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IN VITRO PROPAGATION OF CITRUS AURANTIIFOLIA CV. SAI SHARBATI.

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Abstract:

The present study was undertaken to develop efficient protocol for *in vitro* propagation of *Citrus aurantiifolia* cv. Sai Sharbati through nodal shoot segments. Explants produced multiple buds when cultured on murashige and skoog's medium containing 0.5 mgl^{-1} BAP and 1.0 $mgl^{-1}GA_3$. BAP was recorded to be better than kinetin in terms of multiplication rate, average shoot height and average number of leaves. Separated shoots were rootedon MS medium containing different concentrations of growth hormones NAA and IBA, from which half strength medium supplemented with 1.0 mgl^{-1}IBA was superior to NAA.*In vitro* plantlets were hardened using different potting mixtures, highest survival (90.23 %) success was achieved after transfer to cocopeat. *In vitro* plantlets were successfully acclimatized in greenhouse.

Keywords: Nodal shoot segments, BAP, IBA, MS medium.

Introduction:

Citrus is an important genus of evergreen fruit crops belongs to family Rutaceae, originated in Southeast Asia bordered by Northeastern India and the Yunnan province of China (Gmitter and Hu, 1990; Scora, 1975). In India, citrus occupies an area of 2, 83,000 Ha with production of 32,21,000 MT (Anonymous, 2019).It is considered as 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 (Kour and Singh, 2012).*Citrus aurantifolia* is small fruited acid lime mainly used in daily consumption and in juice production. It is widely used throughout the globe because of its various properties like antibacterial, anticancer, antidiabetic, antifungal, anti-hypertensive, anti-inflammation and antioxidant (Narang and Jiraungkoorskul, 2016).

Acid lime is generally propagated through cutting, air-layering and budding on seedling rootstocks (Rathore *et al.* 2007). Propagation of plants is an often difficult, expensive and season specific when buds are available.Success ratio of these methods of propagation is not affordable. Air layering of plants never yields the required quantity of planting materials. These

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conventional techniques are also not free from risk of in-borne pathogens.Citrus trees also declining on a large scale due to diseases like tristeza, viruses, cancker and various other factors; for example 823 different insects species attacked citrus spp. (Shivankar and Rao, 2001). However, tissue culture methods offer an alternative means of vegetative propagation in short time and can overcome some constraints to citrus improvement and cultivation throughout year with quality fruits and resistance to diseases and environmental stresses (Raja, 2012).There are very few data available about *in vitro* propagation of cv. Sai Sharbati.

The present study was conducted with an objective to develop an efficient protocol for*in vitro* propagation of cv. Sai Sharbati. The studies have focused on establishment of cultures, effects of different cytokinin and auxins on shoot multiplication and *in vitro*rooting and to standardize the culture medium, which is most important factors for the success of tissue culture of cv. Sai Sharbati.

Materials and Methods:

Plant material:

Healthy and high quality fruit yielding plantsof *Citrus aurantiifolia* cv. Sai Sharbati were selected as a mother plant from Savita agro farm, Osmanabad (MH). Shoot cuttings were procured from the selected mother plants in the month of September, 2016. These explants were collected in glass bottle containing tap water to prevent wilting until they were bring to laboratory. Leaves were removed fromshoot cutting and washedcarefully under running tap water to remove micro-flora and dirt before surface sterilization.

Media preparation:

The stock solutions required for Murashige and Skoog (1962), vitamins, growth regulators were prepared well in advance and stored in refrigerator. During medium preparation, each stock solutions after bringing it to room temperature was added one by one in the required quantity to the beaker containing small amount of distilled water. After addition of sucrose (30 g/l), mesoinositol (100 mg/l) and growth regulators, final volume was prepared by adding filtered water. The pH of the medium was adjusted to 5.8 with the help of 1N NaOH and/or 1N HCl. Thereafter, the gelling agent agar was added for solidification of the medium. The medium was then boiled and dispensed in culture glass bottles in equal volume for aseptic manipulations and

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autoclaved at pressure of 15 lbs /inch^{2}and at temperature of 121ºC for 20 min for sterilization. The medium was stored in the dark at 25±2ºC and used after 3-4 days of sterilization.

Surface sterilization and inoculation ofexplants:

Nodal segments excised from the selected healthy tree were used as explants for *in vitro* propagation.Shoot cuttings were immersed in an aqueous solution of liquid detergent containing 2–3 drops of Tween-20 for 10 minand then treated with carbendazime for 30 min. The explants were taken to laminar air flow for surface sterilization. These explants were treated with 70% ethanol for 30 to 40 seconds, followed bydifferent concentration of sterilantslike mercuric chloride ($HgCl_2$) and sodium hypochlorite (NaOCl) (Table-1). After 3-4 times rinsing with sterile autoclaved water, nodal segments of 2.0–3.0 cm were inoculated on MS medium under laminar air flow.

Culture conditions:

Inoculated cultures were kept under controlled conditions of temperature (26±2°C), light for 12 hrphotoperiod and 60% relative humidity. Per cent bud establishment was recorded during four weeks of culture. Sprouted buds were then subculturedon multiplication and rooting medium fortified with different concentration of growth regulators.Observations were recorded regularly for shoot growth, fungal/ bacterial contamination and data was recorded after 4 weeks of culturing.

***In vitro* multiplication:**

Healthy shoots were transferred on MS medium supplemented with different concentrations and combination of growth hormones like BAP, Kin and GA_3 to investigate their effects on enhancement of shoot multiplication. Nodal and shoot tip explants were used for shoot multiplication. Shoots were multiplied by the method of enhanced release of axillary buds (Murashige, 1974). The observations were recorded after 4 weeks of cultures as-rate of multiplication, Quality of shoots, average shoot length and leaves (Table 2). The multiplication medium which showed healthy, long and good quality microshoots during incubation period was selected for further multiplication on same medium.

***In vitro* rooting**:

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Good quality microshoots (20–30 mm long) were used for root induction produced from *in vitro* multiplication stage were individually separated and transferred to full strength and half strengths of MS medium supplemented with various concentrations of IBA or NAA (Table 3).The individual shoot bases were cut transversely. Four such shoots were inoculated in each culture bottle. Three replications of each treatment were made. In all rooting experiments 6 g/l agar was used to easily removal of plantlet during hardening. Data was recorded on Days taken for root initiation, percent rooting, Root length and numbers of roots per shoots after 4 weeks of culturing period.

Hardening and acclimatization of plantlets:

For hardening and acclimatization, *in vitro* rooted plantlets were carefully removed from the culture glass bottles to avoid any damage to delicate root system and kept in a beaker and washed under running tap water for 30 min to remove agar sticking to root surface. Thereafter, the plantlets were kept dipped in solution of 0.2% carbendazim for 15-20 minutes before transferring to different potting mixture. Root portion was placed inside the mixture gently to avoid any injury. In order to maintain high relative humidity, plantlets were covered with glass jars and watered at 2-3 days intervals. Pots were kept at temperature of 28±2ºC in polytunnel.When plants showed initial signs of establishment, humidity was decreased by removing the glass jars for few minutes. Thereafter, the plants were hardened by removing the glass jars for increased time intervals to reduce the relative humidity gradually. Observations were recorded for survival percentage, average number of leaves and average height of plants (Table 4). Eight weeks old primary hardened plants were transferred to polybagscontaining soil media (red soil and FYM, 3:1) for secondary hardening before dispatch to field transfer.

Statistical analysis:

The experiments were repeated three times and subjected to completely randomized design (Gomez and Gomez, 1984). Significance of treatment on various observations was determined using analysis of variance (ANOVA) technique for CRD. If experimental treatments found significant, their relative performance was tested with critical difference ($CD_{0.05}$).

Results and discussion:

Shoot multiplication:

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Nodal explants of cv. Sai Sharbati resulted in 64.43 per cent uncontaminated cultures after four weeks of incubation when treated with 0.1% $HgCl_2$ for 5 min. The results obtained are in conformity with Rana and Singh (2002), surface sterilization is necessary to make explants free from all contaminants (Beura *et al*., 2003). These cultured nodal segments showed 74.80 per cent proliferation after 3 weeks of culture on MS basal medium (Table-1; Fig-1). Proliferated shoots were then cut into small pieces (2-3 nodal segments) and cultured on MS medium supplemented with different concentration and combination of growth regulators. Different concentration and combination of growth regulator showed mark influence on shoot multiplication, length of shoots and leaf numbers. All medium combinations showed multiplication of shoots (Table-2). The highest multiplication rate of 1:2.8 was observed in MS medium supplemented with 0.5 mgl^{-1} BAP and 1.0 mgl^{-1} GA_3 (Table-2 and Figure-2). Similarly, the highest shoot length (4.53 cm) and average number of leaves (5.03) was observed on this medium as compare to other. Similar study of Sujata *et al*., 2010 showed, BAP was best cytokinin for citrus shoot proliferation. The function of BA during *in vitro* shoot multiplication is to break the apical dominance, stimulate growth of new shoots (Muna *et al*., 1999).

***In vitro* rooting:**

Various researchers reported that auxin promotes good quality of roots. Popularly IBA and NAA used as *in vitro* root induction in different citrus species (Carimi and De Pasquale, 2003). For *in vitro* rooting, the shoots were separated and transferred individually to full and half strengths of MS medium fortified with various concentration of IBA and NAA. Incorporation of 1.0 mgl^{-1} IBA resulted in 80.16 % rooting (Table-3; Fig-3). Average 3.43 roots regenerated from each shoot on half strength MS medium. Our results are in contrast to Paudyal and Haq (2000) who found that NAA was superior to IBA for *in vitro* root induction in Pummelo when shoots were transferred into half strength MS medium.

Hardening and acclimatization:

Rooted plantlets obtained from previous experiments were removed from agar gelled medium and transplanted in different potting mixture (Table-4).Physical, chemical and biological properties of potting mixture are greatly influence on *in vitro* produced plantlets. It was observed that out of different potting mixture tried, cocopeat showed highest survival (90.23) with maximum plant height 6.46 cm (Fig-4). These results are in compliance with various

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workers who reported cocopeat to be the best potting mixture with high survival percentage in different tissue culture raised plants of various species (Bharati *et al*., 2018, Chabukswar and Deodhar, 2005, Manjusha and Sathyanarayana, 2008 and Prasad *et al*., 2018).This may be due to better aeration, water holding and nutrient supplying capacity of cocopeat as compared to sand (Prabhuling *et al.,* 2014). The plants were kept in the glasshouse with controlled environmental condition and then transferred to polybags.

Table1: Effect of sterilant on *in vitro* establishment of cv. Sai Sharbati explants.

Sterilants		Time (min)	Survival (%)	Bud sprouting (%)
$HgCl_2$ (%)	NaOCl (%)			
0.05	-	3	0.0 (0.0)	0.0 (0.0)
0.05	-	5	0.0 (0.0)	0.0 (0.0)
0.1	-	3	28.00 (31.93)	49.16 (44.50)
0.1	-	5	64.43 (53.36)	74.80 (59.84)
-	10	5	0.0 (0.0)	0.0 (0.0)
-	10	10	19.00 (25.82)	4.36 (12.04)
-	15	5	26.63 (31.05)	17.70 (24.86)
-	15	10	49.33 (44.60)	30.63 (33.59)
$CD_{0.05}$			1.08 (0.71)	1.08 (0.82)
SE±			0.35 (0.23)	0.35 (0.27)

Values in parenthesis are arc sine transformed values.
CD = Critical difference; SE = Standard error

Table2: Effect of plant growth regulators on *in vitro*shoot multiplication of cv. Sai Sharbati.

Medium Composition MS (Basal medium) + GR (mg/l)			Multiplication rate	Average shoot length (cm)	Average no. of leaves
BA	Kin	GA_3			
0.1	-	1.0	1: 1.5	3.13	4.20
0.5	-	1.0	1: 2.8	4.53	5.03
1.0	-	1.0	1: 2.4	3.63	4.33
1.5	-	1.0	1: 2.5	3.40	4.60
2.0	-	1.0	1: 1.8	3.50	4.46
-	0.1	1.0	1: 1.4	2.76	4.30
-	0.5	1.0	1: 1.5	3.30	4.16
-	1.0	1.0	1: 1.9	3.46	4.20
-	1.5	1.0	1: 1.3	3.63	4.13
-	2.0	1.0	1: 1.6	3.53	3.96
$CD_{0.05}$				0.59	0.46
SE±				0.20	0.15

Values in parenthesis are arc sine transformed values.
CD = Critical difference; SE = Standard error

Table 3:*In vitro* rooting of cv. Sai Sharbati**.**

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Strength	Concentration of growth regulators (mg/l)		Days taken for root initiation	Percent rooting	Root length (cm)	No. of roots per shoot
	NAA	IBA				
Full strength	0.5	-	17	10.23 (18.64)	1.03	1.30
	1.0	-	20	30.23 (33.34)	1.23	1.46
	1.5	-	18	40.36 (39.40)	1.76	1.80
	2.0	-	22	20.26 (26.74)	1.53	1.63
	-	0.5	19	40.43 (39.46)	2.16	1.73
	-	1.0	19	60.23 (50.88)	2.83	2.63
	-	1.5	21	50.56 (45.30)	2.46	2.43
	-	2.0	20	40.30 (39.39)	2.26	2.13
half strength	0.5	-	18	15.50 (23.17)	2.10	1.43
	1.0	-	20	20.16 (26.67)	2.56	1.63
	1.5	-	21	40.46 (39.48)	2.36	2.13
	2.0	-	20	40.40 (39.44)	2.06	1.80
	-	0.5	16	50.40 (45.21)	3.10	2.16
	-	1.0	13	80.16 (63.52)	3.83	3.43
	-	1.5	15	60.16 (50.84)	3.00	3.00
	-	2.0	17	40.26 (39.37)	2.83	2.60
$CD_{0.05}$				0.63 (0.40)	0.51	0.66
SE±				0.22 (0.14)	0.17	0.23

Values in parenthesis are arc sine transformed values.
CD = Critical difference; SE = Standard error

Table 4: Hardening of *in vitro* raised plantlets of cv. Sai Sharbati

Potting mixture	Survival percentage	Average no. of leaves	Average plant height (cm)
Cocopeat	90.23 (71.79)	6.43	6.46
Sand	10.20 (18.61)	4.30	4.46
Cocopeat : sand (1:1)	40.30 (39.38)	4.53	5.36
Cocopeat : perlite (1:1)	50.56 (45.30)	4.50	5.16
Cocopeat : red soil (1:1)	60.46 (51.02)	4.60	5.86
Cocopeat : red soil : FYM (1:1:1)	70.00 (56.76)	5.43	6.16
$CD_{0.05}$	1.15 (0.97)	0.53	0.70
SE±	0.36 (0.30)	0.16	0.22

Values in parenthesis are arc sine transformed values.
CD = Critical difference; SE = Standard error

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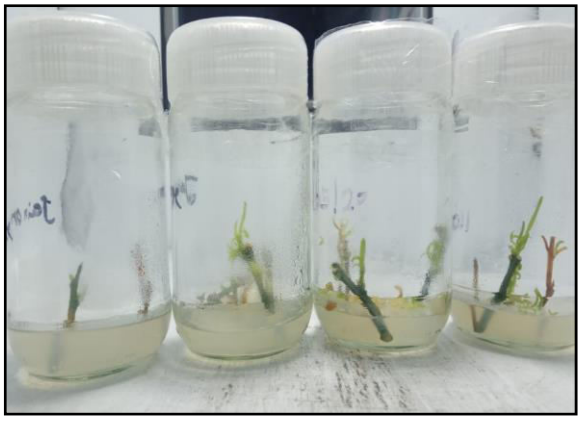

Fig 1: *In vitro* establishment of cv. Sai Sharbati explants.

Fig 2: *In vitro*shoot multiplication of cv. Sai Sharbati.

Fig 3: *In vitro*rooting of cv. Sai Sharbati.

Fig 4: Hardened plantlets of cv. Sai Sharbati in cocopeat

Fig 5: Hardened plantlets in big polybags.

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Conclusions:

In vitro propagation of cv. Sai Sharbati would ensure bulk production of true to type, disease free planting material throughout year. Out of different growth regulators tried BAP (0.5 mgl^{-1}) combination with GA_3 (1.0 mgl^{-1}) showed superior results with multiplication rate of 1:2.8. *In vitro* raised shoots were best rooted on half strength MS medium supplemented with IBA (1.0 mg/L). Hardening of *in vitro* plantlets was done in different potting mixtures, from which highest survival was observed on cocopeat.

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