IN VITRO MULTIPLE SHOOT INDUCTION FROM NODAL EXPLANTS OF CITRUS CULTIVARS

Muhammad USMAN*, SANA Muhammad, B. FATIMA

*Corresponding author: muhammadusman30@yahoo.com
Plant Tissue Culture Cell, Institute of Horticultural Sciences, University of Agriculture, Faisalabad-Pakistan

Manuscript received: April 26, 2005; Reviewed: July 7, 2005; Accepted for publication: August 1, 2005

ABSTRACT

Citrus cultivars were explored for multiple shoot induction and root regeneration in different media. The multiple root and shoot induction was found directly proportionate to the increase in the levels of benzyleaminopurine (BA) and naphthalene acetic acid (NAA) in the modified Murashige and Skoog medium. The study might be promising towards in vitro propagation of sanitated Citrus plant material.

KEY WORDS: Citrus, Kinnow, in vitro, BA, NAA

INTRODUCTION

Citrus propagation by conventional means is restricted to particular season and availability of plant material. It doesn't guarantee trueness of cultivars and mass production of certified Citrus plants throughout the year. Plant tissue culture has emerged as a powerful tool for propagation and improvement of many woody plant species including Citrus. Citrus also stands among difficult to root crops and micropropagation offers rapid propagation of such crops in limited space and time under controlled conditions throughout the year [31]. In vitro culture further eliminates diseases [12], provides scope for the development of new cultivars through somaclonal variation [15] and somatic hybridization [1, 10, 11, 18, 23, 24, 26] that have improved Citrus rootstock resistance against nematode infestation and other pests as well [4, 11, 13, 14].

Reports on Citrus mircopropagation revealed maximum callus induction percentage in Kinnow (86.8%) on Murashige and Tucker's medium supplemented with 0.01mg/L BA, NAA and 500mg/L malt extract [7]. Different concentrations of growth regulators 10mg/L benzyladenine (BA), 0.1mg/L NAA and 500mg/L malt extract caused maximum initiation of shoot buds from Citrus stem explants grown in vitro [9]. The best rooting (100%) in the minimum time (15 days) occurred in the half strength MS medium supplemented with growth hormones (1.0mg/L NAA). The present studies were planned to investigate the effect of growth regulators that enhance growth and development. Further, to induce multiple shoots in Citrus cultivars for mass propagation of certified disease free plant material.

MATERIALS AND METHODS

Source material, sterilization procedures and culture of explants: Fruit of three Citrus cultivars Kinnow (Citrus reticulata L. Blanco), Sweet lime (Citrus limmetoides L.) and Succari (Citrus sinensis Osbeck) was taken from the Experimental Fruit Garden of the Institute. Fruit was dipped in 95% ethanol, flamed for surface sterilization and cut into two halves to get pulp including seeds. Pulp was washed away with tap water to obtain seeds. Seeds were surface sterilized with 70% ethanol for 3 minutes followed by 3-5 times washing with sterilized distilled water. Then seeds were dipped in 0.1% $HgCl_2$ for 1 minute followed by 3-5 times washing with sterilized distilled water. After sterilization, decoated seeds were cultured on Murashige and Skoog [20] or MS medium. Seedlings obtained after six weeks were used as source material for nodal and internodal segments (3-5 mm in length) between cotyledonary leaves and first two leaves. These segments were taken as explant and cultured on following media formulations for shoot and root induction.

Media formulations and sterilization procedures: MS medium consisting of mineral salts, vitamins, and sucrose was used as the basal medium for seed culture.

Shoot induction: MS medium was supplemented with NAA (0.1mg/L) and malt extract (500mg/L). This basal medium was modified by adding different concentrations of BA (0.1, 1.0 and 10mg/L) for shoot induction.

Root induction: The explant derived shoots were excised and sub-cultured on MS medium supplemented with malt extract 500mg/L used as basal medium, further modified with different levels of NAA (0.1, 1.0 and 10mg/L) for root induction.

Sucrose was added in the solution as carbon source at $30gL^{-1}$. Medium pH was adjusted to 5.7 with 1N NaOH. Bacto agar at $8\ gL^{-1}$ (Difco Laboratories, Michigan, USA) was melt at 60°C and added for media solidification. 10 ml of the medium was poured in each of the culture vessel, caped with plastic sheet and autoclaved at 121ºC under 15psi pressure for 20 minutes.

Culture conditions: After inoculation, the cultures were placed in the growth room under 16 hr photoperiod in $35\mu Em^{-2}.s^{-1}$ light intensity at 25 ± 2 °C temperatures.

Experimental layout and data analysis: The experiment was laid out in Completely Randomized Design (CRD) with ten explants per treatment and the experiment was repeated thrice. Data were analysed and means were compared by New Duncan's Multiple Range (DMR) Test [6].

RESULTS AND DISCUSSION

A. Shoot Induction

1. Explant response for shoot induction capacity: Both the explants regenerated shoots in all the cultivars studied on all levels of BA used in the basal MS medium, however, the nodal explant was found significantly ($P \leq 0.01$) better for shoot formation capacity. This response might be attributed to the pre-existing meristems available in the nodal segments.

2. Shoot induction capacity of Citrus cultivars in different media: Sweet lime explants showed higher shoot regeneration (64%) when cultured at the lowest concentration of BA (0.1mg/L) followed by Succari while Kinnow explants depicted the lowest shoot regeneration (47.50%) capacity. Significant ($P \leq 0.01$) increase in shoot regeneration was observed with increase in the concentration of BA in Kinnow explants depicting highest percentage (95.13%) at 10mg/L BA (Table 1). Among cultivars, Kinnow explants gave the

Table 1 Shoot regeneration capacity in *Citrus* cultivars

Cultivars	Media Composition MS + NAA (0.1 mg/L) + Malt extract (500 mg/L)				
	Control	**BA (0.1 mg/L)**	**BA (1.0 mg/L)**	**BA (10 mg/L)**	**Means**
Kinnow	58.75 g	47.50 d	84.00 b	95.13 a	**78.09 a**
Sweet lime	49.50 h	64.00 f	71.50 e	84.00 b	**67.25 b**
Succari	42.50 I	59.25 g	70.00 e	79.00 c	**62.69 c**
Means	**50.25 d**	**65.92 c**	**75.17 b**	**86.04 a**	

Means sharing the same letters are statistically non-significant

highest shoot regeneration (78.09%) followed by Sweet lime and then Succari. Shoot regeneration percentage was found directly proportionate to the addition of BA in the basal medium for shoot induction as the maximum level of BA (10mg/L) supplemented in the medium gave the highest shoot regeneration (86.04%). Goh et al. [8] also reported similar observations that the addition of BA in the growing medium enhanced shoot regeneration in Citrus cv. Succari explants. Our results are also in line with the findings of AlBahrany [1], Morsy and Millet [19] and Singh et al. [29].

3. Effect of BA on regeneration capacity of shoots per explant in Citrus Cultivars: Number of regenerated shoots per explant was found to be dependent on cultivar and concentration of BA, respectively. Study of cultivar response for number of shoots revealed highest number of shoots (4.28) per explant in Kinnow followed by Sweet lime and Succari (3.78 and 3.23 shoots, respectively), however, these results were statistically non-significant (Fig. 1). The findings are inline with the results of Normah et al. [22] who observed multiple shoots on BA from hypocotyl explant of C. halimii. Increase in level of BA in the medium initiated more number of shoots per explant as compared to the basal medium used as control and the highest number of shoots (4.34) was observed at BA (1mg/L). Further increment, however, showed decline in the shoot induction as the highest level of BA (10mg/L) gave 3.1 shoots per explant (Fig. 2). Our results are supported by the findings of AlBahrany [1], Luckman et al. [17], Normah et al., [22] and Tapati et al. [30] for number of shoots regenerated from Citrus cultivars.

4. Number of days to induce shoots in Citrus cultivars: Similar tendency was observed when considering the length of period for shoot formation. The earliest shoot induction (16.13 days) was observed on explants of Kinnow followed by Sweet lime while the basal medium (control) took maximum days (36) for shoot induction in Succari (Fig. 3). Similar tendency was observed when comparing the cultivar means as Kinnow explants showed precocity in shoot regeneration and took significantly less number of days (23.06) followed by sweet lime and then Succari. Increase in the levels of BA was found inversely proportionate to days required for shoot induction as the highest level of BA took less number of days (17.79) to regenerate shoots while medium without BA delayed shoot regeneration upto 33.13 days. These results are in accordance with the observations of Normah et al. [22] and Paudyal and Haq [26].

B. Root Induction

1. Root formation capacity of Citrus cultivars: Kinnow explant derived shoots when sub-cultured on rooting media showed significantly higher rooting percentage (29%) at the lowest level of NAA (0.1mg/L) followed by Sweet lime (24.50%) and Succari. Increase in the level of NAA in the medium significantly increased the rooting percentage as Kinnow showed the best rooting (91%) at 10mg/L NAA followed by sweet lime (79%) as given in Fig., 4. When comparing cultivars, it can be seen that Kinnow shoots exhibited best rooting (57.50%) followed by Sweet lime and then Succari. Comparing levels of NAA showed that the highest level of NAA (10mg/L) gave the highest rooting percentage (79.63%) as shown in Fig. 4. Similar findings are reported by AlBahrany [1], Amin and Akhtar [2], Burgur [5], Pasqual and Ando [25], Sagee et al. [27], Sandra et al. [28] and Singh et al. [29] on different media formulations regarding rooting in Citrus cultivars.

2. Effect of NAA on root induction per shoot in Citrus Cultivars: Increase in level of NAA in the medium significantly ($P \leq 0.01$) increased number of roots per shoot. Maximum roots per shoot were observed at the highest level of NAA (10 mg/L) in cultivars Succari (1.3)

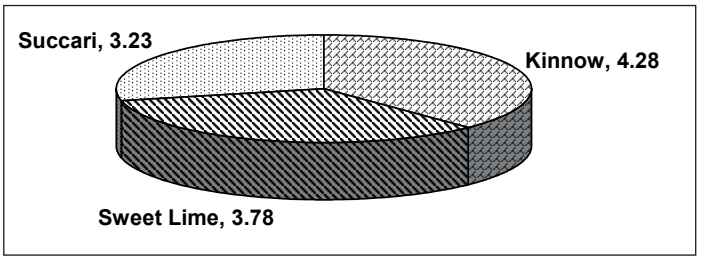

Fig. 1. Number of regenerated shoots per explant in Citrus Cultivars

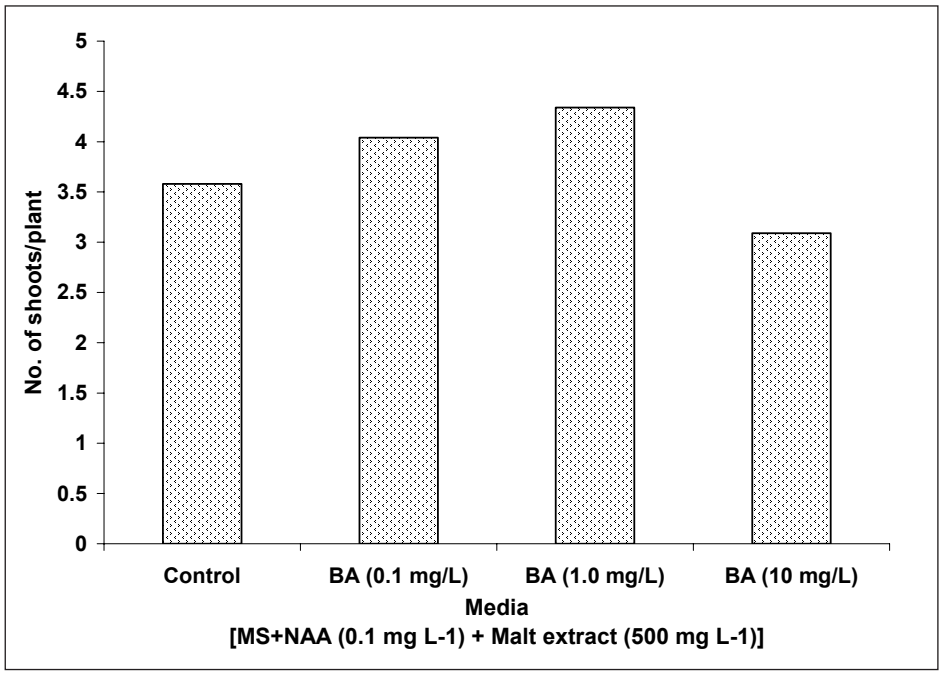

Fig. 2. Effect of BA on shoot regeneration capacity per explant

followed by Kinnow and Sweet lime (Fig. 5). Comparing cultivars it was found that Kinnow induced significant ($P \leq 0.05$) number of roots (1.08) followed by Sweet lime and Succari and the highest number of roots (1.23) was induced at the highest level (10mg/L) of NAA (Fig. 5). These results are in accordance with the findings of James and Stimart [16], Nagro et al. [21], Normah et al. [22] and Paudyal and Haq [26].

3. Number of days to induce roots in Citrus cultivars: The highest level of NAA (10mg/L) in the medium induced the earliest rooting in all the cultivars followed by medium devoid growth regulators (control). Cultivar Kinnow took minimum days (11.75) for root induction followed by Sweet lime (14.43) and Succari (19.50) as illustrated in Fig. 6. Among cultivars, similar trend was observed for root induction as Kinnow shoots showed earliest rooting followed by Sweet lime and Succari. The highest concentration of NAA (10mg/L) reduced the number of days (15.29) required for root induction followed by control and 0.1mg/L NAA. These results are supported by the finding of Amin and Akhtar [2], Gill et al. [7], Sagee et al. [27] and Singh et al. [29]. The observations are also in line with the conclusions of Normah et al. [22] who observed rooting from in vitro shoots of C. halimii on MS medium supplemented with NAA.

Transplantation: After the root formation, the plantlets were removed from the vessels and transplanted to 1-1 plastic pots with drainage holes. The substrate was an autoclaved mixture containing equal parts of leaf mould, sand, peat moss and loam. After 2-3 months in the green house the plants were transplanted under the field conditions.

Conclusion: Genotype dependency regarding root and

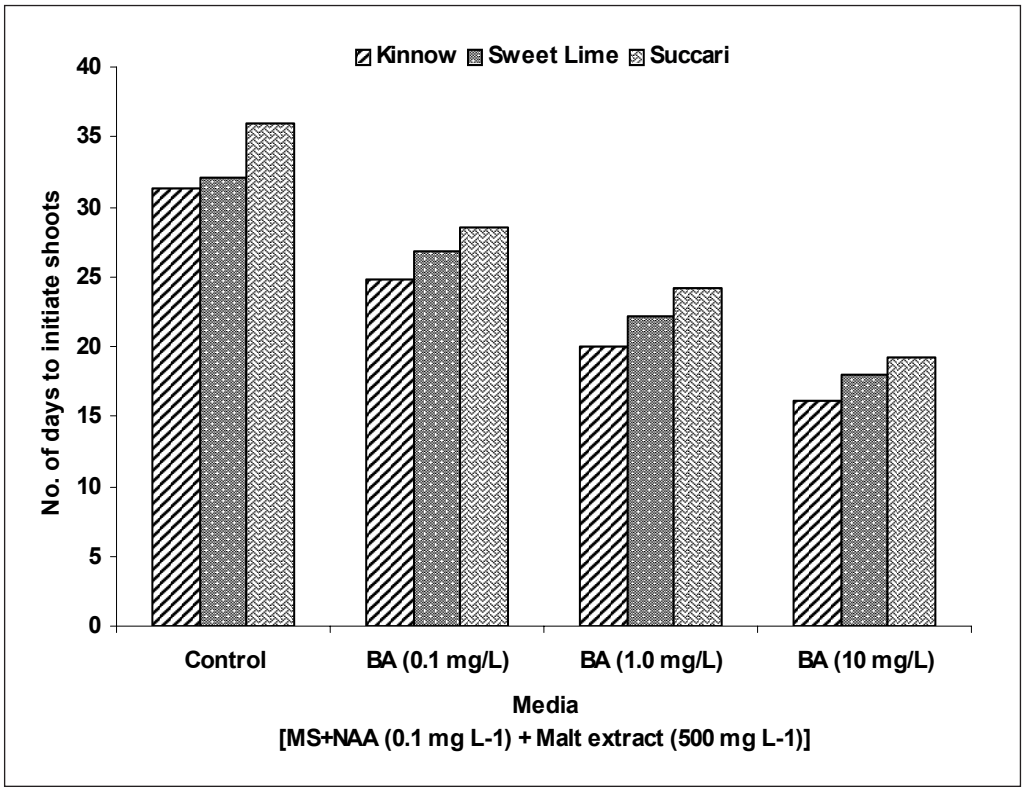

Fig. 3. Number of days to induce shoots in Citrus cultivars

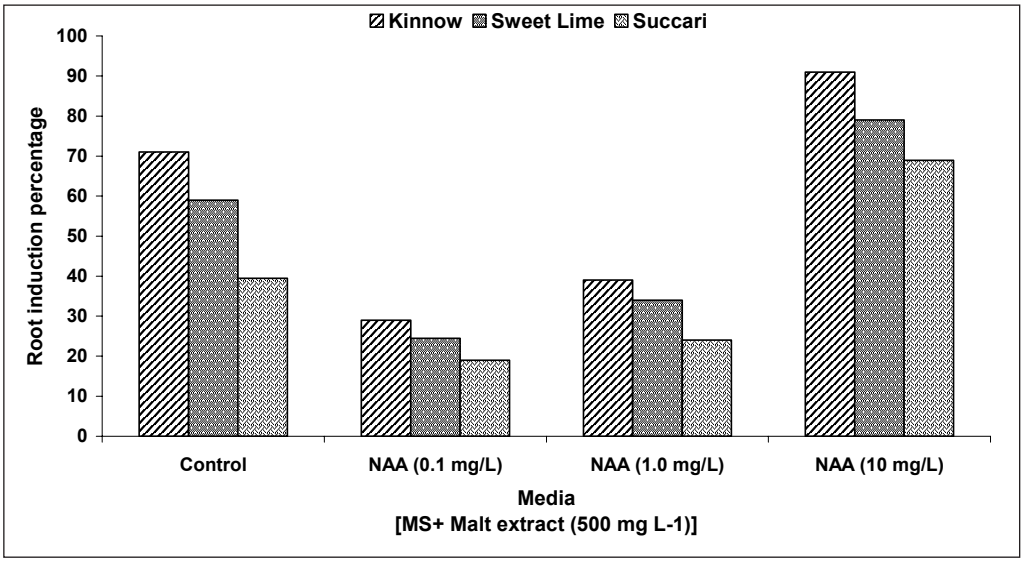

Fig. 4. Root formation capacity of Citrus cultivars

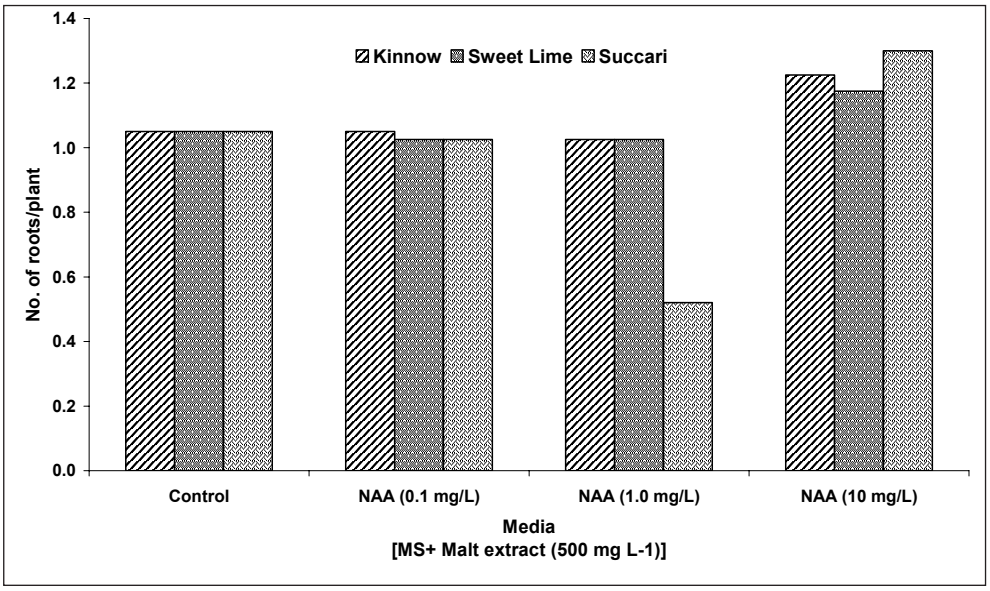

Fig. 5. Effect of NAA on root induction per shoot in Citrus cultivars

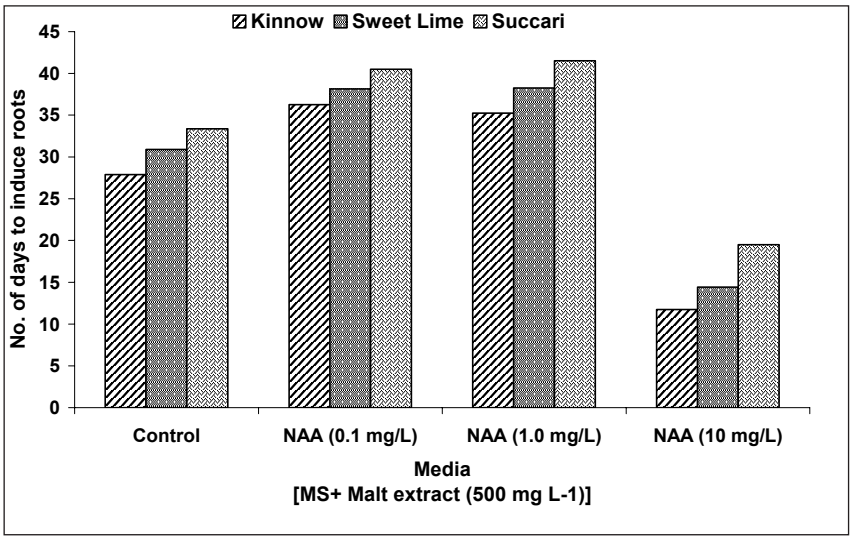

Fig. 6. Number of days to induce roots in Citrus cultivars

shoot induction in vitro exists in case of Citrus cultivars too. Supplement of both BA (1mg/L) and NAA (10mg/L) in the basal media showed multiple shoot and root formation in Citrus cultivars. BA as a cytokinin was found inhibitory at higher levels for shoot induction in all cultivars leading to the fact that hormone sensitivity was similar for the cultivars studied while NAA when used for root formation did not show any inhibitory response. Such studies might be a promising step towards mass production of sanitated plant material of Citrus.

ACKNOWLEDGEMENTS

We are thankful to Dr. Muhammad Jafar Jaskani for his support during studies and critical review of the manuscript.

REFERENCES

[1] Al-Bahrany A.M., Effect of phytohormones on in vitro shoot multiplication and rooting of lime (Citrus aurantifolia Swing.), Scientia Horticulturae (2002) 95: 285-295.

[2] Amin M.N., Akhtar S., Regeneration of plants in vitro from seedling explants of pummelo (Citrus grandis L. Osb.), Plant Tissue Culture (1993) 3: 71-79.

[3] Bordon Y., Guardiola J.L., Garcialuis A, Genotype affects the morphogenic response in vitro of epicotyl segments of Citrus rootstocks. Annals of Botany (2000) 86: 159-166.

[4] Bouquet A., Mark G., Pistanga D., Torregrosa L., Transfer of grape fan leaf virus coat protein gene through hybridization with Xiphinema index resistant genotypes to obtain rootstocks resistant to virus spread, Acta Hort. (2003) 603: 325-334.

[5] Burgur D.W., Regeneration of buds and roots from several Citrus tissues, Proc. Int. Soc. Citriculture (1981) 1: 161-164.

[6] Damon R.A., Harvey W.R., Experimental design, ANOVA and regression, Harper and Row Pub., New York, 1987, pp. 165.

[7] Gill M.I.S., Singh Z., Dhillon B.S., Gosal S.S., Somatic embryogenesis and plant regeneration in mandarin (Citrus reticulata Blanco), Scientia Horticulturae (1995) 63: 167-174.

[8] Goh C.J., Sim G.E., Morales C.L., Loh C.S., Plantlet regeneration through different morphological pathways in pummelo tissue culture, Plant Cell, Tissue and Organ Culture (1995) 43: 301-303.

[9] Grinblet U., Differentiation of citrus stem in vitro, J. Amer. Soc. Hort. Sci. (1997) 5: 599-603.

[10] Grosser J.W., Mourao-Fo F.A.A., Gmitter Jr. F.G., Louzada E.S., Jiang J., Baergen K., Quiros A., Cabasson C., Schell J.L., Chandler J.L., Allotetraploid hybrids between Citrus and seven related genera produced by somatic hybridization, Theor. Appl. Genetics (1996) 92: 577-582.

[11] Grosser J.W., Jiang J., Mourao-Fo F.A.A., Louzada E.S., Baergen K., Chandler J.L., Gmitter Jr. F.G., Somatic Hybridization, an integral component of Citrus cultivar improvement: I. Scion Improvement, HortScience (1998) 33:1057-1059.

[12] Grosser J.W., Chandler J.L., Somatic hybridisation of high yield, cold hardy and disease resistant parents for Citrus rootstock improvement, Hort. Sci. & Biotech. (2000) 75: 641-644.

[13] Guo W.W., Deng X.X., Somatic hybrid plantlets regeneration between Citrus and its wild relative, Murraya paniculata via protoplast fusion, Plant Cell Reports (1998) 18:297-300.

[14] Guo W.W., Deng X.X., Wide somatic hybrids of Citrus with its related genera and their potential in genetic improvement, Euphytica (2001) 118:175-183.

[15] Hammschlag F., Ritchie D., Werner D., Hashmil G., Krusberg L., Meyer R., Huettel R., In vitro selection of disease reistance in fruit trees, Acta Hort. (1995) 392: 19-26.

[16] James F.H., Stimart D.P., Effect of pH and Indole-3-butyric acid (IBA) on rooting of apple microcuttings, Amer. Soc. Hort. Sci. (1996) 121: 1049-1053.

[17] Lukman D.R., Jonard R., Willmer P., Shoot-apices culture of Troyer citrange, Ind. J. Tro. Agri. (1990) 1: 72-74.

[18] Mendes-da-Gloria F.J., Mourao Filho F.A.A., Camargo L.E.A., Mendes B.M.J., Caipira sweet orange + Rangpur lime: a somatic hybrid with potential for use as rootstock in the Brazilian Citrus Industry, Gen. & Mol. Biol. (2000) 23: 661-665.

[19] Morsy A.A., Millet B., Rythemic grwoth and optimization of micropropagation: The effect of excision time and position of axillary buds on in vitro culture of Citrus aurantium L., Annals of Botany (1996) 78: 197-202.

[20] Murashige T., Skoog F., A revised medium for rapid growth and bioassays with Tobacco tissue culture, Phys. Plant (1962) 15: 473-477.

[21] Nagro E.O., Pasqual M., Carralho G.R., Effect of sucrose and IBA on in vitro rooting of explants of Citrus rootstock (P. trifoliata), Coencia-e-Argritechnolgia (1996) 20:160-163.

[22] Normah M.N., Hamidah S., Ghani F.D., Micropropagation of C. halimii-an endangered specie of South East Asia, Plant Cell Tissue & Organ Culture (1997) 50:225-227.

[23] Ollitrault P., Dambier D., Luro F., Somatic hybridization in Citrus, Some new hybrids and cybrids, VIII Congress, Int. Soc. Citriculture Sun City Resort, South Africa, 1996, pp. 98.

[24] Ollitrault P., Dambier D., Lofty S., Rist D., Mass O., Luro F., Frolicher Y., Citrus germplasm exploitation by somatic hybridization, IXth Congress, Int. Soc. Citriculture, Orlando-Florida, 2000, pp. 122.

[25] Pasqual M., Ando A., Micropropagation of Trifoliata through axillary buds in in vitro culture, Pesquisa Agropecuaria Brasileira (1989) 24: 217-220.

[26] Paudyal K.P., Haq N., In vitro propagation of pummelo (Citrus grandis L.Osbeck), In Vitro Cell. & Dev. Biol.-Plant (2000) 36: 511-516.

[27] Sagee O., Raviv M., Medina S., Becker D., Cosse A., Involvement of rooting factors and free IAA in the rootability of Citrus species stem cuttings, Scientia

Horticulturae (1992) 5: 187-195.

[28] Sandra M., King M.I., Morehart A.L., Tissue culture of Osage-orange. HortScience (1988) 23: 613-614.

[29] Singh S., Ray B.K., Bhattacharya S., Deka P.C., In vitro propagation of C. reticulata Blank and C. limon Burm F., HortScience (1994) 29: 214-216.

[30] Tapati D., Mitra G.C., Chatterjee A., Micropropagation of Citrus sinensis. cv. Musambi, Phytomorphology (1995) 45: 57-64.

[31] Usman M., Plant propagation and improvement, in: Usman M., Abbas M., (Eds.), Citrus Nursery Raising: Principles and Practices, Mass Pub., Pakistan, 2005, pp. 23-66.