In Vitro Plant Regeneration Of Mosambi By Direct Organogenesis

Abstract

Exogenous genes can be introduced in plants by genetic transformation techniques. However, an efficient tissue culture system with high rates of plant recovery is necessary for gene introduction. This work aimed to define organogenesis and plant regeneration protocol for sweet orange (Citrus sinensis Osbeck cv Mosambi) which can be used in plant transformation. Testa of seeds was removed, surface sterilized and allowed to germinate in vitro and maintained the dark for three weeks, followed by one week at 16-h photoperiod and 27 ± 2o C temperature. Organogenesis induction was done by introducing epicotyl segments in MS medium with 30 g l-1 sucrose and different BAP concentrations. After adventitious bud growth, the shoots were transferred to MS medium with different concentrations of either NAA or IBA for rooting. The best results were obtained with 1 mg l-1 BAP for bud induction and ½ MS medium supplemented with 1 mg l-1 IBA for rooting. The use of 1mg l-1 BAP followed IBA were the best growth regulator combinations for bud induction and roots, respectively, for Mosambi. The protocols presented in this work are suitable for associations with genetic transformation experiments for this cultivar.

Keywords: Auxins, cytokinins, epicotyl segments, in vitro regeneration, Mosambi,

Introduction

Sweet orange is highly popular and commercially cultivated for its processing quality, fresh consumption and aromatic flavor. Despite its excessive cultivation, citrus plantations still has some problems such as slow growth and long juvenility, insects-pests, diseases, alternate bearing, pre and post-harvest losses, large number of seeds per fruit, short season of supply and short storage life etc. The recent advances of plant biotechnology open a wide scope of introducing exogenous genes in the plant genome, using gene transfer techniques to overcome the various mentioned biotic, abiotic and physiological stresses. However, for efficient transgenic plant production, defined tissue culture system for better plant regeneration, in association with a genetic transformation system for the gene introduction are necessary (Pérez-Molphe-Balch & Ochoa-Alejo, 1998). In citrus, some reports have shown the results of experimentation aiming for the efficient regeneration of plants through organogenesis in vitro, for use with Agrobacterium tumefaciens or particle bombardment for genetic transformation. Pérez -Molphe-Balch and Ochoa-Alejo (1997) studying the effect of BAP and NAA combinations established protocols for plant regeneration of Mexican lime (Citrus aurantifolia Christm.) and Monica mandarin (C. reticulata Blanco) by direct organogenesis in internodal segments of in vitro germinated plantlets. Ghorbel et al (1998), using combinations of the same growth regulators, defined protocols for grapefruit (C. paradisi Macf.), sour orange (C. aurantium L.) and alemow (C. macrophylla Wester) by organogenesis, from internodal segments of juvenile plantlets grown in the greenhouse. Bordón et al (2000) studying the effect of the cultivars and concentrations of BAP and NAA, established plant regeneration protocols through organogenesis from epicotyl segments of seedlings germinated in vitro for sour orange (C.

aurantium L.), Comuna sweet orange (C. sinensis L. Osbeck), Cleopatra mandarin (C. reshi Hort. ex Tan.), Alemow (C. macrophylla Wester), and Troyer citrange (C. sinensis Osbeck x Poncirus trifoliata L. Raf.). However, such protocol for the important sweet orange cultivar of Punjab i.e. Mosambi is not available. Thus, the present work aimed to establish efficient protocols for plant regeneration by organogenesis of sweet orange (C. sinensis Osbeck).

Materials and Methods

Plant material and explant preparation

Seeds of Mosambi were extracted from ripe fruits collected from the experimental orchard of the Department of Fruit Science, Punjab Agricultural University, Ludhiana and Regional Fruit station Abohar. Seed teguments were removed and disinfestation was done in 0.1 % mercuric chloride for 5 minutes. Three washes in distilled and sterilized water were done before the seeds were cultured in test tubes (150 x 25 mm) containing 15 ml of MS medium (Murashig e and Skoog, 1962), supplemented with 30 g l-1 sucrose. The seeds were maintained at 27 ± 2°C in the dark for three weeks, followed by one week under a 16-h photoperiod. After this period, epicotyls approximately 1.0 cm long were excised.

Shoot regeneration

The epicotyl segments were introduced horizontally in Petri dishes (100 x 15 mm) containing 25 ml of MS culture medium solidified with 0.8% agar and supplemented with 30 g l-1 sucrose and different concentrations of benzyl amino purine (BAP): 0.0, 0.25, 0.5, 1.0, 1.5; 2.0 mg l-1 and kinetin: 0.0, 0.25, 0.5, 1.0, 1.5; 2.0 mg l-1. Cultures were maintained at 27 ± 2°C, under 16-h photoperiod for 45 days. The adventitious buds were transferred to MS medium with 30 g l-1 sucrose and 0.8% agar. A completely randomized factorial was used, with 10 different concentrations of growth regulators, and 10 replications. Each replication consisted of six Petri dishes, with 3 segments each. The observations were recorded for the evaluation of suitable shoot regeneration media combination and explant: time taken for initiation and completion of regeneration; number of adventitious buds produced/ explant; number of shoots produced per explant; shoot length; average leaf number per shoot; leaf size; regeneration percentage.

Rooting of adventitious shoots

Culture media with auxins were used for rooting of the adventitious shoots. Full and half strength MS medium with 30 g l-1 sucrose, 0.8% agar supplemented different concentrations was designed with 10 replications and 2 shoots per replication for root induction. The evaluation was done by the determination of time taken for initiation and completion of rooting, rooting percentage, root length and number of roots/ shoot. The data was transformed by (x + 0.5). For both experiments (induction of adventitious buds and rooting of adventitious shoots), data were subjected to ANOVA with 5% significance level.

Results and Discussion

Surface sterilization of seeds: Surface sterilant mercuric chloride (0.1%) was used in this study with exposure time of 2, 3, 4, 5 and 6 minutes for surface sterilization of Mosambi seeds. The data on per cent contamination and seed germination with mercuric chloride are presented in

table 1. The surface sterilization showed an inverse correlation between contamination and the period of exposure. In general, the contamination percentage decreased with an increase in the duration of exposure to the HgCl2 (0.1%). The maximum explant survival of 83.33 per cent was achieved at 5 minutes exposure which consequently reduced the contamination to 8.33 per cent. The contamination percentage reduced further with exposure of 6 minutes; however this proved detrimental and reduced the explant survival to 41.67 per cent compared to survival at 5 min exposure. The reduction in explant survival percentage with increase in the duration of exposure might be due to the phytotoxicity caused by mercury (Hg2+) present in mercuric chloride (Kumar and Tiwari 2001). With respect to contamination percentage and mean explant survival with mercuric chloride (0.1%) with five minutes exposure time proved suitable for both the citrus species. The 0.1 per cent of HgCl2 has proved to be the suitable surface sterilant in rough lemon (Saini and Gill 2010) and three citrus cultivars of Kinnow (Usman et al 2005). In general, the contamination percentage decreased with an increase in the duration of exposure to the HgCl2 (0.1%).

Shoot regeneration: The epicotyl segments cultured on MS + BAP 1.0 mg l-1 induced shoot initiation at 9.40 days and was significantly earlier as compared to other media combinations (Table 2). The lower and higher concentrations of the BAP and kinetin significantly reduced the shoot initiation time compared to the basal, however, the compared to the optimum dose i.e. BAP 1.0 mg l-1 the higher concentrations proved detrimental causing late shoot initiation. The similar trend was observed in completion of shoot regeneration with maximum days required for shoot initiation among the different media combinations were observed in MS + Kin 2.0 mg l-1 and this was at par with MS + BAP 2.0 mg l-1 and MS + Kin 0.25 and 1.5 mg l-1. However, compared to MS basal all the levels of growth regulators proved significantly better in initiation and completion of regeneration. The supplementation of growth regulators in the media had significant effect on the initiation and completion of regeneration. The BAP 1.0 mg l-1 caused initiation 13.3 days early as compared to the MS basal providing an advantage of 19.2 days for the completion of regeneration. The supplementation of 1.0 mg l-1 of BAP was found to be superior as compared to all the other concentrations of the BAP as well as kinetin. Usman et al (2005) observed reduction in the time taken for shoot induction in Kinnow and sweet lime in the BA treated media as compared to the MS basal media. Similar observations have been recorded by Normah et al (1997) and Paudyal and Haq (2000). The higher doses of BAP were inversely proportional to the time required for shoot induction and completion. These results are in tune with those of Usman et al (2005), Normah et al (1997) and Paudyal and Haq (2000).

The number of adventitious buds obtained per explants showed that the highest adventitious buds per explant (4.04) were found on BAP 1 mg l-1 and this was significantly different from other media combinations. The number of adventitious buds was directly proportional to the concentration of different growth regulators in the media. The number of buds increased with the increase in the concentration of BAP or kinetin, however at concentration of >1.0 mg l-1 the number of buds per explants decreased. Sim et al (1981) obtained the highest number of adventitious bud in C. mitis on BAP with concentrations of 0.5 or 1 mg l-1and higher concentrations showed inhibitory effects. Dejam et al (2002) reported that the maximum number of adventitious buds were produced with 4 mg l-1 BAP, but most of these buds were qusicent due to higher concentrations of BAP which had an inhibitory effect on shoot elongation. The isolated effect of BAP on the number of shoots per explant showed that BAP @ 1 mg l-1 provided the best with 3.52 shoots per explants. This response was significantly different from other concentrations. In addition, BAP concentration above 1 mg l-1 caused an antagonistic effect resulting in decrease in the number of shoots per explant. Several reports on these

detrimental effects of growth regulators such as kinetin and BAP have been cited in context to citrus regeneration (Maggon and Singh 1995, Perez-Molphe-Balch and Ochoa-Alejo 1997; Ghorbel et al 1998; Moreira –Dias et al 2000 and Alemdia et al 2002). The shoot length also responded to the serial concentrations of the kinetin and BAP with longest shoots of 2.82 cm observed in BAP 1 mg l-1 which was significantly higher than Kin 1.0 mg l-1 (2.52 cm). Parthasarathy and Nagaraju (1993) obtained maximum shoot length (1.25 cm) on BAP 0.7 mg l-1 in Khasi mandrain. Similarly, Singh et al (1994) obtained shoot length of 1.15-2.60 cm in Khasi mandrain cultured on MS medium, containing BAP (0.05-1.0 mg l-1), kinetin (0.5-1.0 mg l-1) and NAA (0.5 mg l-1). Thus, both genotype and growth regulators might be playing an important role in regulating the shoot length of regenerated tissues. In terms of leaf density also the supplementation of BAP 1 mg l-1 proved to be the most suitable concentration resulting in 3.25 leaves per epicotyls segment. Further, it was significantly higher than all other media combinations except MS supplemented with BAP 0.5 mg l-1 (2.95 leaves/ explant) and kinetin 1.0 mg l-1 (2.80 leaves/ explant). Our studies are in tune with those of Rahaman et al (1996) and Kumar et al (2001) reporting maximum number of leaves (3.93) per explants at BAP mg l-1 in Mosambi. The maximum lead width (1.49 cm) was also observed on BAP 1mg l-1 which was significantly higher than all other media combinations expect Kin 1.0 mg l-1. The level of BAP or kinetin in the medium was directly proportional to leaf density and size which increased with the increase in the concentration of BAP or Kinetin, compared to the basal medium. Although the increase in either BAP or Kinetin level beyond a certain concentration in the medium caused a considerable reduction in the shoot length. The BAP and Kinetin at 1.00 mg l-1 in the media proved to be the optimal level for most of the regeneration attributes.

The highest shooting percentage (83.89%) was observed on BAP 1 mg l-1 which was significantly higher compared to the other media combinations (Fig 1). The increase in the concentration of growth regulators was proportional to increase in the regeneration percentage up to a certain concentration of the growth regulators. However, there was decline in the per cent regeneration at higher levels of growth regulators for both BAP as well as Kinetin and this has been reported by many previous workers (Gloria et al 2000, Paul and Chaudry 2000, Beneditu et al 2000, Chandra et al 2003).

Rooting of adventitious shoots

Shoots were considered rooted when a well-developed primary roots and secondary roots were developed. The shoot-lets cultured on ½ MS + IBA 1 mg l-1 induced rooting as early as 12.90 days compared to 31.90 days on MS basal. Complete rooting was achieved as early as in 25.60 days on ½ MS+ IBA 1 mg l-1 compared to 48.10 days on the MS basal. Thus supplementing with optimum concentration (½ MS+ IBA 1 mg l-1) of growth regulators as well as culture medium concentration provided an advantage of 22.50 days over the basal medium. Dejam et al 2006 also reported better rooting in Bakari micro-shoots on media containing IBA. This has been further supported by the findings of Barless and Skene 1982, Kitto and Young 1981 and Normah et al 1997 reported better rooting with the supplementation of growth regulators such as NAA and IBA in different species of citrus. Among the different levels of IBA and NAA, maximum number of roots (3.70/ planlet) were found on ½ MS+ IBA 1.00 mg l-1 and this was significantly superior as compared to all other media combinations expect MS + IBA 1.0 mg l-1 (3.15 roots/planlet). Kim et al (2002) reported that MS media supplemented with 1.5 mg l-1 of NAA was most effective for root induction in Yooza mandrain. For rooting in Bakrai micro-shoots the best result were obtained with IBA (Dejam et al 2006). The present findings are also in line

with those of Dejam et al (2002) and Mooreira-Dias et al (2000) reporting higher number of roots with NAA and IBA supplimentation. The maximum root length (3.89 cm) was also significantly higher on ½ MS+IBA 1.00 mg l-1 as compared to all the treatments except MS+NAA 0.50 mg l-1 (1.69cm). Similarly Gill et al (1994) obtained 3.1-3.8 cm long roots on MS medium containing NAA (1-2 mg l-1) + IBA (1 mg l-1). Auxins play multiferous role in rhizogenes, which include division of meristematic cells, their elongation and differentiation into primordia (Nanda 1979). Thus in present findings the number of roots produced per shoot, root length and thickness of roots varied with concentration and combination of auxins used as in the media.

Among the different levels of IBA and NAA maximum rooting percentage (95.37%) was observed in ½ MS+IBA 1 mg l-1, which was significantly superior over all the media combinations (Fig 2). Pervious studies on supplementation of MS media with NAA alone (Gill et al 1995, Das et al 2000), IBA alone (Oh et al 1991) or in combination has induced roots in the shoots of different species. Thus based on the present findings the supplementation of BAP @ 1.0 mg l-1 was found to be the most suitable for shoot regeneration. While the combination of ½ MS supplemented IBA @ 1.00 mg l-1 was concluded to be the optimum for rooting in Mosambi. The organogenesis protocol developed will be helpful in tissue culture and genetic transformation of the Mosambi.

References

1. Beneditu, V. A ., Filho, A .M., Mendes, M. J. 2000 Callus induction, somatic embryogenesis and protoplast isolation from sweet orange varities. Sci Agric J, 57:132–38.
2. Bond, J. E., and Rose, M .L. 1998 Agrobactrium-mediated transformation of the commercially important citrus cultivar Washington navel orange. Pl Cell Rep, 18: 229-234.
3. Chandra, A., Gupta, V., Burma, P. and Pental, D .2003 Patterns of morphogenesis from cotyledon explants of Citron (C. medica L.). In Vitro Cell and Dev Biol- Pl 39:514–19.
4. Das, J, M , Moreira ., Molina , R, V ., Bordon ,Y., Guardiola, J ,L., and Luis, A ,Garcia. 2000 Direct and Indirect Shoot Organogenic Pathways in Epicotyl cuttings of Troyer Citrange differ in hormone requirements and in their Response to light. Ann Bot 85:103-10.
5. Dejam, M., Khosh-khui , M .,and Shekafandeh ,A . 2006 Adventitious Bud Induction and Plant Regeneration in Epicotyl Segments of Bakrai (Citrus retioulata Blancox C. Umetta Swing). Intl J Agric Res 1:14-19.
6. Dejam, M., Khoush-Khui, M., and Shekafandeh A .2002 In vitro propagation of sweet orange (Citrus sinensis L.Osbeck) by direct organogenesis in epicotyls segments. Iran J Hortic Sci Technol. 2: 85-94.
7. Ghorbel, R., Navarro, L., and Durán-Vila, N .1998 Morphogenesis and regeneration of whole plants of grapefruit (Citrus paradisi), sour orange (C. aurantium) and alemow (C. macrophylla). J Hort Sci Biotech 73:323–27.
8. Gill, M, I, S., Singh, Z, Dhillon, B, S., and Gosal, S, S., 1994 Somatic embryogenesis and plantlet regeneration on calluses derived from seedling explants of ‘Kinnow’ manadrin (Citrus nobilis Lour X Citrus deliciosa Tenora). J Hort Sci 69:231-36.
9. Gill, M, I, S., Singh, Z., Dhillon, B, S., and Gosal, S, S. 1995 Somatic embryogenesis and plantlet regeneration in mandarin (Citrus reticulata Blanco). Scientia Hort 63:167-74.
10. Gloria, F, J, M., Filho, F, A, A, M., Camargo, L, E, A., and Mendes, B, M, J 2000 Caipira

sweet orange + Rangpur lime: a somatic hybrid with potential for use as rootstock in the Brazilian Citrus Industry. Gen Mol Biol 23:661-65.
11. Kitto, S, L., and Young, M, J. 1981 In vitro propagation of Carrizo citrange. Hort Sci 16:305-306.
12. Kumar, K., Dhatt, A, S., and Gill, M, I, S. 2001 In vitro plant regeneration in sweet orange (Citrus sinensis L. Osbeck.) cv. Mosambi and Jaffa. Indian J Hort 58:208-11.
13. Kumar R and Tiwari J P (2001) Effect of chemicals, light intensity and its duration on in vitro establishment of Chinese guava (Psidium friedrichsthalianum). Prog Hort 33:1-6.
14. Maggon, R .,and Singh, B, D. 1995 Promotion of Adventitious bud regeneration by ABA in combination with BAP in epicotyl and 57 hypocotyls explant of sweet orange (Citrus sinensis L. Osb.). Scientia Hort 63:123-28.
15. Moreira-Dias, J , M ., Molina, R, V., Bordon, Y, J L., Guardiola, J, L., and Garcia-Luis, A., 2000 Direct and indirect shoot organogenic pathways in epicotyl cuttings of Troyer Citrange differ in hormone requirements and in their response to light. Ann Bot 85:103–10.
16. Murashige, T., and Skoog, F. 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-97.
17. Nanda, K, K .1979 Adventitious root formation in stem cuttings in relation to hormone and nutrition. In: S S Bir (ed.) Recent researches in plant sciences. Kalyani Publishers, New Delhi. pp. 461-541.
18. Normah, M, N., Hamidah, S., and Ghani, F, D. 1997 Micropropagation of Citrus halimii – an endangered species of South-east Asia. Pl Cell Tiss Org Cult 50:225–27.
19. Oh, S, D., Song, W, S., and Cho, H, M .1991 Plant regeneration in dangyooza (C. grandis) through embryogenesis. Four effects of plant growth regulators on direct somatic embryogenesis and plant regeneration from immature ovule. Research reports of the Rural Development Administeration. Biotechnol 33:38-43.
20. Paudyal, K, P., and Haq, N. 2000 In vitro propagation of pummelo (Citrus grandis l. osbeck). In Vitro Cell Dev Biol-Pl 36:511-16.
21. Perez-Molphe-Balch, E., and Ochao-Alejo, N .1997 In vitro plant regeneration of Mexican lime and mandarin by direct organogenesis. Hort Sci 32:931-34.
22. Rahman, A, A, S., Nagaraju, V., and Parthasarthy, V, A .1996. Response of embryoids of certain citrus species to two cytokinins. Ann Pl Physiol 10:45-49
23. Saini, H, K., and Gill, M, I, S. 2010 Factors affecting shoot regeneration in rough lemon (Citrus Jambhiri lush) rootstock. Indian J Plant Physiol 15:94-98.
24. Singh, S., Ray, B., K, Bhattacharya., and Deka, P, C. 1994 In vitro propagation of Citrus reticulata Blanco and Citrus limon Burm. Hort Sci 29:214-16.
25. Usman, M., Muhammad, S., and Fatima, B. 2005 In vitro multiple shoot induction from nodal explants of citrus cultivars. J Central European Agri 6:435-42.