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REPORTS ON DIRECT AND INDIRECT ORGANOGENESIS THROUGH TISSUE CULTURE IN CITRUS

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Abstract – Citrus is considered as one of the most essential fruit crop of the world due to its high nutritional value as well as its great production potential; several species of citrus are cultivated in most tropical and subtropical regions of the world. Genus citrus includes more than 162 species which belong to the family Rutaceae and sub family Aurantoideae. Commercially important citrus species includes Kumquat (*Citrus japonica*), Sweet orange (*C. sinensis L. Osbeck),* Grapefruit (*C. paradise Macf.),* Pummelo (*C. grandis Osbeck*), Acid Lime (*C. aurantifolia Christm*) and Lemon (*C. limon (L) Burn F*.) etc. Plant tissue culture is a collection of techniques used to maintain or grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition. This technique involves callus induction from explants, morphogenesis, shoot development and finally root development to regenerate into a complete somaclone. Production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens. Hence, application of tissue culture biotechnology in the field of agriculture seems very crucial so as to increase agricultural productions including citrus for the purposes of feeding the population with no need of international aids. Development of protocols for successful production of callus initiation system through tissue culture for further regeneration of shoots and roots in several species of Citrus have been reviewed in this paper.

INTRODUCTION

Citrus fruits are being considered to be very essential fruits, ranking first with respect to fruit production in the world (Ladanyia, 2008). Citrus species are commonly cultivated in most tropical and subtropical regions of the world and it is nearly grown in 49 countries around the world. Citrus fruit has been cultivated in an ever-widening area since ancient times; with its best-known examples are the oranges, lemons, grapefruit, and limes. Majority of the citrus trees can be propagated in two stages. At the initial stage, rootstock is grown from seeds. After the seedlings are well established, top of the leaf is cut off and bud wood from an existing tree is grafted into root stock which determines the varieties of citrus species that is grown. For commercial production of microbe free plants (Parmessur *et al.,* 2002; Liao *et al.,* 2004) as well as for conservation of germplasm of rare and endangered plant species from extinction (Milkulik, 1999; Chang *et al.,* 2000; Jaime and Da Silva, 2003), biotechnological tools such as techniques of plant tissue culture technology has been successfully used. This technique particularly involves callus induction from explants, morphogenesis, shoot development and ultimately root development to regenerate into a complete plantlet. For development of successful somaclonal variants, techniques of plant tissue culture require several combinations of hormones and nutrient medium. Techniques like *in vitro* culture made it easy to improve citrus against different abiotic stresses, low yield and conserve important citrus genotypes through exploiting somaclonal variations, somatic cell hybridization (Kobayashi *et al.,* 1985), transformation of high yielding cultivars disease free plants. Britters and Murashige (1972) and Kochba and Spiegel Roy (1976) thereby recognized the importance of research in citrus tissue culture. Comparatively with other woody species which are

difficult to culture *in vitro*, future advances with Citrus tissue culture would depend on a better understanding in terms of development of methods for controlling cellular differentiation and organogenesis (Murashige, 1974). Techniques of plant tissue culture offers certain advantages over traditional methods of propagation such as production of exact copies of plants which produces specifically good flowers, fruits or have other desirable traits; for quick production of mature plants; production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds; regeneration of whole plants from plant cells that have been genetically modified; production of plants in sterile containers which allows them to be moved with greatly reduced chances of transmitting diseases, pests and pathogens. In order to increase in the production of agricultural sectors including citrus for the purpose of feeding the population, application of tissue culture biotechnology has proven to be utmost important. Most notable among these are techniques for obtaining virus-free and mycoplasma-free stocks using *in vitro* grafting of apical meristems from infected plants on to decapitated seedlings (Navarro, 1975). For further improving the techniques of Citrus tissue culture, investigations for enhancing the callus culture growth is still under continuation. The far reaching significance of tissue culture in citrus breeding for improvement and augmenting production was discussed by Kochba and Spiegel-Roy, (1977) and various other aspects of citrus tissue culture by Button and Kochba, (1977). This paper specifically highlights about the results related to the development of an efficient callus initiation system of several species of Citrus through tissue culture which further could be used as an efficient and suitable regeneration protocol of Citrus in future.

Regeneration of shoots from callus

Appearance of micro shoot buds on callus is an initiation of callus regeneration. Gradually, morphological changes starts occuring such as turning of callus into dark green coloration followed by its loosening when callus is cultured on regeneration medium. In most of the explants cultured, majority of the shoots regenerates either from the peripheral cells or from the middle cells of the callus. Outer peripheral and middle cells consists of parenchymatous and meristematic origin which helps in further participation in cell division and differentiation as per the reports of histological analysis of callus cultures particularly in *Citrus madurensis* (Grinblat, 1972). In case of callus regeneration, it was observed to be highest (80.52%) in calli cultured on MS medium supplemented with NAA@ 0.5mg/l, BAP@ 3.0 mg/l and Kinetin @ 1.0 mg/l, whereas on the other hand, maximum number of shoots (7.50) was observed when MS medium was fortified with NAA @ 0.5mg/l, BAP @ 2.5 mg/l and Kinetin @ 0.5 mg/l (Kaur, 2018). Similarly, shoot regeneration (76.09%) and number of shoots (8.15) per callus was found to be maximum when callus was inoculated in MS medium supplemented with NAA 0.5mg/litre along with kinetin @ 0.5mg/litre and BA @ 3.0mg/litre (Kumar *et al.,* 2011). Callus obtained from cotyledonary segments of *Citrus jambhiri* when inoculated on MS medium supplemented with BA @ 3 mg/ litre responded maximum in terms of regeneration of shoots (87.50%) (Savita *et al.,* 2011). In several species of citrus, Cytokinins alone or in combination with other plant growth regulators has been considered to be beneficial in case of differentiation and identification of callus cultures into shoots. Regeneration of shoots was obtained from the undifferentiated callus cultures of *Citrus grandis* (Li and Xu, 1993; Begum *et al.,* 2003) and *Citrus sinensis* (Rashad *et al.,* 2005) inoculated in MS medium specifically supplemented with BAP. Another report on shoot regeneration was also obtained from callus of stem segments of *Citrus jambhiri* endowed with BAP @ 3 mg/L supplemented MS medium (Ali and Mirza, 2006). Occurance of redifferentiation in formation of shoots was obtained when callus of *Citrus grandis, Citrus sinensis* as well as *Citrus aurantifolia* was cultured on BAP and NAA supplemented MS medium (Chaturvedi and Sharma, 1988). Highest regeneration of shoots was obtained in BAP supplemented MS medium derived callus in *Citrus sinensis* (Rashad *et al.,* 2005). When callus of Citrus cultivars such as Mosambi, Baramasi lemon and Kinnow were cultured on BAP @ 5 mg/L supplemented ½ strength MS medium, shoots were regenerated (Dhatt and Grewal, 1997). Reports on good regeneration percentage derived from callus of several species of Citrus on MS medium fortified with BA have been opined by several researchers (Pena *et al.,* 1995a, b; Normah *et al.,* 1997; Cervera *et al.,* 1998; Chakraborty and Goswami, 1999; Pena and Navarro 1999; Costa *et al.,* 2002). For identification of explants suitable for regeneration, shoots derived from callus of shoot tips inoculated on MS medium supplemented with

BA (1mg/l) had been considered to be best among the explants (Sharma *et al.,* 2009). Cytokinins such as BA and Kinetin helps in formation and development of shoots when callus is supplemented with it (Raman *et al.,* 1992). Apart from it, similar results with respect to shoot regeneration from callus was also found in Citrus when callus was inoculated in MS medium supplemented with BA and NAA (Chaturvedi and Mitra 1975; Beloualy 1991). In case of indirect regeneration, it was found to be most suitable when MS medium was supplemented with 0.5 mg/l BAP along with 500 mg/l malt extract (Hussain, 2018). These results are also in agreement with some species of citrus such as pummelo and mandarin (Begum *et al.,* 2003; Sarma *et al.,* 2011). Callus obtained from nucellus tissue, shoot apical meristem as well as nodal segments responded well in terms of shoot regeneration which was found to be at the rate of 46%, 20% and 16% when N7 medium consisting of 1.5 mg/l Kin along with 500 mg/l malt extract was fortified to the callus (Hussain, 2018). Similar kind of reports on indirect organogenesis (44%, 40% and 48%) were also obtained when callus was inoculated in MS medium supplemented with BAP @3 mg/L along with NAA @ 0.5 mg/l. During conduction of research in Citrus tissue culture, MS medium fortified with NAA @ 0.5 mg/l along with combination of BA @ 3.0 mg/L and Kinetin @ 0.5 mg/L showed maximum potential of regeneration of shoots (76.09%) (Kumar *et al.,* 2011). A standard protocol was established actually for micropropagation of *Citrus jambhiri* regarding regeneration of shoots through callus induction and it was found that callus derived from leaf segments showed highest regeneration potential (57%) on MS medium supplemented with NAA @ 0.5 mg/l and BA @ 1 mg/l (Savita *et al.,* 2010). Among the explants such as nodal and leaf segments, callus obtained from nodal segments responded well (71.89%) as compared to leaf segments when supplemented with BA (3.0 mg/l). Similar protocol was developed in *Citrus jambhiri* Lush in order to obtain regeneration potential through induction of embryogenic callus (Kaur, 2018). In majority of the species of Citrus, regeneration of shoots obtained from callus has been found to be suitable when MS medium was BA and NAA (Chaturvedi and Mitra, 1974; Beloualy, 1991). Investigations on regeneration of shoots on various species of Citrus via callus have been studied by several researchers by addition of cytokinins such as BA (1 mg/l, 1.5 mg/l) and KIN (1mg/l, 2 mg/l) and BA in combination with KIN (2mg/l+2mg/l, 4mg/l+3mg/l) (Pena *et al.,* 1995 and Dominguez *et al.,* 2000). Callus obtained from epicotyl explants of *Citrus paradise* (Macf.) had given a satisfactory response in terms of shoot regeneration inoculated in MS medium fortified with BA @ 0.5-4 mg/l (Costa *et al.,* 2002). Maximum shooting response (75%) in *Citrus reticulata* Blanco cv Shogun was obtained when different explants of *in vitro* raised seedlings were inoculated on MS medium supplemented with BA @ 0.5 mg/l (Te-Chato and Nudoung, 1998). A medium containing 22 µM BA with or without 5.4 µM NAA was optimum for shoot initiation in all the three Citrus rootstocks (Moore, 1985). Green and healthy friable callus of *Citrus japonica* margarita were cut into small pieces and cultured on MS medium supplemented with various concentrations of BA and Kinetin alone as well as its combination and after 35 days of inoculation, development of shoots were observed which showed 80% response at a concentration of 13.0 µM BA (Hasan, 2016).

Shoot regeneration from cotyledons

Due to the presence of zygotic and nucellar embryos in intact seeds and cotyledons, it has the potential in further formation of shoots naturally (Fig.1). In comparison among the two citrus cultivars, regeneration of shoots from the cotyledons explants of *Citrus nobilis* Lour. of variety Kampar collected from Riau, Indonesia was higher (100%) than the percentage of the highest shoots from cotyledonary explants of *Citrus clementina* cultivar "Monreal", "SRA 63", and "SRA 64" respectively 50%, 33.33%, and 25.93% by fortification of BAP (Lombardo *et al.,* 2011). Regeneration of shoots from cotyledons of *Citrus nobilis* variety Kampar was found to be higher as compared to the shoots developed from cotyledonary segments of 12 day old seedlings of *Citrus reticulata* (84%) inoculated in MS medium fortified with 5 mg/l BAP. Similar protocol was also developed for shoot regeneration from cotyledonary explants of *Citrus reticulata* in MS medium but without an addition of plant growth regulators (Sarma *et al.,* 2011). MS medium supplemented with 2mg/l BAP was considered to be best for regeneration of shoots (88% and 75%) derived from embryo and cotyledonary segment explants (Fig.1) of *Citrus grandis* L. Osbeck (Fatonah *et al.,* 2018). Shoot regeneration does not occur from cotyledonary segment explants on MS medium without an addition of plant growth regulator, but the explants had the potential in formation of callus

(Ibrahim, 2012). MS medium without an addition of plant growth regulators such as cytokinins does not help in the formation of shoots from cotyledons since it results in reduction of meristematic tissues (Sarma *et al.,* 2011).

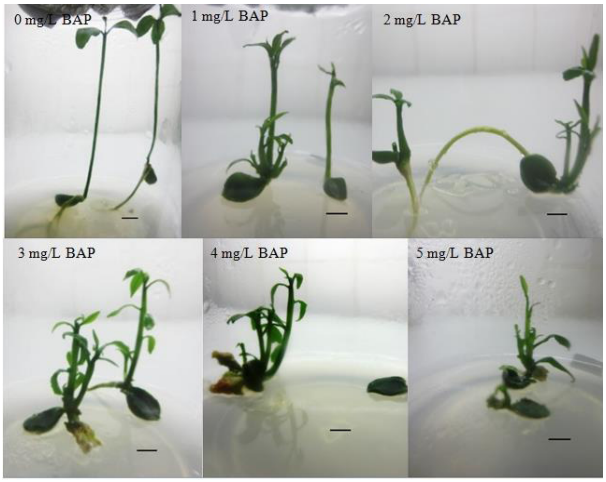

Fig. 1. Regeneration of shoots from cotyledonary explants of *Citrus nobilis* cultured on MS medium fortified with BAP at various concentrations after 49 days of culture (*Source:* Fatonah *et al* 2018)

Regeneration of shoots from roots

Due to the polyembryonic nature of Citrus seeds, it has the potential of regeneration of shoots naturally. In order to regenerate direct multiple shoots from roots, *in vitro* root explants raised from *invitro* grown seedlings were excised and cultured in MSN medium consisting of different kinds of cytokinins such as ADS, BAP, KIN, ZEA along with GA3. After 6-8 weeks of inoculation, a 100% response was obtained in case of regeneration of multiple shoots and among all the plant growth regulators, a combination of MSN+BAP @ 1.0 mg/l along with GA3@ 1.0 mg/l and MSN+BAP @1.0 mg/l along with GA3 @ 2.0 mg/l responded well. During the process of direct regeneration of shoots from roots, nodule like structures were observed on the entire root length which further turned into green colouration thereby leading to the production of shoot buds after 6-8 weeks of culture.MSN medium supplemented with BAP @ 1.0 mg/l along with GA3 @ 2.0 mg/L produced sufficient number of shoots (34.3%) containing 44.4 numbers of leaves after 10-12 weeks of culture. An increase in GA3 concentration in the media (MSN+BAP 1.0+GA3 2.0 mg L^{-1}) resulted in 26.8 shoots and 36.5 leaves per explants (Devi *et al.,* 2021). In *Citrus grandis* (L.) Obseck (pummelo), roots of *invitro* grown seedlings cultured on MS medium supplemented with 2 mg/l BAP produced maximum number of shoots (9.33) (Ibrahim, 2012). Initiation of shoots from root explants have been described in several plant species, indicating a possibility of developing regenerative excised root culture for mass multiplication and their germplasm preservation, viz., *Citrus mitis* (Sim *et al.,* 1989), *Citrus aurantifolia* (Bhat *et al.,* 1992). On the contrary, NAA was found to give better response in *Citrus acida* (Chakravarty and Goswami, 1999).

Root Regeneration from *in vitro* shoots

In case of regeneration of roots from shoots, rooting percent of 88.52 was observed when MS medium was supplemented with NAA@ 1.0 mg/l and IBA @1.0 mg/l (Kaur, 2018). Plant growth regulators such as auxins helps in induction of rhizogenesis thereby leading to the division of meristematic cells which further helps in the process of elongation and differentiation into root primordial (Nanda, 1979). On the other hand, MS medium supplemented with NAA @ 1.0 mg/l and IBA@ 1.0 mg/l helps in formation and development of highest rooting percentage in *Citrus jambhiri* Lush. (Parkash *et al.,* 2005). In *Citrus sinensis* cultivar Mosambi, maximum rooting response was observed when MS medium was supplemented with NAA@1.5mg/l (Rashad *et al.,* 2005) and NAA @ 0.75 mg/l and IBA @ 2 mg/litre (Das *et al.,* 2000). In pectinifera rootstock, average number of roots per shoot was found to be maximum when MS medium was supplemented with NAA @ 2.0 mg/L (Gill and Goshal, 2002). Half strength MS medium fortified with 1.0 mg/L NAA and IBA responded well in case of induction of roots from shoots with maximum rooting percent (83.33) within 4 weeks in *Citrus jambhiri* (Kour and Singh, 2012). In *Citrus jambhiri*, reports on rooting response from regenerated shoots were observed when MS medium was supplemented with IBA @ 1.0 mg/L (Kumar *et al.,* 2011). Among different plant growth regulators tested for root induction from regenerated shoots, NAA at a concentration of 0.5 mg/L was found to give the best rooting response (Savita *et al.,* 2011). In pummelo, root induction (75%) was observed when *in vitro* shoots were inoculated with half strength MS medium supplemented with 1.3, 2.7 and 5.4 5.4 µM of NAA (Paudyal and Haq, 2000). Better response on induction of *invitro* rooting in Mosambi was found to be observed in half strength MS medium supplemented with NAA @ 0.5 mg/l combined with

IBA @ 0.5 mg/l (Krishan *et al.*, 2001). A research was conducted on *invitro* propagation and rooting in some citrus rootstock such as Troyer citrange and Carrizo through tissue culture on MS medium supplemented with BAP@1.0 mg/l along with NAA@1.0 mg/l and GA3 @ 1.0 mg/l and it was observed that it had a maximum induction of shoots as well as its vigorous growth and development was also observed (Kaya and Gubbuk, 2001). In *Citrus reticulata* var. tankan hayata, when shoots of *invitro* raised seedlings were cultured on Murashige and Tucker's medium (MT), fortified with NAA@ 0.5 mg/l, a rooting frequency of 87% was observed (Wang *et al.*, 2002). A study was carried out on *invitro* rooting from *in vitro* raised micro shoots with special effect of bio-regulators in two citrus species such as khasi mandarin and sweet lime on MS medium supplemented with NAA@ 0.1 mg/l and it was reported that it had the highest rooting percentage of 87.71% with its longest root length of about 46.79 mm which was quite a good response (Singh *et al.*, 2003). A rooting response of 70% was observed in *Citrus jambhiri* treated with NAA @0.5 mg/l (Ali and Mirza, 2006). In *in vitro* clonal propagation of *Citrus sinensis*, a 100% rooting frequency was observed when *invitro* raised shoots were treated with various concentrations of IBA (2.64 µM/L, 0.98 to 4.9 µM/L) (Karwa and Chikhale, 2004). In order to induce rooting in citrus, micro shoots that were regenerated from nodal explants were cultured on MS medium supplemented with various concentrations of plant growth regulators such as IBA at 0.0, 0.5 and 1.0 mg/l and NAA at 0.0, 0.5 and 1.0 mg/l. It was observed that shoots treated with MS medium supplemented with NAA @ 0.5 mg/l resulted in best rooting response among all the treatments (EI-Sawy *et al.*, 2006). MS media containing cytokinins such as IBA @ 3.0 mg/l alone or in combination with IAA @ 1.0 mg/l had been considered to be best for induction of rooting from *invitro* grown shoots through biotechnological tools such as tissue culture (Pe´rez-Tornero *et al.*, 2010). Among all the citrus cultivars such as rough lemon, mandarin Pectinifera and Troyer citrange during the conduction of research on *invitro* propagation of citrus rootstocks, it was observed that highest rooting frequency from shoots (1.11%) was found in rough lemon followed by Cleopatra mandarin cultured on half strength MS medium supplemented with growth regulators such as IBA @ 10 mg/l (Sharma *et al.*, 2009). Highest rooting percentage of 77% was observed in *Citrus jambhiri* when *invitro* shoots were cultured on MS medium containing NAA @1.0 mg/L combined with IBA @1.0 mg/l (Saini *et al.*, 2010). An efficient plant regeneration protocol was developed for *Citrus jambhiri* rootstocks and it was observed that callus obtained from *in vitro* shoots, cultured on half strength MS medium supplemented with NAA@ 0.5 mg/l produced maximum percentage of roots (91.67%) (Savita *et al.*, 2011). A 100% response was obtained on induction of roots from *Citrus aurantifolia* treated with MS medium supplemented with NAA @ 0.5 mg/L (Sarker *et al.*, 2015). Rooting of regenerated shoots was highest in MS supplemented with NAA (1.0 mg/l) and IBA (1.0 mg/l) and took minimum number of days to rooting in *Citrus jambhiri* (Kaur, 2018). For rooting development shoots cut off segments of *Citrus*

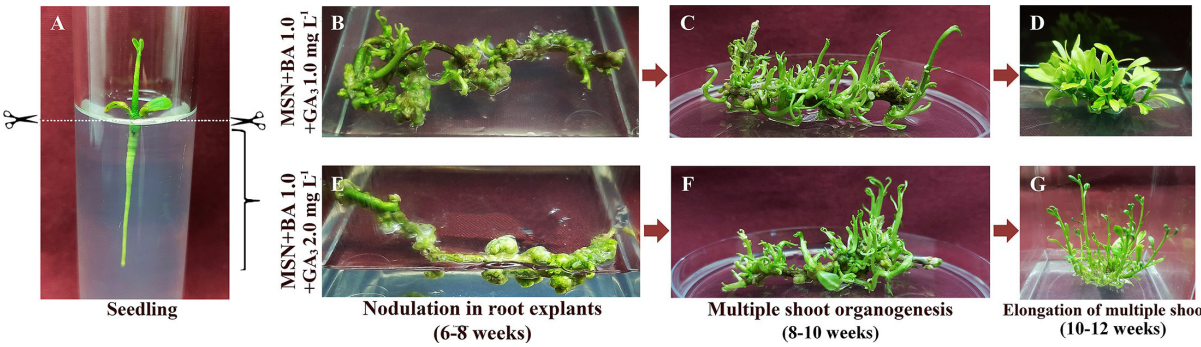

Fig. 2 Direct multiple shoot organogenesis from *in vitro* root explants of *Citrus jambhiri* Lush.
(A) Germination and Induction of Seedling
(B) (C) (D) Occurance of Nodulation in root explants, multiple shoot organogenesis, Elongation of multiple shoots at MSN medium supplemented with BA 1.0+GA3 1.0 mg/L
(E) (F) (G) Occurance of Nodulation in root explants, multiple shoot organogenesis, Elongation of multiple shoots at MSN medium supplemented with BA 1.0+GA3 2.0 mg/L
(Source: Devi *et al* 2021).

japonica Margarita were cultured on rooting medium containing different concentrations of IBA, NAA and their combination. Roots started appearing within 21 days of inoculation. Six different concentrations (5.5, 7, 10, 12, 14, 15 ìM) of IBA and NAA (5, 7.5) were added on MS medium for root initiation. Combination of IBA with NAA was also used. 10 µM IBA and 5 µM of NAA showed 80% and 75% root regeneration respectively (Hasan, 2016). In *Citrus jambhiri*, when *in vitro* micro-shoots were cultured on ½ MSN medium, rooting response ranged between 30-90% whereas on the other hand, in MSN medium, it ranged between 30-80% and among both the concentrations, ½MSN had proven in better rooting response (90%), while poor rooting (40%) was observed in full MSN. Among the auxins, IAA induced significantly higher rooting percentage than NAA in both the culture media as shown in Fig. 2. Early rooting (9.4 d) was observed in the shootlets cultured on ½MSN+IAA (1.0 mg l^{-1}). Moreover, ½MSN+IAA (1.0 mg l^{-1}) medium induced a higher number of roots (3.5) per explant with an average root length of 4.4 cm within 9.4 days followed by the auxins free ½ MSN medium, which induced 3.1 roots having 4.1 cm of mean root length in 9.7 days. On the contrary, rooting was better in MSN medium supplemented with auxins over control as shown in Fig. 2 (Devi *et al*., 2021).

CONCLUSION

Based on the study of various researches carried out by several researchers in terms of plant tissue culture in citrus with its special reference to direct and indirect regeneration through different explants, impregnated with plant growth regulators, it can be clearly concluded that it is an advanced biotechnological tool which includes a collection of techniques and standard protocols in order to maintain or grow plant cells, tissues or organs under aseptic conditions on a nutrient culture medium of various concentrations leading to the conservation as well improvement of citrus germplasm against abiotic stresses, low yield, occurance of somaclonal variations, somatic cell hybridisation and production of disease free plants. It is the only way to conserve the rare and endangered species of citrus thereby protecting it from extinction. On the basis of the reports reviewed in this paper, several techniques such as callus induction from explants, morphogenesis, regeneration of shoot and root and its development into a complete plantlet was involved using several plant growth regulators including callus induction medium, auxins and cytokinins for root and shoot development at various concentrations respectively. Observations on the effects of explants for further regeneration into shoots and roots inoculated on a suitable nutrient media along with plant growth regulators at various concentrations have been highlighted in this paper. On the other hand, identification of the best explants for direct and indirect regeneration suitable on a specific nutrient medium have also been shed lighted. Hence this technique is a necessary prerequisite for genetic improvement and genetic resource conservation.

REFERENCES

Ali, S. and Mirza, B. 2006. Micropropagation of rough lemon (*Citrus jambhiri* Lush.): Effect of explant type and hormone concentration. *Acta Botanica Croatica.* 65(2) : 137- 146.

Begum, F. Amin, M.N. Islam, S. Azad, M.A.K. and Rehman, M.M. 2003. *In vitro* plantlet from cotyledon derived callus of three varieties of pummelo (*Citrus grandis* [L.] Osb. *Online Journal of Biological Sciences.* 3(8) : 751-759.

Beloualy, N. 1991. Plant regeneration from callus of three Citrus rootstocks. *Plant Cell Tissue and Organ Culture.* 24 : 29–34.

Bhat, S.R. Chitralekha, P. and Chandel, K.P.S. 1992. Regeneration of plants from long-term root culture of lime, *Citrus aurantifolia* (Christm.) Swing. *Plant Cell Tissue and Organ Culture.* 29 : 19–25.

Button, J. Kochba, J. and Bornman, C.H.1977. Fine structure of embryoid development from embryogenic ovular callus of 'Shamouti' orange (*Citrus sinensis* Osb.). *Journal of Experimental Botany.* 25 : 446–457.

Cervera, M. Jaurez, J. Navarro, L. Pena, J.A. Duran-Vila N. and Pena, L. 1998. Genetic transformation and regeneration of mature tissues of woody fruit plants by passing the juvenile stage. *Transgenic Research*. 7 : 51–59.

Chakraverty, B. and Goswami, B.C. 1999. Plantlet regeneration from long term callus cultures of *Citrus acida* Roxb. and the uniformity of regenerated plants. *Scientia Horticulturae.* 82 : 159-169.

Chang, C. Chen, C.Tsai, Y. and Chang, Y. 2000. A tissue culture protocol for a rare plant *Lilium speciosum* Var. gloroiosoides Baker. *Botanical Bulletin Academia Sinica.* 41 : 139-142.

Chaturvedi, H.C. and Mitra, G. 1974. Clonal propagation of Citrus from somatic callus cultures. *Hort Science.* 9 : 118–120.

Chaturvedi, H.C. and Sharma, A.K. 1988. Citrus tissue culture. In: *Proc. National. Seminar on Plant Tissue Culture,* ICAR, New Delhi. 36-46.

Chturvedi, H.C. and Mitra, G.C. 1975. A shift in morphogenetic pattern in citrus callus tissue during prolonged culture. *Annals of Botany.* 39(4) : 683-687.

Costa, M.G.C. Otoni, W.C. and Moore, G.A. 2002. An elevation of factors affecting the efficiency of Agrobacterium-mediated transformation of *Citrus paradisi* (macf.) and production of transgenic plants containing carotenoid biosynthetic genes. *Plant Cell Reports.* 21 : 365–373.

Das, A. Paul, A.K. and Chaudhuri, S. 2000. Micropropagation of sweet orange (*Citrus sinensis* Osb.) for the development of nucellar seedlings. *Indian Journal of Experimental Biology.* 38 : 269-272.

Devi, T.R. Dasgupta, M. Sahoo, M.R. Kole, P.C. and Prakash, N. 2021. High efficient *de novo* root to-shoot organogenesis in *Citrus jambhiri* Lush. Gene expression, genetic stability and virus indexing. *PLoS ONE* 16(2): 0246971.

Dhatt, A.S. and Grewal H.S. 1997. Somatic cell culture and shoot regeneration from callus in citrus. *Proceeding of IIIrd Agricultural Science Congress,* March 12-15, Punjab Agricultural University, Ludhiana.213-214.

Dominguez, Guerri, J., Cambra, M., Navarro, L., Moreno, P. and Pena, L. 2000.Efficient production of transgenic citrus plants expressing the coat protein gene of citrus tristeza virus. *Plant Cell Reports.* 19 : 427–433.

El-Sawy, A. Gomaa, A. Reda, A. and Danial, N. 2006. Somatic embryogenesis and plant regeneration from undeveloped ovules of citrus. *Arabian Journal of Biotechnology*. 9 : 189-202.

Fatonah, S. Lestari, W. Isda, M.N. and Purba, L. 2018. *In vitro* shoot regeneration of *Citrus nobilis* Lour from intact seed and cotyled on explants. *SABRAO Journal of Breeding and Genetics*. 50 (2): 168-179.

Gill, M.I.S. and Gosal, S.S. 2002. Micropropagation of pectinifera (*Citrus depressa* Hayata) a potential citrus rootstock for sweet orange. *Indian Journal of Citriculture.* 1 : 32-37.

Grinblat, V. 1972. Differentiation of Citrus stem *in vitro*. *Journal of American Society of Horticultural Science.* 97:599–603.

Hasan, M.R. Gupta, A. Hasan, M.N. Fahim, S.M. Rejwan, H.M. Shamim, M.A. Siddique, M.A.T. and Prodhan S.H. 2016. Efficient Callus Initiation and Plantlet Regeneration of *Citrus japonica* Margarita. IOSR *Journal of Pharmacy and Biological Sciences.* 11(4):72-78.

Hussain, M. Raja, I.N. Rashid, H. Mashwani, Z.R. Mehmood, A. and Iqbal, M. 2018. Establishment of an efficient protocol for plantlets regeneration via direct and indirect organogenesis in Citrus reticulate Blanco (Kinnow mandarin) *Pakistan Journal of Botany.* 50(3): 1203-1210.

Ibrahim, M.A. 2012. *In vitro* plant regeneration of local pummelo (*Citrus grandis* (L.) Osbeck.) via direct and indirect organogenesis. *Genetics and Plant Physiology.* 2: 187–191.

Jaime, A. and Da Silva, T. 2003. Chrysanthemum: Advances in the tissue cultures, cryopreservation, post-harvest technology, genetics and transgenic biotechnology. *Biotechnology Advances.* 21: 715-766.

Karwa, A.S. and Chikhale, N.J. 2004. Effect of various growth hormones on *Invitro* clonal propagation of *Citrus sinensis* Osbeck. *Recent Trends in Biotechnology*. Scientific Publishers (India), Jodhpur, India, pp. 192-195.

Kaur, S. 2018. *In vitro* somatic embryogenesis and regeneration from epicotyl segments of Rough lemon (*Citrus jambhiri* Lush.) *International Journal of Chemical Studies.* 6(1): 2082-2091.

Kaya, B. and Gubbuk, H. 2001. Investigation on the propagation of some citrus rootstocks by tissue culture. *Ziraat Facultesi Dergisi, Akedeniz University*.14 (2): 69-76.

Kobayashi, S. Ikeda, I. and Uchimiya, H. 1985.Conditions for high frequency embryogenesis from orange (*Citrus sinensis* Osb.) protoplasts. *Plant Cell Tissue Organ, Culture*. 4 : 249–259.

Kochba, J. and Spiegel-Roy, P. 1976. Improvement of plant production in Citrus by tissue culture. *Plant Propagator.* 22 : 11-12.

Kochba, J. and Spiegel-Roy, P. 1977. Embryogenesis in gamma-irradiated habituated ovularcallus of the Shamouti orange as affected by auxin and by tissue age, *Environmental and Experimental Botany. 17*:151-159.

Kour, K. and Singh, B. 2012. *In vitro* multiplication of rough lemon (*Citrus jambhiri* Lush.). *IOSR Journal of Agriculture and Veterinary Science.* 1(4):5-9.

Krishan, K. Dhatt, A. S. and Gill, M. I. S. 2001. *In-vitro* plant propagation in sweet orange (*Citrus sinensis* L. Osbeck.) cv. Mosambi and Jaffa. *Indian Journal of Horticulture.* 58 : 208-211.

Kumar, K. Kaur, H. Gill, M.I.S. Rattanpal, H. Kanika, S. and Gosal S.S. 2011.An efficient regeneration protocol from callus culture in rough lemon (*Citrus jambhiri* Lush.). *Indian Journal of Agricultural Sciences.* 81(4) : 324-329.

Ladanyia, M. 2008. *Citrus Fruit, Biology, Technology and Evaluation.* Academic press, San Diego, USA, pp: 576.

Li, M.Y. and Xu, C. 1993. Cotyledon culture and plantlet regeneration of Shimeichen orange (*Citrus sinensis*). *Journal of South west Agricultural University Sichuan China.* 14:51-53.

Liao, Z. Chen, M. Tan, F. Sun, X. and Tang, K. 2004. Micropropagation of endangered Chinese Aloe. *Plant Cell Tissue Organ Culture.* 76: 83-86.

Lombardo, G. Alessandro, R. Scialabba, A. and Sciandra, M. 2011. Direct organogenesis from cotyledons in cultivars of *Citrus clementina* Hort. Ex Tan. *American Journal of Plant Sciences.* 2(2) : 237-244.

Milkuk, J. 1999. Propagation of endangered plant species by tissue culture, *Acta Universitalis Palackianeae Olomucensis Facultas Rerum Naruralium Biologia*.37: 27-33.

Moore, G.A. 1985.Factors affecting *in vitro* embryogenesis

from undeveloped ovules of mature citrus fruit. *Journal of American Society of Horticultural Science.* 110: 66-70.

Murashige, T. 1974. Plant propagation through tissue cultures. *Annual Review of Plant Physiology.* 25 : 135-66.

Nanda, K.K.1979. Adventitious root formation in stem cuttings in relation to hormones and nutrition. *In: Recent Researches in Plant Sciences.* 441-41.

Navarro, L. Roistacher, C.N. and Murashige, T. 1975. Improvement of shoot tip grafting in vitro for virus-free citrus. *Journal of American Society of Horticultural Science.* 100 : 471-479.

Nicolosi, E. Deng, Z.N. Gentile, A. La Malfa, S. Continella, G. and Tribulato, E. 2000. Citrus phylogeny and genetic origin of important species as investigated by molecular markers. *Theoretical and Applied Genetics.* 100 (8) : 1155–1166.

Normah, M.N. Hamidoh, S. and Ghani, F.D. 1997. Micropropagation of Citrus halimii-an endangered species of South-east Asia. *Plant Cell Tissue and Organ Culture.* 50 : 225-227.

Parmessur, Y.S. Alijanabi, S. Saumatally, S. and Dookun-Samutually, A. 2002. Sugarcane yellow virus and sugarcane yellow phytoplasma: elimination by tissue culture. *Plant Pathology.* 51 : 561-569.

Paudyal, K.P. and Haq, N. 2000. *In vitro* propagation of pummelo (*Citrus grandis* L. Osbeck). *In Vitro Cellular Developmental Biology Plant.* 36 : 511-516.

Pena, L. and Navarro, L. 1999. Transgenic Citrus. *Biotechnology in Agriculture and Forestry.* 44 : 39–54.

Pena, L. Cervera, M. Juarez, J. Navarro, A. Pina, J.A. Duran-Villa, N. and Navarro, L. 1995. Agrobacterium-mediated transformation of sweet orange and regeneration of transgenic plants. *Plant Cell Reports*. 14 : 616–619.

Pena, L., Cervera, M., Juarez, J., Navarro, A., Pina, J.A., Duran-Vila, N. and Navarro, L. 1995a. Agrobacterium-mediated transformation of sweet orange and regeneration of transgenic plants. *Plant Cell Reports.* 14 : 616–619.

Pena, L., Cervera, M., Juarez, J., Navarro, A., Pina, J.A., Duran-Vila, N. and Navarro, L. 1995b. High efficiency Agrobacterium-mediated transformation and regeneration of Citrus. *Plant Science.* 104 : 183–191.

Perez-Tornero, O. Tallon, O.C. and Porras, I. 2010. An efficient protocol for micropropagation of lemon (*Citrus limon*) from mature nodal segments. *Plant Cell, Tissue and Organ Culture.* 100 : 263-271.

Prakash, O. Rattanapal, H.S. and Dhaliwal, H.S. 2005. Studies on *in vitro* plantlet production in rough lemon (*Citrus jambhiri* Lush.). *Journal of Research PAU.*42 (3): 285-89.

Raman, H. Gosal, S.S. and Brar, D.S. 1992. Plant regeneration from callus cultures of *Citrus limon* and *Citrus jambhiri*. *Crop Improvement.* 19 : 100-103.

Rashad, M. Khan, M.M. Ramzan, R. Adnan, S. and Khan, F.A. 2005. *In vitro* regeneration and somatic embryogenesis in *Citrus aurantifolia* and *Citrus sinensis*. *International Journal of Agricultural Biology.* 7(3) : 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 Journal of Biotechnology.* 9(4) : 419-423.

Sarker, I. Islam, J. Shaekh, M.P.E. Rahman, M.M. Khan, H. Chowdhury, A.S.M.H.K. Mukim, M.S.I. Islam, R. and Haque, R. 2015. Establishment of a Standard Protocol for *In Vitro* Meristem Culture and Plant Regeneration of *Citrus aurantifolia*. *Journal of Pharmacy and Biological Sciences*. 10 (2) : 61-69.

Sarma, C. Borthakur, A. Singh, S. Modi, M.K. and Sen, P. 2011. Efficient *In vitro* plant regeneration from cotyledonary explants of *Citrus reticulata* L. Blanco. *Annals of Biological Research.* 2(6): 341-348.

Savita Singh, B. Virk, G.S. and Nagpal, A.K. 2011. An efficient plant regeneration protocol from callus cultures of *Citrus jambhiri* Lush. *Physiology and Molecular Biology of Plants.* 17(2) : 161-169.

Savita Virk, G.S. and Nagpal, A. 2010. Effect of explant type and different plant growth regulators on callus induction and plantlet regeneration in *Citrus jambhiri* Lush. *Environment and We-An International Journal of Science & Technology.* 5 : 97-106.

Sharma, S. Prakash, A. and Tele, A.2009.*In vitro* propagation of Citrus rootstocks. *Notulae Botanicae Horti Agrobotanici Cluj Napoca.* 37(1) : 84-88.

Sim, G.E., Goh, C.J. and Loh, C.S. 1989. Micropropagation of *Citrus mitis* Blanco multiple bud formation from shoot and root explants in the presence of 6-benzylaminopurine. *Plant Science.* 59 : 203-210.

Singh, I. P. Parthasarthy, V. A. and Handique, P. J. 2003. Effect of bio-regulators on *In-vitro* raised microshoots of economically important *Citrus species* of the NEH region. *Indian Journal of Horticulture.* 60 : 16-21.

Te-Chato, S. and Nudoung, S. 1998. Tissue culture of *Citrus reticultata* Blanco cv. Shogum and gene transformation by Agrobacteria. *Proc. Abstracts IMT-GTUNITE Conference Songkxhla.*

Wang, S. Huang, Z. Yu, R. Zou, W. and Song, F.S. 2002. Studies on the induction of embryogenic callus, establishment of cell suspensions and plant regeneration in *Citrusretuculata* var. *tanaka* Hayata. *Journal of South China Agricultural University*. 23 : 52-55.