The Pharma Innovation Journal 2020; 9(11): 18-21

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2020; 9(11): 18-21

www.thepharmajournal.com
Received: 16-09-2020
Accepted: 20-10-2020

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Plant bioregulators and its effect on growth and development of Kachai lemon (*Citrus jambhiri* Lush.) *in-vitro*

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Abstract
In the present study, micropropagation of Kachai lemon was attempted through direct regeneration from micro-cuttings of the *in-vitro* germinated seedlings followed by rhizogenesis and subsequently acclimatization. MS medium supplemented with Kinetin (0.5 mg/l) and GA_3 (1.0 mg/l) (T_{11}) sprouted lemon seeds earlier (9.87 days). Highest microshoot length (9.55 cm) was observed in treatment T_{12} (MS+ 1.0 mg/l Kn + 1.0 mg/l GA_3), while treatment T_{13} (MS+ 1.5 mg/l Kn + 1.0 mg/l GA_3) produced maximum number of microshoots per culture (5.93) at 90 DAI. MS + 0.5 mg/l Kn produced maximum number of leaves per microshoot (8.73). Earlier root initiation (10.07 days) was observed in MS basal medium containing 0.5 mg/l IBA + 1.0 mg/l NAA (T_9). Maximum root length (5.54 cm each) of Kachai lemon was observed in MS medium fortified with 0.5 mg/l IBA + 1.0 mg/l NAA (T_9) and 1.25 mg/l IBA + 1.0 mg/l NAA (T_{11}). Treatment T_2 (MS + 0.25 mg/l IBA) produced maximum number of roots per microshoot while the rooting percentage was recorded maximum in treatment T_{13} (MS+ 2.0 mg/l IBA + 1.0 mg/l NAA). Least performance was shown by control (T_1) in all the cases. Acclimatization of *in vitro* regenerated plantlets of Kachai lemon using vermiculite and vermicompost at equal proportion (1:1) showed earliest adaptation (H_3) with better survivability rate (92.89%) whereas, use of equal proportion of soil and vermicompost showed inferior result (H_5) with least plantlet survivability (57.39%).

Keywords: Kachai lemon, plant bioregulator, *in vitro*

Introduction
The genus Citrus belonging to the plant family Rutaceae, includes more than 150 species. Among the different Citrus species, *Citrus jambhiri* Lush. commonly known as 'rough lemon', is the most favourite rootstock for lemons, oranges, mandarins, grape fruits and kinnows because of its high vigour and well adaptation to warm, humid areas with deep sandy soils. (Vij and Kumar, 1990) [1]. Kachai lemon (*Citrus jambhiri* Lush.) locally known as *Champra*, indigenous to Kachai village of Ukhrul District, Manipur, India, is one of the largest horticultural commodities produced in the state which contains the highest quality of ascorbic acid in the realm of citrus fruits. It is a seedless lemon. It is grown in the sub-tropical climate with a thick fog or mist in the morning during the month of Dec-Jan. The important factor that makes the Kachai lemon unique is due to the fog or mist prevailing in that particular area. The mean annual temperature ranges from 19-21^{0}C and rainfall varies from 1,300 to 1,500 mm. This village produces 5737 MT lemons in 840 ha annually. The uniqueness of Kachai lemon has been accorded geographical indication (IG) registration (GI-446). It is the pride of Manipur as it has its distinctive identity at national and international platform. It contains 51% ascorbic acid while other citrus contain 20-30% of ascorbic acid that is found in India. The juice content is 36-56 ml per fruit. The flavor is different from other varieties. The Kachai lemon also have medicinal properties that is used in the treatment of various health problems like blood pressure, gastritis, dry scalp, fat reduction, and also used in making soaps. It has an antioxidant compounds and a high content of vitamins.
Kachai lemon is commercially propagated by air-layering but the rate of multiplication is slow. The limitations in propagation of citrus through nucellar seedlings are excessive thorniness and bears fruit very late. Plants raised through nucellar seedlings may require about 6-7 years before coming to bearing stage like that of sexual seedlings. Air layering is generally followed which is not satisfactory because of low rooting percentage and slow in growth. These limitations can be easily overcome by *in vitro* propagation which offers the advantage of rapid

multiplication of clones of elite genotypes in terms of time and space with no limitations of growth season.

In vitro propagation is also useful in seedless citrus which bear fruits but never produce seeds (Bowman, 1994) [2]. Non availability of seeds in some species and low percentage of polyembryony in other species necessitates the application of the micro propagation technique (Kitto and Young, 1981; Edriss and Burger, 1984; Moore, 1986) [3-5]. A many fold increase in the rate of multiplication over conventional method has been achieved very recently through *in vitro* techniques. A few scientists have tried to obtain differentiation of shoot-buds and plantlets from *in vitro* cultured explants of different citrus species (Altaf *et al*., 2008; Altaf *et al*. 2009; Laskar *et al*., 2009; Pe'rez-Tornero *et al*. 2010; Singh and Rajam, 2009; Sharma *et al*., 2019) [7-12].

A little work has been carried out on the *in-vitro* propagation of *C. jambhiri* using seeds (Khawale and Singh, 2005; Ali and Mirza, 2006; Savita *et al*. 2010) [13-15], nodal segments (Kour and Singh, 2012[16]), and *in vitro* grown root and stem segments (Ali and Mirza, 2006) [14] as explants. However, no works have been reported yet in the *in vitro* propagation of Kachai lemon. Hence, the following objectives were framed to develop an efficient and reproducible protocol for regeneration of *Citrus jambhiri* cv. Kachai lemon *in-vitro*:

1. To standardize the protocol for *in vitro* multiplication of Kachai lemon.
2. To find the best hardening medium for *ex vitro* acclimatization of plantlets.

Materials and Methods

The present investigation was conducted during the year 2017-2019 in the Department of Horticulture, PDDUIAS, Utlou. The plant materials required for the study were collected from the Kachai village, Ukhrul, Manipur. The experiment was conducted on newly emerging shoot tip explants obtained from the *in-vitro* germinated seeds. The explant used in micropropagation study was the shoot tips obtained from the axenic seedlings of 10-30 days. After removing the axenic seedlings from culture bottles, they were washed with sterile distilled water. The explants of optimum size were surface sterilized in 0.1 per cent $HgCl_2$ solution for 2-3 minutes for avoiding bacterial and fungal growth followed by 2-3 washings with sterile distilled water. The explants whether taken from *in vitro* grown seedling were cut aseptically in Laminar Air Flow cabinet to get explants of appropriate size and shape. The shoot tips of 0.5-1.0 cm having 2-3 leaf primordial, nodal segments of 1.0-1.5 cm with at least one axillary bud and then transferred to the aseptic conditions. Microshoots formed in the culture tubes, slant tubes and jam bottles were taken out after four weeks of inoculation. The shoots were separated by dissecting them in the sterile environment of laminar air flow cabinet with sterile dissecting needle and forceps. They were again placed in the respective tubes and jam bottles containing fresh media. The microshoots of more than 3.0-5.0 cm in height were taken out and placed in the tubes containing media with different concentrations of IBA and NAA for *in-vitro* rooting. Then, the plantlets were taken for acclimatization.

Results and Discussion

The data obtained from table 1 revealed that the least number of days (9.87) for shoot regeneration was observed in the treatment T_{11} (MS + Kn 0.5 + GA_3 1.0), while the highest microshoots length (9.55 cm) was found in the treatment T_{12} (MS + Kn 1.0 + GA_3 1.0), found statistically at par with the treatments T_7 (6.62 cm), T_8 (6.66 cm), T_9 (8.49 cm), T_{10} (8.76 cm), T_{11} (7.45 cm), T_{13} (8.26 cm). Amongst all the treatments, maximum number of microshoots (5.93) was found in the treatment T_{13} (MS + Kn 1.5 + GA_3), which was found statistically similar with the treatment T_8 (MS+0.5 Kn+0.5GA_3) (5.36). MS basal medium containing 0.5 mg/l Kn (T_2) observed highest number of leaves per plantlet (8.73) at 90 DAI. Control treatment devoid of any plant hormones (T_1) recorded the least results in all the cases. The higher *in-vitro* growth and development of microshoots in Kachai lemon occurred due to the growth promotive action of the plant growth regulators used in the culture media as well as absence of apical dominance. Both gibberellins and cytokinin are having active role in growth and development of meristematic tissues.

Data pertaining to table 2 showed that the minimum days (10.07 days) taken for root initiation was recorded in the treatment containing MS + 0.5 mg/l IBA+ 1.0 mg/l NAA (T_9) which was found statistically at par with the treatments T_3 (MS+0.5 mg/l IBA) (11.00 days), T_6 (1.5 mg/l IBA) (14.13 days), T_{10} (1.0 mg/l IBA + 1.0 mg/l NAA) (12.80 days), T_{11} (1.25 mg/l IBA+1.0 mg/l NAA) (13.40 days) and T_{12} (1.5 mg/l IBA+1.0 mg/l NAA) (14.13days). Amongst all the treatments, the maximum root length (5.54 cm) were recorded in MS+ 0.5 mg/l IBA+ 1.0 mg/l NAA (T_9) and (MS + IBA 1.25 mg/l + NAA 1.0 mg/l (T_{11}) (5.54 cm) found statistically at par with T_2 (5.14 cm), T_3 (4.39 cm), T_4 (4.55 cm), T_5 (5.15 cm), T_6 (5.18 cm), T_7 (5.44 cm), T_8 (4.54 cm), T_{10} (10.42 cm), T_{12} (5.38 cm) and T_{13} (4.91 cm) respectively. Meanwhile, maximum number of roots was found highest in the treatment T_2 (MS + IBA 0.25 mg/l) (7.22) which is statistically at par with the treatments T_3 (6.62), T_4 (5.87), T_8 (5.64), T_{10} (5.47). Among the different treatment combinations, T_{13} (MS + IBA 2.0 mg/l + NAA 1 mg/l) was found to have the highest rooting percentage (89.16%). In all the cases, control treatment recorded the least results in all the cases. The reason of IBA for producing better rooting parameters might be because of the involvement of IBA in ethylene biosynthesis. Hudge (1989) suggested that auxin induced ethylene which may result in inducing adventitious root formation instead of auxin itself.

Acclimatization of *in-vitro* regenerated plantlets *ex-vitro* is one of the most important criteria in tissueculture. The data in Table-3 depicts that amongst all the hardening medium tested, treatment H_3 [Vermiculite + Vermicompost, (1:1)] took the least number of days (11.33) for hardening, which is found statistically at par with the treatment H_2 [Garden soil + Sand + Vermicompost, (1:2:1)] (12.11 days). The same treatment even recorded the highest percentage of hardening (92.89). Meanwhile, treatment H_5 [Soil + Vermicompost (1:1)] takes the maximum number of days (19.70 days) to acclimatize *ex-vitro*, while the minimum hardening percentage (57.39) was recorded in the treatment H_5 [Soil + Vermicompost (1:1)]. The reason may be due to the availability of optimum moisture, aeration and nutrients to the plantlets for sufficient time period of the plantlets.

Table 1: Effect of plant bio-regulators on vegetative growth of Kachai lemon

Treatment details	Shoot regeneration (days)	Length of microshoots (cm)	Number of microshoots per culture	Number of leaves (days)
T_1 (Control)	25.40	5.67	2.78	4.53
T_2 (0.5 mg/l Kn)	14.13	5.61	3.83	8.73
T_3 (1.0 mg/l Kn)	11.40	6.07	4.24	8.13
T_4 (1.5 mg/l Kn)	13.93	5.75	3.62	7.27
T_5 (0.5 mg/l GA_3)	11.33	5.76	4.44	6.10
T_6 (1.0 mg/l GA_3)	13.40	5.96	4.12	7.27
T_7 (1.5 mg/l GA_3)	12.47	6.62	4.44	5.87
T_8 (0.5 mg/l Kn + 0.5 mg/l GA_3)	14.08	6.66	5.36	6.90
T_9 (1.0 mg/l Kn + 0.5 mg/l GA_3)	13.47	8.49	3.42	6.40
T_{10} (1.5 mg/l Kn + 0.5 mg/l GA_3)	12.80	8.76	4.09	7.67
T_{11} (0.5 mg/l Kn +1.5 mg/l GA_3)	9.87	7.45	4.13	7.00
T_{12} (1.0 mg/l Kn + 1.5 mg/l GA_3)	14.00	9.55	5.19	7.07
T_{13} (1.5 mg/l Kn + 1.5 mg/l GA_3)	13.47	8.26	5.93	7.40
S.Ed (±)	3.09	1.53	0.63	1.53
C.D. (0.05%)	6.35	3.03	1.30	3.02

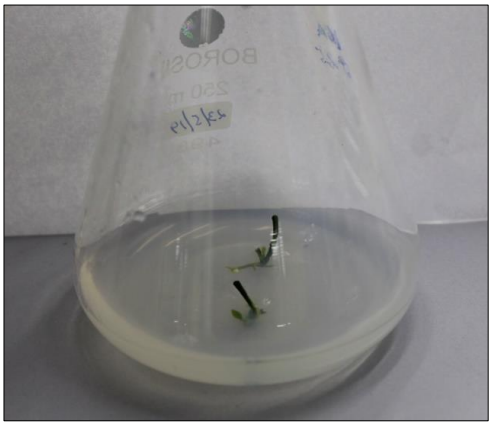
Table 2: Effect of plant bio-regulators on root development of Kachai lemon

Treatment details	Number of days for root initiation	Root length(cm)	Number of roots per microshoot	Rooting Percentage
T_1 (Control)	23.33	2.57	1.88	42.87
T_2 (0.25 mg/l IBA)	15.93	5.14	7.22	70.63
T_3 (0.5 mg/l IBA)	11.00	4.39	6.62	75.38
T_4 (1.0 mg/l IBA)	16.20	4.55	5.87	76.56
T_5 (1.25 mg/l IBA)	14.53	5.15	5.13	79.30
T_6 (1.5 mg/l IBA)	14.13	5.18	5.09	77.40
T_7 (2.0 mg/l IBA)	18.60	5.44	5.07	74.73
T_8 (0.25 mg/l IBA+1.0 mg/l NAA)	13.27	4.54	5.64	72.79
T_9 (0.50 mg/l IBA+1.0 mg/l NAA)	10.07	5.54	5.27	67.42
T_{10} (1.0 mg/l IBA + 1.0 mg/l NAA)	1 a) *In-vitro* seed germination 2.80	5.42	5.47	78.45
T_{11} (1.25 mg/l IBA + 1.0 mg/l NAA)	13.40	5.54	4.96	74.26
T_{12} (1.5 mg/l IBA+1.0 mg/l NAA)	14.13	5.38	4.88	76.97
T_{13} (2.0 mg/l IBA+1.0 mg/l NAA)	14.80	4.91	4.89	89.16
S.Ed (±)	2.11	0.68	1.09	3.09
C.D. (0.05%)	4.35	1.39	2.25	6.35

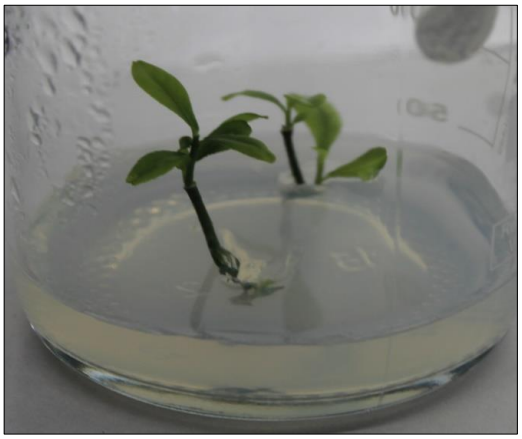
Table 3: Effect of different hardening media on acclimatization of plantlets *ex-vitro*

Treatment details	Days required to hardening	Percentage of hardening (%)
H_1 [Vermiculite + sand (1:1)]	14.86	70.19
H_2 [Garden soil + sand + vermicompost (1:1:1)]	12.11	89.95
H_3 [Vermiculite + Vermicompost (1:1)]	11.33	92.89
H_4 [Garden soil + sand (1:1)]	13.82	78.49
H_5 [Soil + Vermicompost (1:1)]	19.70	57.39
S.Ed (±)	1.09	1.09
C.D. (0.05%)	2.25	2.25

a) *In-vitro* seed germination



b) Shoot regeneration from micro-cutting



c) *In-vitro* shoot multiplication

d) *In-vitro* Rooted microshoots

e) Rooted microshoot ready for hardening

f) Hardened plantlets

Plate 1: Phases of micropropagation of Kachai lemon

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