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# *In vitro* Plant Regeneration in Cleopatra (*Citrus reshni* Hort. ex Tan.) by Direct Organogenesis

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*Key words:* Shoot regeneration, *Citrus reshni,* Direct regeneration

## Abstract

Maximum shoot regeneration (87.85%) and number of shoots per explant in the epicotyl segments having longitudinal cut in *Citrus reshni* var. Cleopatra were obtained on MT medium supplemented with BAP (2 mg/l) + NAA (0.2 mg/l). Maximum shoot length (5.10 cm), average leaf number per shoot (12), leaf length (2.12 cm) and width (1.5 cm) in Cleopatra were obtained on MT medium supplemented with BAP (2 mg/l) + NAA (0.2 mg/l). The maximum *in vitro* rooting (78.88 %) in the excised shoots occurred in liquid medium on MS supplemented with BAP (0.5 mg/l) + NAA (0.5 mg/l).

## Introduction

Rootstocks are a major contributor to tree performance and longevity, as it determines yield, fruit quality and reaction to various biotic and abiotic stresses. However, the citrus genotype, the culture conditions and media determine the regeneration pathway (Garcia-luis et al. 1999). The main citrus rootstocks are usually propagated by growing open-pollinated seed (Wutscher 1979), but propagation through seeds in cross-pollinated species like citrus lead to huge variability. Though seeds, because of their polyembryonic nature, give rise to several vigorous and virus free seedlings, which are genetically similar among themselves and to mother tree, but difficulty in elimination of zygotic seedlings necessitates the application of *in vitro* micropropagation. Micropropagation through tissue culture ensures rapid, true to type and mass multiplication of disease free plants and is one of the highly successful examples of commercial exploitation of tissue culture technology. The growth of small plant parts under

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sterile and constant environment conditions enables year round fast multiplication without any loss due to biotic and abiotic stresses. Rootstocks like Cleopatra which increase fruit quality and have desirable traits like salt tolerance could play crucial role in saving the citrus industry.

In several citrus genotypes, the formation of adventitious shoots has been reported from epicotyls or stem internodes (Barlass and Skene 1982). Oh et al. (1991), Vestri et al. (2003) and Kim et al. (2002) found that variations in concentration of different growth hormones significantly affect the shoot formation percentage in citrus cultivars and is genotype specific (Barlass and Skene 1982). However, this information is not available for most of the rootstocks recommended under Punjab conditions. So to undertake citrus rootstocks improvement programme, an effective regeneration system is the prerequisite. In order to develop this base the present study was undertaken with the objectives to develop *in vitro* regeneration system for *citrus* rootstock Cleopatra.

## Materials and Methods

The fresh fruits of *Citrus reshni* var. Cleopatra were washed with water containing 1 to 2 drops of Teepol and then washed thoroughly with running tap water. After squeezing off the juice, the seeds were removed from the fruit and testa of the seeds was peeled off. The seeds were disinfected with freshly prepared 0.1% mercuric chloride for 5 min followed by rinsing with sterile distilled water thrice to remove the toxic effects of the sterilant. The de-coated seeds were cultured in MS supplemented with 3% sucrose and 0.8% agar. MS was supplemented with 100 mg/l myo-inositol. The pH of the medium was adjusted to 5.8 before autoclaving. The epicotyl explants with three different cuts (i.e. transverse, oblique and longitudinal, hypocotyls and cotyledon explants from these seedlings were used for regeneration studies.

The *in vitro* grown etiolated seedlings (8 - 10 cm long) were chosen as the source of explants. The epicotyls were sectioned into about 1 cm long pieces with three different cuts, the hypocotyls and cotyledons were placed horizontally on MT medium supplemented with different phytohormones for regeneration studies. Cultures were incubated in light conditions at 26 ± 0.5°C with a 16 hrs photoperiod. The different explants (about 5) were placed horizontally in each Petri plate with 5 replicates for each treatment and the experiment was repeated three times. Experiments were set up in completely randomized design. The data on number of shoots produced per explants, shoot length, leaf density, leaf size and breadth were recorded.

To determine the combined influence of BAP and NAA on regeneration, the different explants were cultured in media containing varying concentrations of

BAP in combination with NAA. All explants were incubated in identical conditions after which they were scored for the number of responsive explants exhibiting shoot regeneration.

In order to determine the influence of different auxins on rooting capability of *in vitro* shoots, individual shoots were excised from clusters and cultured on rooting medium comprising MS supplemented with varying concentration of IAA ranging from 0.2 to 1 mg/l. The rooted plantlets were removed from the culture medium and acclimatized under controlled conditions after washing the roots under tap water. The plantlets were then planted in plastic cups containing Soilrite under light intensity for 7 - 10 days in incubation room for establishment of roots and hardening of plants. Well rooted plantlets were thus transferred to polythene bags containing garden soil and farmyard manure (FYM) in 1 : 1 ratio for acclimatization.

## Results and Discussion

With respect to the effect of medium composition on shoot regeneration, significant variation was observed for shoot regeneration from different explants *viz.,* epicotyl (transverse cut, oblique cut and longitudinal cut), hypocotyls and cotyledons (Table 1). Among the different explants, the maximum mean shoot regeneration (51.17%) was obtained in epicotyl segments with longitudinal cut which was significantly higher over other explants *viz.,* cotyledons (9.83%), hypocotyl segments (27.84%), epicotyls segments with transverse cut (46.17%) and epicotyls segments with oblique cut (45.25%). The explant × medium interaction was found significant with respect to shoot regeneration in Cleopatra (Table 1). The overall maximum shoot regeneration percentage (87.85) was obtained in epicotyl segments having longitudinal cut on MT + BAP (2 mg/l) + NAA (0.2 mg/l), followed by transverse cut (84.83) on the same medium (Fig. 1C, D). This might be because longitudinal cut increases the wound area and more cells become competent for differentiation of epicotyl explants, resulting in more shoot regeneration as compared to the transverse cut. In citrus, longitudinal cut gave the highest number of buds per explant followed by oblique cut and transverse cut (Duan et al. 2007, Yu et al. 2002, Kayim et al. 2004, Saini and Gill 2010).

Response of shoot induction in the respective explants increased with increase of hormone level. However, shoot induction was optimum at 2 mg/l of BAP which decreased with the increase of hormone concentration. The lowest shoot regeneration in all the explants was recorded on MT + BAP (4 mg/l) + NAA (0.2 mg/l). These results find support from previous workers like Gloria et al. (2000), Paul and Chaudhri (2000), Beneditu et al. (2000) and Chandra et al.

(2003). The addition of growth regulators to culture media supply the endogenous levels of hormones in explants that are separated from the plant hormone production sites.

**Table 1. Effect of medium composition on per cent shoot regeneration from different explants in Cleopatra.**

| Media* | Shoot regeneration (%) | | | | | |
|---|---|---|---|---|---|---|
| | Epicotyl | | | Hypo-cotyl | Coty-ledons | Mean |
| | Transverse cut | Oblique cut | Longitudinal cut | | | |
| MT | 21.95 (27.93) | 17.46 (24.69) | 28.18 (32.04) | 15.00 (22.77) | 6.00 (14.17) | 17.72 (24.32) |
| MT + BAP(1) | 40.00 (39.21) | 44.12 (41.60) | 48.00 (43.83) | 15.10 (22.85) | 8.86 (17.30) | 31.21 (32.96) |
| MT + BAP(1) + NAA(0.1) | 41.12 (39.86) | 48.00 (43.83) | 49.12 (44.47) | 17.00 (24.34) | 7.42 (15.80) | 32.53 (33.66) |
| MT + BAP(1) + NAA(0.2) | 52.29 (46.29) | 59.33 (50.35) | 62.00 (51.92) | 17.46 (24.69) | 7.00 (15.33) | 39.61 (37.72) |
| MT + BAP(2) | 74.83 (59.86) | 70.00 (56.76) | 80.25 (63.60) | 51.95 (46.10) | 9.15 (17.59) | 57.23 (48.78) |
| MT + BAP(2) + NAA(0.1) | 75.33 (60.20) | 69.00 (56.14) | 77.00 (61.31) | 84.83 (67.08) | 19.25 (26.01) | 65.08 (54.15) |
| MT + BAP(2) + NAA(0.2) | 84.83 (67.08) | 74.08 (59.37) | 87.85 (69.62) | 62.46 (52.19) | 25.00 (29.98) | 66.84 (55.65) |
| MT + BAP(3) | 62.00 (51.92) | 64.11 (53.20) | 72.16 (58.14) | 28.12 (32.01) | 9.23 (17.68) | 47.12 (42.59) |
| MT + BAP(3) + NAA(0.1) | 44.08 (41.58) | 41.16 (39.89) | 46.66 (43.07) | 16.54 (23.99) | 8.00 (16.42) | 31.29 (32.99) |
| MT + BAP(3) + NAA(0.2) | 37.33 (37.64) | 38.09 (38.09) | 40.33 (39.41) | 12.75 (20.91) | 8.00 (16.42) | 27.3 (30.49) |
| MT + BAP(4) | 27.33 (31.50) | 24.65 (29.76) | 28.12 (32.01) | 26.90 (31.22) | 6.85 (15.16) | 22.77 (27.93) |
| MT + BAP(4) + NAA(0.1) | 21.17 (27.38) | 20.85 (27.15) | 25.59 (30.37) | 7.26 (15.63) | 6.59 (14.87) | 16.29 (23.08) |
| MT + BAP(4) + NAA(0.2) | 18.00 (25.09) | 17.46 (24.69) | 20.00 (26.55) | 6.59 (14.87) | 6.53 (14.79) | 13.71 (21.20) |
| Mean | 46.17 (42.73) | 45.25 (41.96) | 51,17 (45.87) | 27.84 (30.66) | 9.83 (17.81) | - |
| CD (5%) | Explant : (0.31)<br>Media : (0.50)<br>Explant × media : (1.12) | | | | | |

*Values in parenthesis are in mg/l.

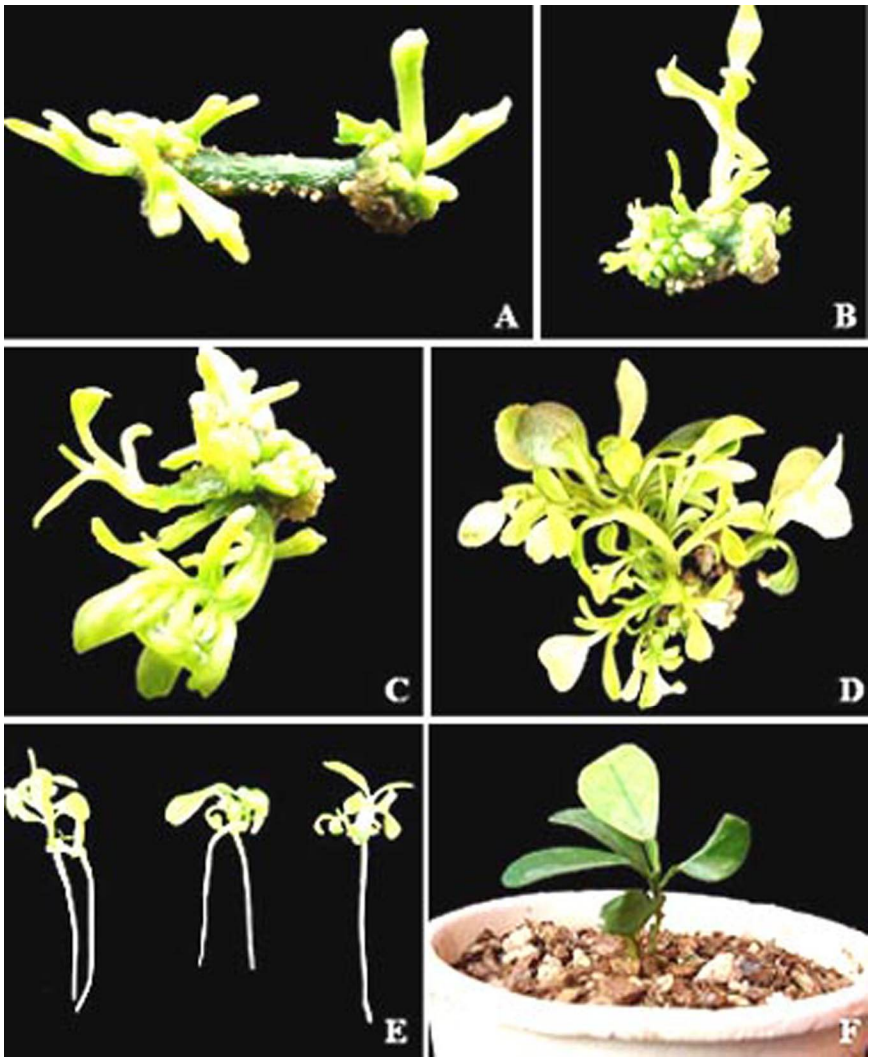


Fig. 1. Direct induction of adventitious shoot buds and plant regeneration via culture of different explants from *in vitro* grown seedlings in Cleopatra. (A) Initiation of shoot formation. (B) Emergence of shoot buds from the longitudinal cut of epicotyl segment. (C) Shoot development from epicotyl segment on MT + BAP (2 mg/l) + NAA (0.2 mg/l). (D) High frequency of regeneration of multiple shoots from epicotyl segment. (E) Rooted plants. (F) *In vitro* hardened plantlet.

Increase in level of BAP in the medium initiated more number of shoots and shoot length per explant as compared to the basal medium used as control (Table 2). Maximum number of shoots (9) and shoot length (5.10 cm) was observed in BAP (2 mg/l) + NAA (0.2 mg/l) in epicotyl segments. Further increment, however, showed decline in the shoot induction and shoot length progressively.

**Table 2. Effect of medium composition on shoot number and shoot length per explant in Cleopatra.**

| Media* | Epicotyl | | | | | | Hypocotyl | | Cotyledon | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Shoot no./explant | | | Shoot length (cm)/explant | | | Shoot no./ explant | Shoot length (cm)/explant | Shoot no./ explant | Shoot length (cm)/explant |
| | Transverse cut | Oblique cut | Longitudnal cut | Transverse cut | Oblique cut | Longitudnal cut | | | | |
| MT | 1.85 | 2.00 | 2.12 | 1.00 | 1.3 | 2.12 | 2.00 | 1.3 | 1.00 | 1.00 |
| MT+BAP(1) | 6.13 | 8.14 | 6.25 | 2.10 | 2.0 | 2.85 | 2.12 | 1.4 | 2.25 | 1.14 |
| MT+BAP(1)+NAA(0.1) | 4.45 | 4.45 | 5.23 | 2.85 | 2.10 | 3.00 | 3.07 | 2.12 | 2.00 | 1.12 |
| MT+BAP(1)+NAA(0.2) | 6.05 | 4.25 | 5.16 | 2.85 | 2.85 | 3.00 | 3.85 | 1.41 | 3.00 | 1.00 |
| MT+BAP(2) | 5.00 | 4.14 | 7.00 | 2.58 | 3.16 | 4.00 | 4.00 | 2.00 | 2.54 | 1.28 |
| MT+BAP(2)+NAA(0.1) | 4.54 | 5.12 | 6.88 | 3.51 | 3.28 | 4.00 | 7.23 | 4.12 | 2.16 | 1.40 |
| MT+BAP(2)+NAA(0.2) | 8.26 | 5.85 | 9.00 | 5.00 | 4.33 | 5.10 | 4.76 | 4.05 | 3.28 | 3.12 |
| MT+BAP(3) | 6.73 | 5.66 | 7.00 | 3.70 | 4.00 | 4.14 | 4.13 | 3.85 | 3.42 | 2.85 |
| MT+BAP(3)+NAA(0.1) | 4.12 | 3.85 | 5.85 | 3.88 | 3.12 | 4.00 | 3.00 | 3.00 | 2.85 | 1.51 |
| MT+BAP(3)+NAA(0.2) | 5.23 | 7.25 | 6.00 | 2.75 | 3.33 | 3.00 | 6.12 | 3.15 | 2.33 | 3.00 |
| MT+BAP(4) | 4.00 | 3.85 | 4.51 | 2.00 | 2.88 | 2.88 | 3.00 | 1.3 | 2.88 | 0.88 |
| MT+BAP(4)+NAA(0.1) | 3.46 | 4.05 | 3.76 | 1.85 | 1.3 | 1.85 | 1.54 | 1.14 | 1.00 | 0.72 |
| MT+BAP(4)+NAA(0.2) | 2.66 | 2.63 | 3.88 | 1.18 | 1.14 | 1.31 | 2.41 | 0.72 | 1.00 | 0.54 |
| CD (5%) | Shoot number/explants- Explant : (0.71)<br>Media : (0.11)<br>Explant × media : (0.25) | | | | | | Shoot length/explants- Explant : (0.52)<br>Media : (0.84)<br>Explant × media : (0.18) | | | |

*Values in parenthesis are in mg/l.

Similar results were observed by Usman et al. (2005) in three citrus cultivars Kinnow, sweet lime (*Citrus limmetoides* L.) and Succari (*Citrus sinensis* Osbeck). Among the different cut modes in epicotyl segments, longitudinal cut gave maximum shoot length followed by transverse cut and oblique cut (Table 2). Similar to our findings Yu et al. (2002) reported that bud formation was significantly affected by different cut modes (i.e. transversal, oblique and longitudinal cut) and the number of regenerated buds increased with the enlarged cut area.

**Table 3. Effect of medium composition on leaf density in Cleopatra.**

| Media* | Leaf density | | | | | |
|---|---|---|---|---|---|---|
| | Epicotyl | | | Hypo-cotyl | Cotyle-dons | Mean |
| | Transverse cut | Oblique cut | Longitudi-nal cut | | | |
| MT | 4.63 | 4 | 5.18 | 6 | 3.0 | 4.56 |
| | (2.35) | (2.23) | (2.48) | (2.64) | (2.0) | (2.34) |
| MT + BAP(1) | 5.56 | 6.6 | 6 | 5 | 4.6 | 5.55 |
| | (2.54) | (2.74) | (2.64) | (2.44) | (2.34) | (2.54) |
| MT + BAP(1) + NAA(0.1) | 6.0 | 4.6 | 6 | 3.6 | 3.3 | 4.72 |
| | (2.64) | (2.37) | (2.64) | (2.11) | (2.07) | (2.37) |
| MT + BAP(1) + NAA(0.2) | 6.6 | 4.72 | 5.85 | 3.7 | 3.63 | 4.90 |
| | (2.74) | (2.39) | (2.61) | (2.17) | (2.12) | (2.41) |
| MT + BAP(2) | 6.0 | 4.63 | 9.51 | 5.56 | 4.0 | 5.94 |
| | (2.64) | (2.35) | (3.24) | (2.54) | (2.23) | (2.60) |
| MT + BAP(2) + NAA(0.1) | 5.0 | 5.33 | 5.0 | 9.51 | 4.72 | 5.91 |
| | (2.44) | (2.51) | (2.44) | (3.24) | (2.39) | (2.60) |
| MT + BAP(2) + NAA(0.2) | 10 | 9.66 | 12 | 5.85 | 5.0 | 8.50 |
| | (3.31) | (3.26) | (3.60) | (2.61) | (244) | (3.05) |
| MT + BAP(3) | 5.85 | 6.18 | 10 | 4.6 | 4.63 | 6.25 |
| | (2.61) | (2.67) | (3.31) | (2.34) | (2.35) | (2.66) |
| MT + BAP(3) + NAA(0.1) | 4.33 | 5.00 | 5.0 | 4.0 | 4.33 | 4.53 |
| | (2.30) | (2.44) | (2.44) | (2.23) | (2.30) | (2.34) |
| MT + BAP(3) + NAA(0.2) | 5.0 | 6.10 | 8.17 | 9.0 | 4.33 | 6.52 |
| | (2.44) | (2.66) | (3.02) | (3.16) | (2.30) | (2.72) |
| MT + BAP(4) | 5.6 | 5.0 | 6 | 8.0 | 5.51 | 6.02 |
| | (2.55) | (2.44) | (2.64) | (2.99) | (2.55) | (2.63) |
| MT + BAP(4) + NAA(0.1) | 4.51 | 4.0 | 4.6 | 3.3 | 3.33 | 3.95 |
| | (2.34) | (2.23) | (2.34) | (2.07) | (2.07) | (2.21) |
| MT + BAP(4) + NAA(0.2) | 3.60 | 3.0 | 3.66 | 5.0 | 3.10 | 3.67 |
| | (2.11) | (2.20) | (2.15) | (2.44) | (2.02) | (2.15) |
| Mean | 5.59 | 5.30 | 6.69 | 5.62 | 4.11 | - |
| | (2.54) | (2.48) | (2.74) | (2.54) | (2.24) | |
| CD (5%) | Explant : (0.83)<br>Media : (0.13)<br>Explant × media : (0.30) | | | | | |

*Values in parenthesis are in mg/l.

The maximum mean leaf density in Cleopatra was noted in epicotyl segments with longitudinal cut (6.69) which was followed by hypocotyl segments (5.62), epicotyl segments with transverse cut (5.59), oblique cut (5.30) and cotyledon segments (4.11, Table 3). The media differed significantly for their effect on leaf density. The maximum mean average leave number per shoot length (8.50) was noted with MT medium with BAP (2 mg/l) + NAA (0.2 mg/l). This was significantly better over all other treatments. Similarly, Oliveira et al. (2010) observed that the combination of BAP and NAA was essential for maximum shoot production in all cultivars, although the balance between these growth regulators for optimal shoot regeneration varied among cultivars. The increase in the BAP level beyond 2 mg/l in the medium resulted in the progressive decline in the average leaf number per shoot length. The lower leave number per shoot length of cultures in medium with higher concentration of BAP might be due to the phytotoxicity of BAP at higher concentration.

**Table 4. Effect of medium composition on *in vitro* rooting of the shoots in Cleopatra.**

| Culture medium* | Rooting % | Number of roots/shoot | Av. root length (cm) | Mean |
|---|---|---|---|---|
| MS + BAP(0) + IAA(0) | 30.04 | 0.45 | 1.18 | 0.81 |
| | (33.22) | (1.20) | (1.47) | (1.34) |
| MS + BAP(0) + IAA(0.25) | 46.18 | 1.05 | 2.06 | 1.56 |
| | (42.79) | (1.43) | (1.75) | (1.59) |
| MS + BAP(0) + IAA(0.5) | 50.41 | 1.10 | 2.45 | 1.78 |
| | (45.22) | (1.45) | (1.85) | (1.65) |
| MS + BAP(0.5) + IAA(0) | 52.75 | 1.56 | 2.06 | 1.81 |
| | (46.55) | (1.59) | (1.74) | (1.67) |
| MS + BAP(0.5) + IAA(0.25) | 60.76 | 1.73 | 3.26 | 2.49 |
| | (51.19) | (1.65) | (2.06) | (1.85) |
| MS + BAP(0.5) + IAA(0.5) | 78.88 | 2.00 | 4.45 | 3.22 |
| | (62.61) | (1.73) | (2.33) | (2.03) |
| Mean | - | 1.31 | 2.58 | - |
| | | (1.51) | (1.87) | |
| CD (5%) | (0.7) | No. of roots/shoot :(0.25) | | |
| | | Root length : (0.25) | | |
| | | Media : (0.44) | | |
| | | No. of roots/shoot × media : (0.63) | | |
| | | Root length × media : (0.63) | | |

*****Values in parenthesis are in mg/l.

Individual shoots separated from the cluster of shoots were cultured on liquid MS supplemented with BAP and IAA, as solid medium (½MS + IBA + NAA) was unable to produce roots. The regenerated shoots were placed on the

improved filter-paper bridges for root induction. Similar filter paper bridge technique was used by Zeng et al. (2009). The maximum rooting percentage (78.88 ) was observed on MS supplemented with BAP (0.5 mg/l) and IAA (0.5 mg/l) and it was significantly higher over control and all other treatments (Table 4, Fig. 1E).

The maximum rooting percentage (78.88) was observed on MS supplemented with BAP (0.5 mg/l) and IAA (0.5 mg/l) and it was significantly higher over control and all other treatments (Table 4, Fig. 1E). Similar combination of BAP with auxins was used by Germana et al. (2008) for rooting of *Citrus macrophylla* in ½ MS and Jajoo (2010) for rooting of *Citrus limonia* in MS.

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