The Bioscan 11(4): 2127-2131, 2016
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.com

IN VITRO MULTIPLE SHOOTS INDUCTION FROM NODAL EXPLANTS OF SOUR ORANGE (*CITRUS AURANTIUM* L.)

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KEYWORDS
BAP
Kinetin
IBA
IAA
Sour Orange

Received on :
11.06.2016

Accepted on :
26.10.2016

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ABSTRACT
The investigation was conducted to describe a micro propagation technique for Sour Orange, *Citrus aurantium* (L.) using nodal explants of mature trees were cultured on Murashige and Skoog medium containing 6-benzylaminopurine (BAP) and 6-furfurylaminopurine (Kinetin) both were at different concentrations.Best results for multiplication shoot formation, 7.4 shoots per node were obtained with BAP 1.0 mg l^{-1} combined with Kinetin 0.5 mg l^{-1}. Transfer of shoots to a rooting medium containing Auxins, IBA and NAA were at various levels. The highest percentages of rooting (90.00 %) were appeared in treatment IBA and NAA both were at 0.5 mg l^{-1}. Plantlets shifted to hardening media containing Cocopeat, Vermiculite, Perlite, Fine sand and Garden Soil in the ratio of 2 : 1 : 1 : 1 : 1 parts given maximum survival percent (74.00 %).

INTRODUCTION

Citrus is considered as the number one fruit of the world due to its high nutritional value, great production potential and preparation of large number of fruit products from them. Citrus species are cultivated in most tropical and subtropical regions of the world. Sour orange, C*itrus aurantium* (family Rutaceae) is an important cultivar of Citrus commonly used rootstock in south Asia and it is an excellent rootstock for warm, humid areas with deep sandy soils. In these environments trees budded on this rootstock grow rapidly, remain productive for longer period and produce high quality fruit (Kour and Singh, 2012). 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. It also has long juvenile phase and stands among difficult to root crops. Here, micro propagation offers rapid propagation of such crops in limited space and time under controlled conditions throughout the year (Mohammad *et al*., 2005). Sour Orange being polyem bryonic in nature, give rise to several vigorous and virus free nucellar seedlings which are difficult to differentiate from zygotic seedling, and are also difficult to eliminate from zygotic seedlings, which necessitate the application of *in vitro* micro propagation (Edriss and Burger, 1984), however, very little work has been carried out on the tissue culture of this plant (Ali and Mirza, 2006). Rapid and cost effective *in vitro* methods of reproducing this rootstock would ensure bulk production of true to type and disease free planting material.

Therefore, in vitro micro propagation constitutes a feasible alternative to overcome these problems. Such process involves bud multiplication from shoot tips, nodes or axillary buds, possesses less probability of somaclonal variation among regenerates in comparison with callus mediated regeneration pathway. Several research work on plant regeneration, callusing, embryogenesis have been achieved in number of citrus species like *C. reticulata* Blanco (Gill *et al*., 1995), Kinnow (Praveen *et al*., 2003), (Altaf, 2006), *Citrus jambhiri* (Ali and Mirza, 2006), *etc.* The aim of this work was to set a protocol for establishment, regeneration, rooting and hardening of Sour Orange from nodal explants for commercially multiplication.

MATERIALS AND METHODS

Fruits of Sour Orange was taken from the Experimental Fruit Garden of the Department of Horticulture, College of Agriculture, SKRAU, Bikaner (Rajasthan). 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 placed vertically on individually in 25 x 150 mm culture tubes containing 25ml of Murashige and Skoog (1962) or MS media containing 5% sucrose and solidified with 0.8% agar. For germination the culture tubes were incubated in darkness at a constant 27ºC temperature for 2 weeks and then at 25ºC,

in a growth chamber with 16h of photoperiod, illumination of 45 mEm-2 s-1and 60 % relative humidity for 3 weeks (Pena *et al.*, 1995). Seedlings obtained after six weeks were used as source material for nodal and internodal segments (1-2 cm in length). These segments were taken as explant and cultured on following media formulations for shoot and root regeneration.

Shoot regeneration

Shoot regeneration was performed in 25 x 150mm culture tubes containing 25ml of MS medium containing 3% sucrose mineral salts, vitamins and different concentrations of BAP and Kinetin both were at 0, 0.5, 1.0 and 2.0 mg l^{-1} and solidified with 0.8% agar.

Rooting of regenerated shoot

The explant derived shoots were excised and transfer to 25 x 150 mm culture tubes containing 25 ml of MS medium supplemented with 3% sucrose, mineral salts, vitamins and different concentrations of NAA and IBA at 0, 0.1, 0.5, 1.0 and 2.0 mg l^{-1} and solidified with 0.8% agar.

The pH of media adjusted to 5.6- 5.8 with 1N NaOH, 1N HCl and autoclaved at 121ºC temperature and 15 lbs pressure for 20 minute. After inoculation, the cultures were placed in the growth room under 16 hr photoperiod in 35ìEm-^{2}s-^{1} light intensity at 25 ± 2ºC temperatures. Visual observations were taken every three days and the effect on different shoots was quantified on the basis of percentage of shoots showing response for rooting.

Hardening

Plantlets were planted in pots with different combination of autoclaved hardening media (Table 3). The potted plants were kept for 2 weeks , coverend with clear plastic sheet in the culture room conditions, after which they were transplanted to a green house and kept covered for additional 2 weeks. The cover was gradually removed during following 2 weeks.

The experiment was laid out in Completely Randomized Design (CRD) with ten explants per treatment and the experiment was repeated thrice.

RESULTS AND DISCUSSION

Shoot induction

Nodal explants were inoculated on MS media supplemented with different concentrations of BAP and Kinetin for optimization of the best phyto hormonal combination and concentration for shoot multiplication. Sour orange explants showed higher percent survival (93.00 %) when cultured at the concentration of BAP at 2.0 mg l^{-1} with Kinetin at 0.5 mg l^{-1} (Table 1 and Fig. 1). Shoot percent survival was found directly proportionate to the addition of BAP from 0.5 - 2.0 mg l^{-1} with Kinetin 0.5 mg l^{-1} in the basal medium. A further increase in kinetin concentration reduces the survival. Similar shoot percent response was also observed by Sharma *et al*. (2012) and Ali and Mirza (2006) reported higher shoot regeneration response 89% and 83 on MS medium fortified with 3.0 mg/L BAP and 0.5 mg/L NAA. Increase in the levels of BAP and Kinetin was found inversely proportionate to days required for shoot induction. The combined effect of BAP 1.0 mgl^{-1} + Kinetin 0.5 mgl^{-1} observed significantly ($P<0.01$) minimum days to bud break (19.2 days) and maximum took by control (22.4 days). Similarly, Singh *et al*. (1994) and Kour and Singh (2012) observed minimum number days to bud break in *Citrus reticulate*, *Citrus limon* and Rough lemon with 17, 18 and 4.3 days, respectively, when explants were cultured in MS medium modified by BAP 1.0 mgl^{-1} + kinetin 0.5 mgl^{-1} + NAA 0.5 mgl^{-1} and BAP 1.5 mgl^{-1}.

The data revealed that high concentration of cytokinin and low concentration of auxin is required for shoot multiplication. The MS medium supplemented with 1.0 mg/L BAP and 0.5 mg/ L Kinetin proved to be best medium for shoot multiplication recorded maximum number of shoots (7.4), While maximum length of shoot (4.2 cm) was observed with

Table 1: Effect of BAP & Kinetin, added in combination on basal medium on shoots parameters of Sour Orange

Treatments (Mg L^{-1})			Percent Survival (%)	Number of days taken to bud break	Number of shoots	Length of shoot (cm)
Sym	BAP	KN				
T_0	0	0.0	15 (22.55)	22.4	1.2	1.6
T_1	0	0.5	58 (49.32)	21.6	4.6	2.0
T_2	0	1.0	40 (39.09)	21.8	3.2	3.1
T_3	0	2.0	30 (33.09)	22.6	2.4	2.2
T_4	0.5	0.0	58 (49.32)	20.6	4.0	1.7
T_5	1.0	0.0	63 (52.32)	18.8	5.0	3.5
T_6	2.0	0.0	50 (44.98)	19.8	4.6	1.4
T_7	0.5	0.5	58 (49.53)	20.4	4.8	2.9
T_8	0.5	1.0	60 (50.88)	20.6	4.6	3.4
T_9	0.5	2.0	35 (36.09)	21	2.8	2.0
T_{10}	1.0	0.5	70 (62.14)	19.2	7.4	3.8
T_{11}	1.0	1.0	60 (50.88)	19.4	4.8	4.2
T_{12}	1.0	2.0	48 (43.53)	19.6	3.8	2.1
T_{13}	2.0	0.5	93 (77.55)	20.4	6.2	2.6
T_{14}	2.0	1.0	68 (55.32)	23.4	5.4	2.9
T_{15}	2.0	2.0	50 (44.98)	21.2	4.0	1.1
S. Em. ±			3.17	0.78	0.39	0.39
CD (1%)			8.92	2.02	1.09	1.09

*Figures given in parentheses are angular transformed values

Table 2: Effect of IBA & NAA concentrations used in combination in basal medium on root parameters of microshoots of Sour Orange

Treatments ((Mg L^{-1})			Percent success (%)	Number of days to root initiation	Number of roots	Length of roots (cm)
Sym	NAA	IBA				
T_0	0	0.0	0 (00.00)	0	0	0
T_1	0	0.1	53 (46.43)	19.2	4.2	3.3
T_2	0	0.5	78 (61.83)	17.0	6.2	5.1
T_3	0	1.0	48 (43.53)	17.9	3.8	3.4
T_4	0	2.0	45 (42.09)	21.0	3.6	3.3
T_5	0.1	0.0	53 (44.98)	19.6	4.2	3.7
T_6	0.5	0.0	83 (65.55)	16.8	6.6	5.8
T_7	1.0	0.0	45 (42.09)	20.2	3.6	4.4
T_8	2.0	0.0	43 (40.64)	20.6	3.4	4.5
T_9	0.1	0.1	50 (44.98)	19.2	4.0	4.1
T_{10}	0.1	0.5	55 (47.88)	17.2	4.4	4.3
T_{11}	0.1	1.0	50 (44.98)	17.6	4.0	4.3
T_{12}	0.1	2.0	40 (39.09)	19.6	3.2	4.2
T_{13}	0.5	0.1	75 (60.28)	17.4	6.0	4.9
T_{14}	0.5	0.5	90 (75.69)	16.2	7.2	6.2
T_{15}	0.5	1.0	60 (50.98)	16.4	4.8	5.4
T_{16}	0.5	2.0	48 (43.53)	17.6	3.8	4.8
T_{17}	1.0	0.1	48 (43.53)	20.2	3.8	4.6
T_{18}	1.0	0.5	53 (46.43)	17.4	4.2	5.0
T_{19}	1.0	1.0	43 (40.54)	19.8	3.4	4.6
T_{20}	1.0	2.0	40 (39.09)	20.6	3.2	4.3
T_{21}	2.0	0.1	48 (43.53)	17.2	3.8	4.6
T_{22}	2.0	0.5	45 (41.98)	17.6	3.6	4.2
T_{23}	2.0	1.0	43 (40.54)	17.4	3.2	4.2
T_{24}	2.0	2.0	40 (39.09)	19.2	1.8	3.8
S. Em. ±	2.95	0.41	0.36	0.08		
CD (1%)	8.30	1.12	1.02	0.22		

Figures given in parentheses are angular transformed values

Table 3: Effect of different hardening media combination on different growth parameters of Sour orange (*C. aurantium* L.)

Treatment details	Plant Height (cm)	Number of new leaves appear	Number of old leaves drop	Number of new shoots cm	Length of new shoots cm	Number of days taken to new sprout	Percent success %
Cocopeat	6.4	4.0	6.4	1.8	2.4	10.2	46 (42.68)
Cocopeat (1) : Sand (1)	7.0	4.2	4.8	2.0	2.1	9.4	50 (44.98)
Garden soil (1) : Soilrite (3)	6.8	4.4	4.4	2.6	2.4	8.0	48 (43.83)
Cocopeat (2) : Vermiculite(1) :Perlite(1)	5.8	4.4	4.8	2.0	2.4	8.2	50 (44.93)
Cocopeat (1) : Fine Sand (2) : Garden Soil (1)	7.0	6.2	4.2	3.0	3.4	7.4	64 (53.16)
Cocopeat (2) : Vermiculite (1) : Perlite (1) : Fine Sand (1) : Garden Soil (1)	9.2	6.4	3.0	3.6	3.1	7.4	7.4 (59.55)
SEm ±	0.32	0.35	0.27	0.16	0.15	0.42	2.31
CD at 5 %	0.93	1.03	0.78	0.47	0.44	1.22	6.75

*Figures given in parentheses are angular transformed values

medium fortified by BAP 1.0 mgl^{-1} + Kinetin 1.0 mgl^{-1}. The results of the present study were very close to that reported by Al-Khayri and Al-Bahrany (2001) in lime Kour and Kher, (2010) and Singh and Patel (2014) in Rough Lemon, they observed maximum number of shoots when explants were inoculated on MS medium fortified with BAP 2.0 mgl^{-1} + kinetin 0.5 mgl^{-1}. We have obtained better regeneration response than those cited in these reports. This increase in culture establishment in medium supplemented with BAP and Kinetin showed that cytokinines are the most obligatory component as observed as earlier workers (Te-Chato and Nudoung, (1998); Cervera *et al.,* 2000; Dominguez *et al.,* 2000; Costa *et al*. (2002); Praveen *et al.*, 2003 and Usman (2005). This may be due to the reason that BAP suppressed apical dominance and induced lateral branches. However, endogenous levels of cytokinin play a very important role. Depending on the endogenous level, requirement of the exogenous application vary to get the optimum response. In this investigation reduction in the culture establishment percentage due to presence of Kinetin indicated that endogenous level of kinetin was near to optimum. This may be due to the different endogenic cytokinin levels of different species Morever, it is specific endo – ectogenous ratio which control shoot formation in tissue culture (Costa *et al*., 2002).

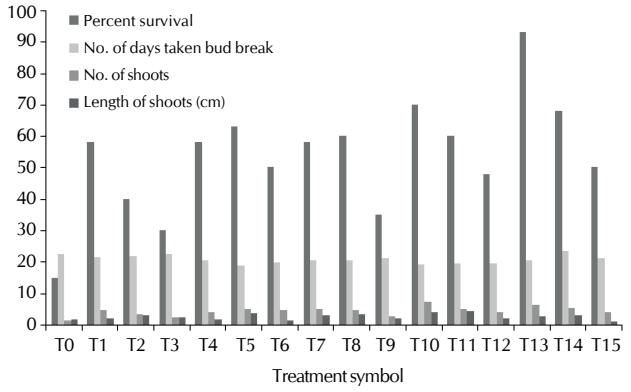

Figure 1: Effect of BAP and Kinetin on shoot parameters of sour Orange

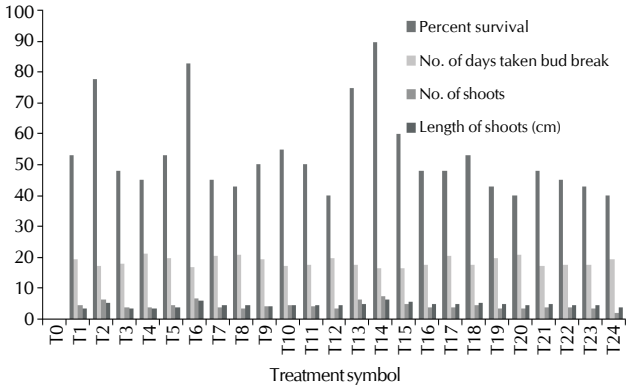

Figure 2: Effect of IBA and NAA on root parameters of micro shoot of Sour Orange

Root induction

Sour orange explants derived shoots when sub cultured on rooting media showed significantly ($P<0.01$) higher rooting percentage (90.00 %) at the lowest level of IBA and NAA at 0.5 mgl^{-1} (Table 2 and Fig 2). Ali and Mirza,(2006) developed shoots cut off segments were cultured on MS medium supplemented with either 0.5 mg L^{-1} NAA or 1 mg L^{-1} 2,4-D obtained 70% and 50% rooting results in these two media, respectively. In interactions a minimum of 16.2 days in Sour orange were needed to root induction was observed at NAA (0.5 mgl^{-1}) + IBA (0.5 mgl^{-1}), and no root initiation was noticed in medium devoid of auxin. Improved rooting in lower strength media was attributed to reduction in nitrogen concentration (Hundman *et al*, 1982). Similar response were also reported by Syamal *et al*. (2007) in Kagzi Lime and Kour and Singh, (2012) in Rough Lemon, who reported in that a minimum of 18.79 days and 16.51 were required to root induction on NAA and IBA (0.5 mgl^{-1}) and NAA and IBA (1.0 mgl^{-1}), respectively. Among various concentrations of IBA and NAA, the maximum number of roots (7.2) and length (6.2 cm) per micro shoots was observed on MS culture medium supplemented with NAA and IBA each 0.5 mg l^{-1}. These results are similar with the findings of Syamal *et al*. (2007) and Lalrinsanga *et al*. (2013) they reported maximum 3.41 roots per micro shoot in MS medium supplemented by NAA and IBA at 0.5 mgl^{-1}. The results of present study are also in close conformity with the findings of Al-Khayri and Al-Bahrany, (2001), Kim *et al*. (2002) Ling *et al*. (2002), Chandra *et al*. (2003) Kour and Singh, (2012) and Lalrinsanga *et al*. (2013). The effect of IBA and NAA on rooting of microshoots was found significant and synergitic. Such studies might be a promising step towards mass production of sanitated plant material of Citrus.

Hardening

The perusal of data presented in table 3 revealed that treatment containing Cocopeat, Vermiculite, Perlite, Fine sand and Garden Soil in the ratio of 2 : 1 : 1 : 1 : 1 parts exerted positive effect on the parameters of acclimatization except maximum shoot length (Table 1). The plant height (9.2 cm), new leaves (6.4 leaves), new shoots (3.6) minimum number of old leaf drop (3.0 leaves) and number of days to bud sprout (7.4) and Success of 74.00 per cent was significantly higher compared to minimum in the treatment combinations containing Cocopeat, Cocopeat and Sand in the ratio of 1 : 1 and Garden Soil and Soilrite in the ratio of 1 : 3. Whereas, maximum length of shoots was observed with media containing Cocopeat, Fine sand and Garden Soil in the ratio of 1 : 2 : 1. Better results may be due to performance of FYM to improve biological properties of the soil. Sand may be responsible for providing sufficient aeration. Cocopeat and vermiculite provide nutrition and moisture due to having high water holding capacity. Physical chemical and biological properties of the mixture are important for the establishment of *in vitro* produced plantlets. Similar results report by Navarro (1988), Kumar (2001) in citrus species and Karunakaran (2006) in Coorg mandarin, who reported 95.00 per cent survival of grafts in green house.

ACKNOWLEDGEMENT

Authors are thankful to ARS, Sriganganagar for providing experiment facilities during studies.

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