Journal of Agricultural Technology 2012 Vol. 8(2): 625-638
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Interspecific morphogenic ability differences in citrus

M. Vibhute[1], M.K. Tripathi[1*], R. Tiwari[2], B. S. Baghel[3] and S. Tiwari[4]

[1]Horticultural Biotechnology Laboratory, KNK-College of Horticulture, Mandsaur, Rajmata Vijayraje Scindia Krishi Vishwa Vidaylaya, Gwalior 482 004, India, [2]Department of Fruit Science, KNK-College of Horticulture, Mandsaur, M. P., India , [3]KNK-College of Horticulture, Mandsaur, M. P., India, [4]Division of Transgenic and Tissue Culture, Biotechnology Centre Jawaharlal Nehru Agricultural University, Jabalpur 482 004, India

M. Vibhute, M.K. Tripathi, R. Tiwari, B.S. Baghel and S. Tiwari (2012). Interspecific morphogenic ability differences in citrus. Journal of Agricultural Technology 8(2): 625-638.

Mature cotyledons of three species of *Citrus* were cultured on different fortifications of MS media to assess their *in vitro* response. Induction medium MS5D.5B (MS+ 5.0 mg.l^{-1} 2, 4-D + 0. 5 mg. l^{-1} BAP) proved well for callus initiation (84.18%). Nutrient medium MS4B.5N (MS + 4.0 mg.l^{-1} BAP + 0.5 mg.l^{-1} NAA) performed convincingly for formation of morphogenic calli (31.89%) as well as regeneration of plantlets (79.09%). Higher root proliferating efficiency (77.64%) was recorded on rooting medium MS.5I (MS + 0.5 mg. l^{-1} IBA). Roots in higher numbers (3.53)were documented on culture medium MS2I.5Kn (MS + 2. 0 mg. l^{-1} IBA + 0. 5 mg. l^{-1} Kn), while culture media MS.5I.5B (MS + 0.5 mg. l^{-1} IBA + 0. 5 mg. l^{-1} BAP) enhanced mean root length (3.55 cm). In terms of interspecific *in vitro* response, in general, Acid lime followed by Mandarin and Sweet Orange responded determinedly for the most of the culture phases. The *in vitro* raised plantlets were acclimatized and established successfully in the field.

Key words: *Citrus,* mature cotyledons, callus, morphogenesis, plant regeneration.

Abbreviations: MS- Murashige and Skoog's medium; WP - Llyod and McCown medium; BA- 6-benzylaminopurine; TDZ-Thidizuron; NAA- α-Naphthalene acetic acid; 2,4-D- 2,4-dichlorophenoxyacetic acid; IBA-Indole-3-butyric acid

Introduction

Among the fruit crops, the genus *Citrus* and its relatives are horticulturally important. This group is highly popular and commercially cultivated for its processing quality, fresh consumption and aromatic flavor. In *Citrus*, most of the collections are conserved in field gene banks in different citrus-growing countries. Such collections are vulnerable to different biotic and abiotic hazards (Damania, 1996). Traditional genetic plant improvement offers limitation for the production of new varieties of scion and root stocks, and the

* Corresponding author: M.K. Tripathi; e-mail: ms_1tripathi@yahoo.com

new varieties produced so far were originated form natural selection and mutation. The first barrier met by researcher was related to the complex citrus biology, which has high nucellar polyembryony, high heterozygosity, auto-incompatibility and a long juvenile period (Gmitter *et al*., 1992). Advances in biotechnology have generated new opportunities for citrus genetic improvement. *In vitro* propagation has therefore been a great potential tool to overcome problems related with the field culture for such species (Hidaka and Omara, 1989). Techniques like *in vitro* culture made it easy to improve citrus against different biotic and abiotic stresses, low yield and conserve important citrus genotypes; however, these techniques requires the presence of highly responsive regeneration protocol.

Although, all plant cells are derived from the fertilized egg cell and contain identical information, callus derived from somatic cells varies in competence to express totipotency (*i.e.* their genetic ability to produce plants). Mostly, explants that contain immature, meristematic cells develop callus, which are competent to express totipotency. In *Citrus*, previously diverse explants have been used to obtain proficient and reproducible regeneration system including mature cotyledons (Gill *et al.,* 1995; Miah *et al*., 2002; Ramkrishna and Singh, 2005; Rashad *et al*., 2005; Saini *et al*., 2010) with varying degree of success.

This study was conducted with the aim to explore regenerative ability, appropriate media from the cotyledonary explant excised from mature seeds of three species of citrus *viz*: Acid Lime (*Citrus aurantifolia*), Mandarin (*Citrus reticulata*) and Sweet Orange (*Citrus sinensis* L.).

Materials and methods

Three species of citrus *viz: Citrus aurantiafolia*, *Citrus reticulata* and *Citrus sinensis* were chosen for the *in vitro* studies. Seeds were collected from 4-5- years-old trees planted at local areas and Sewage Farm, KNK, College of Horticulture, Mandsaur (M. P.).

To begin with a preliminary experiment, two dissimilar fortifications of basal media *viz*: MS (Murashige and Skoog, 1962) and WP (Llyod and McCown, 1980) were recognized to find out better *in vitro* response. During the preliminary investigation, MS basal medium was found more receptive than WP medium (data not presented), consequently, for subsequently experimentations basal MS medium was employed. Three different sets of plant growth regulators were added to fortify MS basal media. In first set: two different auxins, *namely*: 2, 4-D and NAA (alone), in second set: two diverse cytokinins *viz*: BAP and TDZ (sole) and in third set: different auxins (2,4-D/NAA) with a cytokinin (BAP) in varying concentrations and combinations

were supplemented. All media were supplemented with 3% sucrose and solidified with 0.75% agar. Before autoclaving (121°C for 20 minutes), the pH of the medium was adjusted to 5.7±0.1 and 30 ml aliquot was poured in 100 x17 mm Petri dishes. Readymade MS basal medium, plant growth regulators and other ingredients were procured from Hi-media Laboratories, Mumbai, India.

Seed were collected from fruit and kept for 24 hours for draying. Dried seed then washed with 2% Tween 20 (v/v) (a commercial detergent) for 15-20 minutes and then washed thoroughly with running tap water for 20 minutes to remove dirt and residues followed by a treatment with 70% (v/v) ethanol for 1 minute. Then seed were subjected to treatment of 1% Bavistin® (BASF, Germany) for five minutes followed by 0.2% $HgCl_2$ for seven minutes. Finally seed were rinsed 4-5 times with sterile double distilled water and inoculated in culture tubes containing agar gelled water (7.5 $g.l^{-1}$ agar) and kept under diffused luminance of 16 μ mol m^{-2} s^{-1} provided with white fluorescent lamps. Mature cotyledon was obtained from 7-10 days-old germinated seeds. Zygotic and nucellar embryos were removed aseptically and cotyledons separated out with minimum damage. In 100 x 17 mm glass petridishes, 6-8 pieces of mature cotyledons were plated. Petridishes containing cultures sealed with Lab film (Para film®) were incubated under complete darkness at 25 ± 2°C for one week. Later *in vitro* cultured explants were subjected to photoperiod regime of 16 hours light and 8 hours dark at an intensity of 2000-lux luminance provided by PAR lamps. After 4-5 weeks of initial culturing, calli were subcultured on same medium (initial medium) for regeneration of plantlets. Cultured baby food bottles /culture tubes were subjected to 25 ± 2°C temperature and photoperiod regimes of 60 μ mol m^{-2} s^{-1} luminance provided by cool fluorescent tubes for 16 hrs. When root formation was not obtained on regeneration medium, plantlets were subsequently transferred to MS rooting medium amended with different concentrations of IBA, NAA and Kn (alone) as well as IBA in combination with BA and Kn, 15.0 g. l^{-1} sucrose and 7.5 g. l^{-1} agar powder. For rooting, reduced level of sucrose was used on the basis of work conducted by various scientists as well as preliminary experiments conducted in this laboratory. Rooted plants were thoroughly washed with running tap water to remove the adhering agar and were planted in 2.5 cm root trainers filled with 1:1:1 sand, soil and FYM sterilized mixture. Root trainers with transplanted plants were placed under 30±2°C and 60±5% RH for 15-20 days in a green house for acclimatization. Finally, acclimatized plants were transferred to field. The experiment was laid out in Factorial Completely Randomized Design. Experiment had two factors, first species and second different culture media combinations. Design was used to find out the significance of species, culture

medium combination and their interactive effect. Each treatment was consisting of two replications. Per replication approximately 80-100 mature cotyledon segments were excised and cultured on each media. The arc-sine transformation was made before the analysis of data, since all data were in percentage. The data were analyzed as per method suggested by Snedecor and Cochran (1967).

Results and discussions

Major factors that produced considerable variation in the pattern of development in culture were nature of explants, species and culture media combinations. The constituents of culture medium play an important role in the process of tissue differentiation. During present investigations, basal MS medium was used throughout the experiment, as this has been found more responsive than WP in course of beginning experiments. During present investigation, three different sets of culture media were formulated by amending two different auxins (alone), two cytokinins (sole) and two different auxins (2,4-D and NAA) in combinations with a cytokinin (BAP) to basal MS media to achieve the best *in vitro* response.

Cultured mature cotyledons followed mostly indirect pathway of plantlet regeneration (*via* callus formation). The first response of cultured mature cotyledons was similar after 7-10 days and mostly independent from explant and culture media. All explants became swollen and no callus proliferation was evident during first few days. Callus initiation was usually started from cut portions not in contact with the culture medium and callus proliferation started usually from the edges (Fig.1A). Cultured mature cotyledons followed either indirect somatic embryogenesis or organogenesis route. In indirect embryogenesis, embryoid formation started approximately 10 days from initial culturing on callus surface (Fig.1B-C). However, the duration varied from culture to culture and in a few cases embryoids developed after 35-40 days of apparently undifferentiated growth. The embryoid like structures were rounded with irregular out lines usually appeared in clusters (Fig.1B). These somatic embryos germinated after transferring into regeneration medium (Fig.1D). In indirect organogenesis, shootlets developed from the buds arising on the surface of the callus (Fig.1E-F). Shoot formation started approximately 15 days from initial culturing. However, the duration varied from culture to culture and in a few cases shoots formed after 45 days (Fig.1G). Most of the calli, after prolonged culturing on the induction media gave rise to plants (Fig.1G).

Various shoot forming calli were able to produce one (Fig.1G) or many plantlets (Fig.1H) at a time. Complete plantlets regenerated *via* embryogenesis and shoots developed *via* organogenesis were counted as regenerated plantlets. Regenerated shoots alone were also counted as plantlets, since they gave rise to

complete plants after rhizogenesis on rooting medium (Fig.1I). Root trainers with transplanted plants were transferred for 25-30 days in the glasshouse conditions (Fig.1J) and field (Fig.1K) for acclimatization. The plants, after survival, were evaluated visually on the basis of appearance. Although the traits were not scored quantitatively, regenerated plants were phenotypically normal and true to the type.

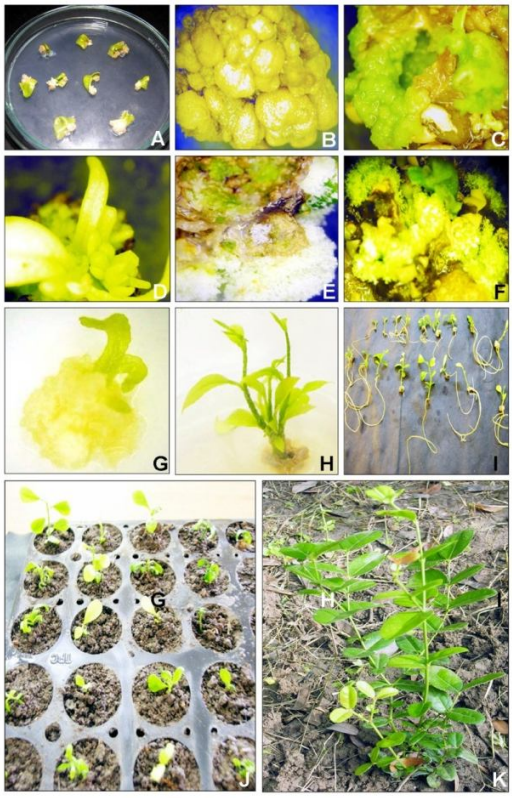

Fig. 1. Plant regeneration from mature cotyledon culture of citrus: **A**. Cultured mature cotyledons after 10 days in culture; **B.** Initiation of globular somatic embryos; **C-D** Germination of somatic embryo; **E**. Initiation of protuberances on surface of callus mass; **F**. Multiple shoot initiation *via* indirect organogenesis; **G**. Formation of shootlet; **H.** Elongated shootlet; **I.** *In vitro* rooting of regenerants; **J**. Hardening of regenerants in Green House and **K**. Plant transferred in Field**.**

The analysis of variance presented in Table 1-4 revealed that there were highly significant ($p<0.01$) differences exist among the response of species, culture media combinations as well as their interactions in terms of overall callus induction, formation of morphogenic calli, plantlet regeneration, root proliferating efficiency, number of root (s) and mean root length. It indicates

the presence of the considerable amount of variability amongst the species and culture medium as well as their interaction.

For growth regulators, large variations have been found in our experiment, where some combinations led to morphogenesis from cultured explants, while other produced only callus with high or low growth rate. Not all the cells within an explant or callus raised from them went on to form organs or embryoids. Also, not all the explant responded equally to conditions congenial for morphogenesis. For mature cotyledon cultures, higher concentration of an auxin (2, 4-D/NAA) in combination with lower concentration of BAP (a cytokinin) facilitated higher degree of callus initiation. Higher callus initiation frequency was observed on culture media MS5D.5B / MS5N.5B as compared to MS5B.5D / MS5BN that suggested that relatively higher concentration of an auxin in combination with a lower concentration of a cytokinin is required for this purpose. Similar results were also reported by Gitarani *et al.* (2003), Haoa *et al.* (2004) and Ali and Mirza (2006) in citrus for different explants cultures. Much lower results with 2, 4-D, NAA or BAP alone on Media MS5D, MS5N or MS5B (even in higher concentrations) as compared to combination of 2, 4-D / NAA with BAP revealed that cytokinins as well as an auxin (alone) were insufficient for higher degree of callus initiation. However, culture medium fortified with auxins (alone) initiated calli in higher frequencies (more than 60%) when culture medium supplemented with NAA in range of 0.5- 4.0 $mg.l^{-1}$ and 2,4-D in range of 1.0- 5.0 $mg. l^{-1}$ as compared to culture medium containing a cytokinins (alone). Among different cytokinins tested, BA induced calli in maximum numbers (~20%) in range of 0.5 -1.0 $mg. l^{-1}$. Similar results were also reported by Parthasarathy *et al.* (2001).

A higher concentration of a cytokinin (BAP) in combination with lower concentration of auxin (NAA) was supported for formation of higher degree of morphogenic calli as well as plantlet regeneration. Higher morphogenic calli formation frequency was observed on culture media MS4B.5N/MS4B.5N as compared to MS4D.5B/ MS4N.5B that suggested that higher concentration of a cytokinin in combination with a lower concentration of an auxin is required for this purpose. Moreover, higher morphogenic potential as well as plantlet regeneration was investigated on culture media MS4B.5N / MS3B.5N as compared to MS4B.5D / MS3B.5D that suggested that only higher concentration of BAP in combination with NAA instead of 2, 4-D enhance *in vitro* morphogeneic potential. Much lower results with 2, 4-D (5-8%); NAA (10-11%) or BAP (5-12%) alone even in higher concentrations as compared to combination of BAP with NAA revealed that a cytokinin as well as an auxin (alone) was insufficient for higher degree of morphogenic callus formation when supplemented in culture medium as solely. Moreover, among different

tested auxins and cytokinins, only NAA and BAP were found responsive for promoting *in vitro* morphogenesis. Similar response of plant growth regulators were observed for regeneration of plantlets, where, lower plantlet regeneration efficiency was documented with application of 2, 4-D (40-50%), NAA (~ 55%) and BAP (~70%) as sole. The beneficial effect of BA has been reported in the production of shoot bud primordia in different crop plants (Lesham, 1973) which may be due to more synthesis of nucleic acids and proteins required for morphogenesis (Torres, 1988). The cytokines are generally added to induce shoot formation and to inhibit root formation. In citrus also the role of cytokinins in shoot regeneration has been well documented (Gill and Gosal, 2002; Rashad *et al*., 2005; Jajoo, 2010; Saini *et al*., 2010). However, during present investigation mature cotyledon cultures responded better on combination of an auxin with cytokinin. Similar results have been obtained by various other scientists for mature cotyledon culture (Ramakrishna and Singh, 2005) nodal segment culture (Usman *et al*., 2005; Murkute *et al*., 2008) shoot tip culture (Murkute *et al*., 2008) and ovule culture (Vijayakumari and Singh, 2001) in citrus.

During present investigation, *in vitro* rooting frequency was found to be higher after transferring shootlets into rooting medium. Auxins play multifarious role in rhizogenesis, which includes division of meristmatic cells, their elongation and differentiation into root primordia (Nanda, 1979). In *Citrus*, auxins like IBA (Syamal *et al*., 2007; Murkute *et al*., 2008), IAA (Jameel *et al*., 2001) as well as NAA (Starrantino and Caruso, 1988) were found effective for inducing *in vitro* rooting. In present study, full strength MS medium supplemented with either IBA alone in range of 0.5-2.0 $mg.l^{-1}$ or in combinations with cytokinins (Kn/BA) at the concentration of 0.5 $mg.l^{-1}$ was found to be optimum for enhancing *in vitro* root proliferating ability. Higher root proliferation efficiency was exhibited by culture media MS.5I and MS.5I.5B as compared to MS3I, MS3Kn or MS3N suggested that auxins (especially IBA) required in lower concentration for this purpose. The number of roots increased when higher concentration of IBA (2.0 $mg.l^{-1}$) in combinations with lower concentration (0.5 $mg.l^{-1}$) of a cytokinin (Kn/BAP) was added into rooting medium. The root of higher length was recovered from medium fortified with equal proportion of IBA (0.5 $mg.l^{-1}$) and 0.5 $mg.l^{-1}$ cytokinin (BAP/Kn). Similar results were reported by Gill *et al*. (1995) and Syamal *et al.* (2007) in citrus. Culture media supplemented with IBA responded better as compared to culture media fortified with other auxin (NAA) even in higher concentrations suggested that IBA promotes *in vitro* rooting of shootlets.

Among auxins, IBA was the most effective than any other plant growth regulators in the most of the cases, apparently because it is not smashed by IAA

oxidase or other enzymes and thus persists longer. The above results are in agreement with the earlier findings of Rana and Singh (2002) and Kumar *et al*. (2001) for diverse explant cultures of citrus. However, in some other reports the maximum rooting response was recorded with application of NAA in case of *C. sinensis* (Rashad *et al*., 2005) and *Pectinifera* root stock (Gill and Gosal, 2002).

During present investigations considerable variability for *in vitro* response was observed among three species. In general, Acid lime species followed by Mandarin and Sweet orange responded well for the most of the culture phases which is due to differences between the endogenous hormone levels (Norstog, 1970) amongst the species. In *Citrus*, interspecific differences have also been reported for various explant cultures by Parthasarathy *et al.* (2001).

During present investigation interactions of species with culture media also varied considerably. Species on either MS5D.5B or MS5N.5B or in both induction media induced more than 80 per cent callus. Interactions of all species with medium MS4B.5N and MS3B.5N also exhibited similar results for formation of morphogenic calli (more than 20% explants induced morphogenic calli). For regeneration of plantlets, either regeneration medium MS4B.5N or MS3B.5N or both were found more responsive (More than 75% plantlets were regenerated) from all species. This revealed that in addition to different responses of species for culture medium, particular species does not necessarily respond in the similar manner to each of diverse culture media combinations investigated. This suggests that a particular species selected for advance work can be cultured on the most suitable medium to obtain maximum response. Also, the possibility exists for improvement of *in vitro* efficiency of a particular species by further modifying the culture medium.

It was concluded from present study that under appropriate conditions mature cotyledon explant cultures of *Citrus* in contrast to other fruit crops gave rise to higher number of shoots. Higher plantlet formation was recovered *via* indirect organogenesis as well somatic embryogenesis; therefore, organogenic route of morphogenesis can be used to obtain multiple plants and germplasm conservation. The embryogenic frequency and the conversion rate of embryoids into plantlets obtained by our process were also higher than that described by earlier findings, which can be used for *Agrobacterium*-mediated genetic transformation and studying the possible role of plant growth regulators and other addenda on *in vitro* morphogenesis. A system for plant regeneration in *Citrus* is thus available, which has potential to improve this species *via* breeding and biotechnological means.

References

Ali, S. and Mirza, B. (2006). Micropropagation of rough lemon (*Citrus jambhiri* Lush.). Effect of explant type and hormone concentration. Acta Bot. Croat. 65:137-146.

Damania, A.B. (1996). Biodiversity conservation: A review of options complementary to standard *ex situ* methods. Plant Genet. Resour. Newsl. 107: 1–18.

Gill, M.I.S. and Gosal, S. S. (2002). Micropropgation of Pectinifera (*Citrus depressa* Hayata) - A potential citrus stock for sweet orange. Indian J. Citriculture 1:32-37.

Gill, M.I.S., Singh, Z., Dhillion, B.S. and Gosal, S.S. (1995). Somatic embryogenesis and plantlet regeneration in mandarin (*Citrus reticulata* Blanco). Scientia Hort. 63:167-174.

Gitarani, G.S.V. and Vinash, N.A. (2003). Callus induction and plantlet regeneration in Sweet orange (*C. sinensis* L.) and Lime (*C. aurantifolia*). *In vitro* Cell Dev. Biol. 39: 468–474.

Gmitter Jr, F.G., Grosser, J.W. and Moore, G.A. (1992). Citrus. In: Hammerschlag F.A. and Litz, R.E. (Eds). Biotechnology of perennial fruit crops. Biotechnology in Agriculture n.8. CAB International, Cambridge P. 335-369.

Haoa, Y.J., Wen, X.P. and Deng, X.X. (2004). Genetic and epigenetic evaluations of citrus calluses recovered from slow growth culture. J. Plant Physiol. 161: 479–84.

Hidaka, T. and Omura, M. (1989). Control of embryogenesis in citrus cell culture regeneration protoplasts and attempts to callus bank. Bulletin of the Fruit tree Research Station, Series Okitsu, 16: 1–17.

Jajoo, A. (2010). *In vitro* propagation of *citrus limonia* Osbeck through nucellar embryo culture Current Research J. Biological Sciences. 2: 6-8.

Jameel, M., Khayri, A., Baharany, A. and Abdulaziz, M. (2001). *In vitro* micropropagation of lime (*Citrus aurantifolia*). Cur. Sci., 81: 9 -10.

Kumar, K., Dhatt, A.S. and Gill, M.I.S. (2001). *In vitro* plant regeneration in kinnow mandarin (*Nobolism Loure* × *Citrus deliciosa* Tenora). Ind. J. Hort. 58: 299-302.

Lesham, Y. (1973). Molecular and hormonal basis of plant growth regulation. Pergamon Press, New York. 35-86.

Lloyd, G. and McCown, B. (1980). Commercially feasible micropropagation of mountain laurel (*Kalmia latifolia*) by use of shoot tip culture. Proceeding Int. Plant Prop. Soc.30: 421-427.

Miah, M.N., Islam, S. and Hadiuzzaman, S. (2002). Regeneration of plantlets through somatic embryogenesis from nucellus tissue of *Citrus macroptera* Mont.*var*. *anammesis* (Sat Kara). Plant Tiss.Cult & Biotech 12: 167-172.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. 15:473-497.

Murkute, A.A., Sharma, S. and Singh, S.K. (2008). Rapid clonal *in vitro* multiplication. Ind. J. Horti. 65: 127-133.

Nanda, K.K. (1979). Adventitious root formation in stem cuttings in relation to hormones and nutrition, in Recent researchers in pant sciences, edited by S.S. Bir, Kalyani Publisher, New Delhi.461-541.

Norstog, K. (1970). Induction of embryo-like structure by kinetin in cultured barley embryos. Dev. Biol. 23:665-670.

Parthasarathy, V.A., Nagaraju, V. and Parthasarathy, U. (2001). Citrus In: *Biotechnology of Horticultural Crops*. (Eds. Parthasarathy, V.A., Bose, T.K., Deka, P.C., Das, P., Mitra, S.K. and Mohandas, S.). Published by Naya Prokash, Calcutta.119-165.

Ramakrishna N.K. and Singh, S.K. (2005). *In vitro* adventitive embryony in *Citrus*: A technique for *Citrus* germplasm exchange Cur.Sci.88: 1309-1311.

Rana, J. S. and Singh, R. (2002). *In vitro* clonal propagation of kagzi lime (*Citrus aurantifolia* Swingle) through shoot tip. Prog. Hort. 34: 27-34.

Rashad, M., Khan, M.M., Ramjan, R., Adnan, S. and Khan, F. A. (2005). *In vitro* regeneration and somatic embryogenesis in (*Citrus aurantifolia* and *Citrus sinensis).* Int. J. Agri Biol. 7: 518-520.

Saini, H.K., Gill, M.S. and Gill, M.I.S. (2010). Direct shoot organogenesis and plant regeneration in rough lemon (*Citrus jambhiri* Lush.) Indian J. Biotechnol. 9: 419-423.

Snedecor, G. W. and Cochran, W. G. (1967). Statistical methods. VI Ed. Oxford IBH Pub. Co. Delhi.

Starrantino, A. and Caruso, A. (1998). *In vitro* culture for citrus micropropagation. Acta Horti. 227: 444-446.

Syamal, M.M., Upadhyay, S. and Biswas S. (2007). *In vitro* clonal propagation of kagzi lime (*Citrus aurantifolia* Swingle). Ind. J. Hort. 64: 84-86.

Torres, K.C. (1988). Tissue culture technique for horticultural crops. Vam Mostrand Reinhold, New York.295-298.

Usman, M., Muhammad, S., and Fatima, B. (2005). *In vitro* multiple shoot induction from nodal explants of citrus cultivars J. Central European 6:435-442.

Vijayakumari, N. and Singh, S. (2001). Effect of growth regulators on somatic embryogenesis, morphogenesis and plantlet regeneration in *Citrus reticulata* Blanco. Ind. J. Hort. 58: 294-298.

(Published in March 2012)

Table 1. Effect of different auxins (alone) on *in vitro* response of mature cotyledons

Culture media ▼ Species ▶	Callus initiation (%)				Morphogenic calli (%)				Plant regeneration (%)			
	Acid lime	Mandarin	Sweet orange	Mean	Acid lime	Mandarin	Sweet orange	Mean	Acid lime	Mandarin	Sweet orange	Mean
MS.5N	65.17	62.10	68.10	65.12^{e}	10.96	10.53	10.62	10.70^{b}	54.11	52.23	54.44	53.59^{b}
MSN	64.33	72.11	71.02	69.15^{c}	13.32	10.52	11.52	11.78^{a}	54.51	54.11	53.09	53.90^{a}
MS2N	71.62	69.12	75.12	71.95^{b}	12.20	11.22	10.30	11.24^{ab}	50.35	52.06	52.41	51.62^{d}
MS3N	58.12	53.31	72.35	61.26^{h}	10.53	10.00	10.49	10.34^{bc}	47.41	48.11	49.39	48.30^{f}
MS4N	61.01	58.11	56.12	58.41^{i}	5.99	6.01	6.11	6.03^{h}	47.05	44.00	44.41	45.15^{h}
MS5N	45.21	43.32	48.37	45.63^{j}	4.91	5.98	5.65	5.51^{i}	42.11	31.21	38.10	37.14^{j}
MS.5D	65.11	68.30	65.65	66.35^{de}	7.50	8.65	7.93	8.02^{e}	54.45	54.06	53.12	53.87^{b}
MSD	73.09	72.35	74.18	73.20^{b}	8.86	8.59	7.93	8.46^{d}	52.31	53.11	53.06	52.82^{c}
MS2D	78.52	74.35	70.88	74.58^{a}	9.72	8.56	8.03	8.77^{c}	50.11	51.06	52.06	51.07^{de}
MS3D	62.33	72.59	64.18	66.36^{d}	6.50	5.50	6.98	6.32^{f}	48.09	49.11	50.05	49.08^{e}
MS4D	62.05	65.70	60.66	62.80^{f}	5.01	6.87	6.65	6.17^{f}	47.12	48.30	47.06	47.49^{g}
MS5D	60.32	65.12	59.12	61.52^{g}	5.33	5.96	5.08	5.45^{i}	44.11	43.25	42.05	43.13^{i}
Mean	63.90^{c}	64.70 ^{b}	65.47^{a}		8.40^{a}	8.19^{a}	8.10^{a}		49.30	48.38	49.09	
CD 0.05												
Species				0.66				0.59				NS
Media				1.32				1.17				1.16
S x M				2.28				2.03				2.01

Values within column followed by different letters are significantly different at 5% probability level.

Table 2. Effect of different cytokinins (alone) on *in vitro* response of mature cotyledons.

Culture media	**Callus initiation (%)**				**Morphogenic calli (%)**				**Plant regeneration (%)**			
▼ **Species►**	Acid lime	Mandarin	Sweet orange	Mean	Acid lime	Mandarin	Sweet orange	Mean	Acid lime	Mandarin	Sweet orange	Mean
MS.5B	32.39	28.52	30.20	30.37[a]	12.12	11.50	10.33	11.31[a]	67.22	62.10	70.11	66.47[b]
MSB	27.12	28.95	30.88	28.98[b]	10.95	9.53	9.86	10.11[ab]	72.31	65.42	62.44	66.72[b]
MS2B	28.01	26.50	27.53	27.34[cd]	9.98	9.53	9.01	9.50[b]	68.01	67.13	69.09	68.07[a]
MS3B	30.01	28.05	26.37	28.14[bc]	7.31	6.93	6.77	7.00[d]	64.12	65.01	66.02	65.05[c]
MS4B	24.06	25.19	27.39	25.54[e]	5.53	6.76	6.90	6.39[e]	57.71	67.25	64.33	63.09[d]
MS5B	20.51	18.99	22.68	20.72[g]	5.60	5.50	5.75	5.61[h]	60.11	62.01	60.06	60.72[e]
MS.1Td	28.12	26.23	27.93	27.42[c]	9.60	9.59	9.33	9.50[b]	52.06	46.15	46.10	48.10[i]
MS.2Td	26.92	26.39	24.64	25.98[d]	8.88	8.73	8.01	8.54[c]	55.04	46.23	50.12	50.46[h]
MS.3Td	25.02	23.96	22.25	23.74[f]	5.43	6.01	6.98	6.14[f]	60.01	56.30	62.11	59.47[f]
MS.5Td	22.17	21.66	20.55	21.46[g]	6.66	5.60	5.99	6.08[g]	57.02	60.42	55.32	57.58[g]
MSTd	20.63	19.89	21.73	20.75[g]	5.01	4.93	4.97	4.97[i]	55.11	60.06	54.06	56.95[g]
MS2Td	27.98	18.86	16.01	20.95[g]	5.93	4.01	4.19	4.71[j]	40.11	36.11	42.14	39.45[j]
Mean	26.07[a]	24.43[b]	24.84[b]		7.75[a]	7.38[b]	7.34[b]		59.06	57.84	58.49	
CD 0.05												
Species				0.69				0.57				NS
Media				1.37				1.15				1.22
S x M				2.38				1.99				2.12

Values within column followed by different letters are significantly different at 5% probability level.

Table 3. Combined effect of different added auxins and cytokinins in varying concentrations and combinations on *in vitro* response of cultured mature cotyledons

Culture media ▼ Species ▶	**Callus induction (%)**				**Morphogenic calli (%)**				**Plant regeneration (%)**			
	Acid lime	Mandarin	Sweet orange	Mean	Acid lime	Mandarin	Sweet orange	Mean	Acid lime	Mandarin	Sweet orange	Mean
MS.5N.5B	62.10	72.66	68.33	67.69^{h}	8.33	9.05	8.85	8.74^{j}	79.06	75.07	76.08	76.73^{b}
MSN.5B	65.05	68.23	68.30	67.19^{h}	9.92	8.03	8.64	8.66^{j}	77.34	75.35	71.18	74.62^{b}
MS2N.5B	68.32	68.59	69.01	68.64^{g}	8.93	10.53	10.60	10.02^{h}	77.09	74.15	72.05	74.43^{b}
MS3N.5B	82.59	80.77	76.36	79.90^{c}	15.32	14.98	14.01	14.77^{f}	70.15	74.23	65.44	69.94^{c}
MS4N.5B	83.33	79.12	82.53	81.66^{b}	17.02	17.32	17.11	17.15^{de}	63.32	63.34	64.00	63.55^{f}
MS5N.5B	80.11	82.09	85.03	82.41^{ab}	9.43	10.01	10.76	10.06^{h}	68.11	67.10	54.16	63.12^{f}
MS.5D.5B	69.93	66.52	63.22	66.55^{i}	5.90	6.19	5.65	5.91^{l}	64.47	61.31	58.11	61.29^{i}
MSD.5B	67.33	71.03	61.11	66.49^{i}	6.69	6.64	5.99	6.44^{k}	62.16	66.01	56.05	61.40^{h}
MS2D.5B	84.13	80.15	81.16	81.18^{b}	9.23	9.48	9.50	9.40^{i}	60.30	63.12	63.15	62.19^{g}
MS3D.5B	79.25	78.52	77.31	78.36^{d}	11.30	12.30	12.54	12.04^{g}	64.28	60.11	61.48	61.95^{g}
MS4D.5B	82.18	83.39	81.13	82.23^{b}	16.93	16.01	16.05	16.33^{e}	54.06	53.16	54.01	53.74^{l}
MS5D.5B	85.23	84.10	83.22	84.18^{a}	14.01	14.95	14.26	14.43^{f}	58.54	56.45	53.11	56.03^{k}
MSBN	75.29	79.22	70.88	75.13^{e}	16.93	17.55	14.79	16.42^{e}	78.11	74.06	77.26	76.47^{b}
MS2BN	75.09	73.12	73.32	73.84^{ef}	17.32	18.50	18.19	18.00^{cd}	77.09	78.19	76.06	77.11^{b}
MS3BN	69.55	65.21	66.96	67.24^{h}	22.52	24.59	23.06	23.39^{b}	75.06	77.04	80.01	78.03^{ab}
MS4BN	67.10	65.66	63.20	65.32^{j}	32.12	33.01	30.56	31.89^{a}	80.13	79.05	78.11	79.09^{a}
MS5BN	59.12	57.33	59.02	58.49^{l}	16.50	18.97	16.56	17.34^{d}	74.13	73.31	72.15	73.19^{bc}
MSB.5D	82.19	79.31	77.11	79.53^{cd}	12.90	14.95	14.53	14.12^{fg}	62.05	60.33	58.10	60.16^{j}
MS2B.5D	75.10	71.52	69.10	71.91^{f}	15.02	14.35	14.98	14.78^{f}	70.05	66.31	64.05	66.80^{e}
MS3B.5D	73.22	72.20	59.32	68.24^{gh}	16.92	15.58	16.03	16.17^{e}	70.05	68.06	68.16	68.75^{d}
MS4B.5D	71.33	60.12	65.22	65.55^{j}	18.10	19.63	19.90	19.21^{c}	66.16	60.06	60.11	62.11^{g}
MS5B.5D	63.98	61.91	58.22	61.37^{k}	15.02	14.93	14.36	14.77^{f}	57.10	67.33	63.16	62.53^{g}
Mean	73.70^{a}	72.75^{a}	70.86^{b}		14.38^{a}	14.88^{a}	14.39^{a}		68.35^{a}	67.87^{a}	65.72^{b}	
CD 0.05												
Species				1.25				0.66				1.95
Media				3.39				1.80				5.28
S x M				5.88				3.11				9.14

Values within column followed by different letters are significantly different at 5% probability level.

Table 4. Effects of different plant growth regulators on *in vitro* rooting of shootlets

Culture media	Root proliferating shootlets (%)				Number of roots				Root length (in cm)			
▼ Species ►	Acid lime	Mandarin	Sweet orange	Mean	Acid lime	Mandarin	Sweet orange	Mean	Acid lime	Mandarin	Sweet orange	Mean
MS.5I	82.12	77.15	73.66	77.64[a]	2.99	2. 07	2.37	2.47[b]	3.33	3.30	3.34	3.32[a]
MSI	76.09	71.32	72.09	73.16[cd]	2.18	3.10	3.33	2.87[b]	3.19	3.15	3.19	3.17[b]
MS2I	71.22	69.12	66.33	68.89[e]	3.19	2.29	2.93	2.80[b]	2.99	2.31	2.25	2.51[bc]
MS3I	61.32	62.05	60.12	61.16[g]	1.12	1. 08	1.29	1.16[b]	2.12	2.30	2.02	2.14[c]
MS.5N	75.12	71.19	74.04	73.45[c]	2.20	3.12	3.98	3.10[b]	3.20	3.39	3.12	3.23[ab]
MSN	70.66	70.60	71.10	70.45[de]	1.98	1.58	2.22	1.92[b]	2.12	2.30	2.02	2.14 [c]
MS2N	69.52	68.12	69.16	68.93[e]	1.10	1. 09	1.87	1.35[b]	3.09	3.19	3.03	3.10[b]
MS3N	61.33	59.72	58.79	59.94[h]	1.20	1. 07	1.10	1.12[b]	1.78	1.70	1.01	1.49[c]
MS.5Kn	79.12	75.10	76.66	76.96[b]	3.19	2.29	2.93	2.80[b]	3.59	3.23	3.08	3.30[a]
MSKn	67.03	61.01	65.10	64.38[f]	2.19	2. 09	2.10	2.12[b]	2.97	2.78	2.12	2.62[b]
MS2Kn	75.42	77.03	72.33	74.92[b]	3.33	3.13	3.27	3.24[ab]	2.11	2.61	2.03	2.25[c]
MS3Kn	58.17	60.66	56.10	58.31[i]	1.20	1.97	1.33	1.50[b]	1.99	1.60	1.49	1.69[c]
MS.5I.5B	79.10	77.32	75.12	77.18[ab]	2.91	3.22	3. 06	3. 06[b]	3.60	3.78	3.29	3.55[a]
MSI.5B	75.22	75.10	71. 09	73.80[bc]	3.71	2.97	2.77	3.15[b]	3.53	3.33	3.45	3.43[a]
MS2I.5B	72.10	75.11	70.33	72.51[d]	2.11	1.81	2. 03	1.98[b]	3.12	3.03	2.99	3.04[b]
MS.5I.5Kn	70.11	71.92	73.10	71.84[d]	2.91	2.92	2.98	2.98[b]	3.47	3.52	3.42	3.47[a]
MSI.5Kn	73.01	71.34	75.91	73.42[c]	2.98	2.22	2.87	2.69[b]	3.42	3.49	3.39	3.43[a]
MS2I.5Kn	79.12	75.10	76.66	76.96[b]	3.97	3.12	3.50	3.53[a]	3.59	3.23	3.08	3.30[a]
MSI.5Kn	67.03	61.01	65.10	64.38[f]	1.13	1.19	1.93	1.41[b]	2.97	2.78	2.12	2.62[b]
Mean	71.90[a]	70.35[b]	69.69[b]		2.38	2.24	2.52		2.95[a]	2.89[a]	2.65[a]	
CD (0.05)												
Species				1. 07				NS				0.40
Media				2.69				2.32				1.00
S x M				NS				NS				1.74

Values within column followed by different letters are significantly different at 5% probability level.