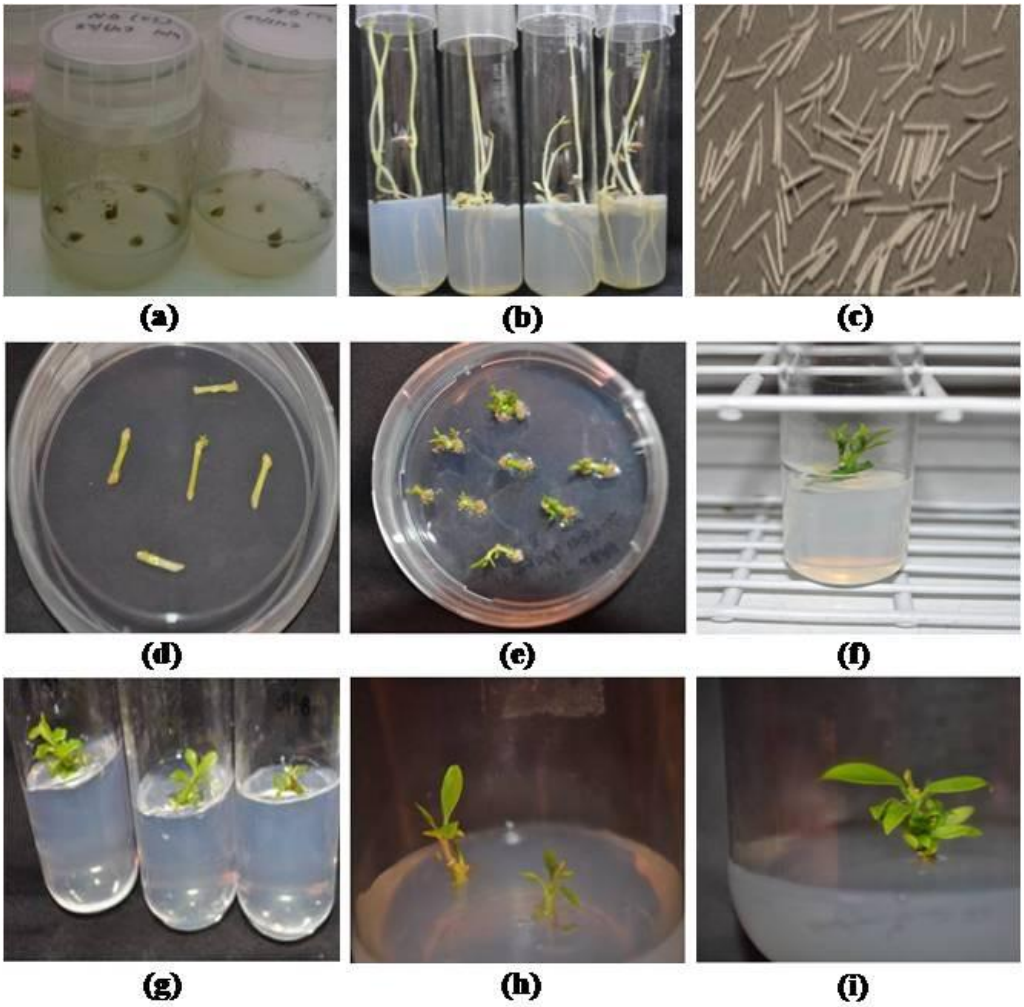

Fig. 1 Different steps of regeneration of Kagzilime explant; (a) surface disinfected seeds inoculated in MS basal solid medium in dark; (b) etiolated *in vitro* raised seedling for explant preparation; (c) 0.75-1.0 cm long epicotyl segment dissected from 21 days old *in vitro* grown etiolated Kagzilime seedling; (d) initiation of shoot regeneration from epicotyl explants incubated in shoot regeneration medium (MS +2mg/l BAP) in dark; (e) shoot regeneration; (f) multiple shoot induction from epicotyl explant; and (g,h,i) different stages of shoot maturation

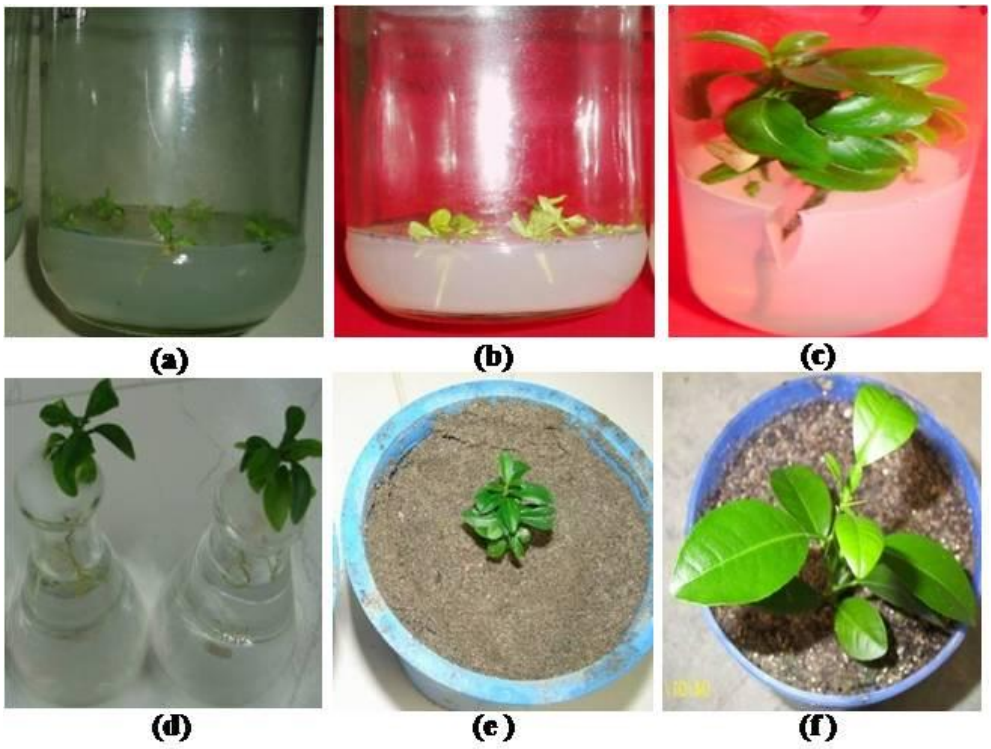

Fig. 2 Different step of root induction and hardening of Kagzilime; (a) root induction from regenerated Kagzilime shoots in rooting medium (MS+0.5 mg/l NAA); (b) rooted microshoots; (c) rooted plants after 5 weeks in rooting medium; (d) plants in Hoagland medium; (e) transfer of plant to sand in culture room; and (f) mature 3-4 months old plant in pot in greenhouse

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