Micro propagation of *Annona muricata* using different concentration of 6-Benzylaminopurine (BAP)

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Abstract- A commercially feasible technique of micro propagation requires the successful appearance of healthy shoots and an improved multiplication rate, which heavily depends on the make-up of the media employed as well as the kind of explant used, reaction, growth of new shoots. In the current study, the impact of BAP on *invitro* propagation of nodal and leaves explants of *Annona muricata* was seen. Among the different concentration of BAP T_5 (0.75 mg/L), T_6 (1.0 mg/L) and T_7 (1.50 mg/L) treatments respectively were selected as it showed higher response with minimum days for shoot initiation, higher shoot induction per cent, maximum number of shoots and higher length compared with other treatments for green nodal explants and same treatments were selected for leaves as it possesses least contamination, good health better survival of explants.

Key words: Micro propagation, Annona muricata, 6-Benzylaminopurine

INTRODUCTION

The Annonaceae family, which has over 130 genera and 2300 species, the genus *Annona* is the most economically important containing 120 species. Out of five edible species, four have originated in South or Meso-America and one is from Eastern Africa. *Annona cherimola* Mill (cherimoya), *Annona muricata* L. (soursop), *A. reticulata* (custard apple) and *A. squamosa* L. (sugar apple) included in first group and *A. senegalensis* Pers. (wild soursop) is from Africa (Pinto *et al.*, 2010). It was once found in the warmest tropical regions of South and North America (Moghadamtousi *et al.*, 2015). As it becomes more well known, the exotic commodity *A. muricata* is currently making its way into commercial markets (Sanusi and Bakar 2018). Different portions of *A. muricata* are used to cure disorders like diabetes, coughs, skin issues and cancer in developing tropical nations like Africa. Moreover, it has been described as uterine stimulant, cardiac depressant, antiamoebic, antibacterial, hypertensive, spasmogenic, vasodilator, smooth muscle relaxant, anti-inflammatory and anti-protozoan (Rady *et al.*, 2018). Micropropagation and plant improvement programmes require plant regeneration from tissue culture of any crop species. There are numerous issues in getting the desired reaction from any explants. The requirements of plant growth regulators for successful plantlet regeneration are as diverse as explant types. Although any explant, cell, or protoplast can be employed for *in vitro* propagation, the apical or auxiliary growth sites are the most often used explants. Even though, a varieties of tropical tree species have been the subject of extensive tissue culture research, the Annonaceae have got little attention and showed uneven results (Bejoy and Hariharan 1992). Numerous research targeting micro propagation that have been carried out using meristems, buds, or nodal segments (Telles and Biasi 2005; Costa and Aloufa 2006).

MATERIAL AND METHODS

The present research work was done on standardization of PGRs for shoot regeneration in *in vitro* micro propagation of Soursop (*Annona muricata*

L.)." which was carried out at Tissue culture laboratory, College of Horticulture, Sirsi (University of Horticultural Sciences, Bagalkot), Uttar Kannada district, Karnataka during the year 2020-21 and 2021-22.

Seedlings of *Annona muricata* were brought from the nursery from Shivamogga and the mother plants were maintained in nursery of Department of Fruit Science in COH, Sirsi. Seedlings were maintained in polybag consisting of soil, vermicompost and FYM at 1: 1: 1 (v/v) ratio. Pots were drenched with Carbendazim (2g/ L) and plants were sprayed with Streptomycin (0.5 g/L) along with Cetrimide (0.5g/L) at weekly intervals to reduce the pathogen load. Plant growth was maintained by weekly spray of macro nutrients (19:19:19) and regular watering is done. After the establishment of the plants, the healthy, new sprouts were collected and used as an explant source. *Annona muricata* cuttings which possess more than one bud have more chances to encourage new *in vitro* shoots (Bridg 2000).

Surface sterilization and preparation of explant (green wood and leaves)

Nodal explants and leaves from the seedlings of *Annona muricata* grown in a greenhouse were sterilized by the following procedure.

1. To lower the bacterial and fungal load, the mother plant was frequently sprayed with Carbendazim (0.2%) and cetrimide (0.5 g/L) at weekly intervals and streptomycin sulphate (0.5 g/L) at 10 days interval.
2. Using a scissors that had been swabbed with cotton dipped in 70 per cent ethanol, the healthy and freshly sprouted stem was removed from the mother plant that was grown in a polybag under greenhouse conditions.
3. Leaves of the stem were removed to their half and then nodal explants were prepared.
4. Stems measuring 10 cm were washed for 10 minutes under running tap water, then soaked for three hours in solution containing Carbendazim (0.2%), cetrimide (0.5g/ L) and two drops of Tween 20.
5. Initial sterilization with Sodium hypochlorite or mercuric chloride has been taken up and then washed with distil water for four times. Further sterilization was carried out under sterile laminar hood, as per the treatments. Explants were subjected to sterilization treatments in a closed container and repeatedly washed with sterile water inside the laminar air flow chamber for five times.
6. Treated nodal explants were cut with sterile blades to remove any dead tissues and were fragmented into 3-4 cm length containing two nodes. Leaves were fragmented then both the explants were placed over the MS media containing growth regulators.

During the study, Murashige and Skoog's (MS) media with a combination of supplements were employed as basal medium. By adjusting the volume to the required amount, stock solutions (MS-A, MS-B, MS-C, and MS-D) of known concentration were prepared. Growth regulator stock solutions of BAP with known concentrations were made by dissolving the necessary amount of the chemicals in a few drops of NaOH (1N) then volume was made up by distilled water and stored in refrigerator at 4-5 ℃. The pH of the media was adjusted to 5.71 to 5.75 by adding of HCl/NaOH (0.1 N) dropwise and distill water added to make up the final volume. Agar (6.25 g) was added to the medium solution and heated to around 50–55 ℃ to prevent the formation of agar clumps. The media solution (30-35 ml) was dispensed into culture bottle (250 g capacity) and it was tightened with cap, sterilized and stored in growth room. Sterile explants were aseptically added to sterilised culture bottles containing MS media and various concentrations of plant growth regulators.

Treatment details

First experiment was done with different concentrations of 6-Benzylaminopurine (BAP) on shoot multiplication. The experimental design used was CRD with 11 treatments, three replications and 10 explants per replication and following treatments were used.

Treatment No.	Treatment
T_1	Control
T_2	0.1mg/l BAP
T_3	0.25mg/l BAP
T_4	0.5mg/l BAP
T_5	0.75mg/l BAP
T_6	1.0mg/l BAP

T_7	1.5mg/l BAP
T_8	2.0mg/l BAP
T_9	2.5mg/l BAP
T_{10}	3.0mg/l BAP
T_{11}	3.5mg/l BAP

Note: The best tree treatments were selected for carrying out further experiments.

Statistical analysis

As all the studies were conducted in the laboratory under well-defined condition of medium of growth, temperature and light, complete randomized design (CRD) was employed for analysis of the experiments. Critical difference (C.D.) values given in the table were at one per cent level of significance, where the F test was significant and used to compute the means. Values in percentages were subjected to arc sine transformation to ensure homogenity. Wherever values were 0 per cent or 100 per cent, arcsin (1/4n) and arcsin (100-1/4n), where n is the number of observations that make up the percentage, were substituted respectively (Panse and Sukhatme 1967). The significance of differences among means was carried out using Duncan's multiple-range test at P= 0.01.

Results

Initiation of response and days taken for shoot induction

In the present experiment, there was no response in leaf explants, significantly the highest initiation of response in green nodal explants was noted in T_7 (95.00%) which was on par with T_6 (90.00%) both after 15 and 30 days of initiation. The least response was noted in T_1 (36.37% after 15 days and 53.33% after 30 days). Early shoot emergence in green nodal explants was noted in T_6 (15.00), T_7 (15.00) and T_5 (15.33) and late shoot emergence was recorded in T_1 (21.67) (Table 1). (Plate 1)

Contamination (%)

Significantly the highest bacterial contamination in green nodal explants was noted in T_9 (50.00%) followed by T_3 (40.00%) and the lowest was recorded in T_{10} (0) followed by T_6 and T_{11} (20%) after 30 days of initiation. The highest fungal contamination was recorded in T_4, T_5 of 50 per cent followed by T_9 and T_{10} of 40 per cent and rest shows zero fungal contamination after 30 days (Table 2).

No bacterial contamination in leaf explants was noted in 15 days of initiation, but found in T_2 (50.00%), T_8, (30.00%), T_{10} (30.00%) and in T_7 (20.00%) after 30 days. There were significant results for fungal contamination and the highest was recorded in T_4 and T_{11} (50%) followed by T_9 (40%), T_3 (30.00%) and rest shows zero fungal contamination after 15 and 30 days of initiation (Table 4).

Health (+, ++, +++)

The healthy green nodal explants were noted in T_2 to T_8 (+++) and moderate healthy in T_1, T_9 and T_{10} (++) (Table 3). Moderate health was noted in T_4 to T_7 (++) and least health was noted in T_1 to T_3, T_8 to T_{10} (+) in leaf explants (Table 4).

Shoot induction (%)

Data was significant in 1st 15 days for shoot induction in green nodal explants however, the highest was recorded in T_7 (50.00%) followed by T_6 (40.00%). Later, at 30 days significant results were obtained in shoot induction and the highest was recorded in T_7 (96.67%) which was on par with T_6 (93.33%) and no shoot induction was noted in control (T_1) (Table 4).

Number of shoot /explants and Shoot length (cm)

Significantly the highest shoots per explant in green nodal explants after 15 days of initiation was recorded in T_5 (1.0), T_7 (1.0) and T_6 (1.0) and no shoot induction in rest of the treatments. Significantly the highest number of shoots /explants was recorded in T_7 (1.67) followed by T_6 (1.58) and T_5 (1.50) and no shoots were noted in control (T_1) after 30 days (Table 5).

Shoot length in green nodal explants had significant results among the treatments. The highest was recorded in T_7 (1.33 cm) followed by T_6 (1.00 cm) and T_5 (0.92 cm) after 15 days of initiation. After 30 days of initiation, the maximum shoot length was recorded in T_7 (2.08 cm) which is followed by T_6 (1.83 cm) and T_5 (1.67 cm) and no shoots were found in control (T_1) (Table 5).

DISCUSSION

In the experiment, there was less response in control as the explants swelled and were necrotic after four weeks in the hormone-free medium

(Shah *et al.,* 2008). On the MS medium without PGRs, there was no evidence of either growth or differentiation. After one week of incubation, the addition of several PGRs to the MS medium accelerated the induction of shoot buds (Naaz *et al.,* 2019). The shoot multiplication rate nearly quadrupled when both cytokinins were present (shah *et al.,* 2008). BAP has a higher physiological ability than Kinetin to end apical dominance and encourages shoot growth at low doses (Wolella 2017). Axillary node of juvenile plants of soursop in MS with BAP (2.0 mg/L) recorded reactivity rate of 91.67 per cent (Ba *et al.,* 2021).

Guava nodal segments took five days for shoot bud induction by Mishra *et al.* (2005) in MS media with BAP (3.0 mg/L). *Annona squamosa* nodal explants show shoot induction within 4 to 6 days in BAP (1.00 mg/L) found by Farooq *et al.* (2002). Amin *et al.* (2002) found nodal segments of *Annona squamosa* took 15 to 18 days to sprout from the axillary buds in BA (1.5 mg/L).

The present experiment results are in line with Lemons and Blake (1996) who found *Annona squamosa* nodal explants in WPM medium with BA (35 µM) had buds producing shoots of 87.9 per cent. Three different clones of *Annona muricata* shoot segments on WPM medium with BAP (0.2 mg/L) showed the maximum axillary bud induction (Alzate *et al.,* 2002). Guava shoot tips in MS with BAP (1.0 mg/L) and glutamin (500 mg/L) recorded 72 per cent shoot development (Zamir *et al.* 2007). According to Venkatachalam *et al.* (2007) the number of shoots grew as the concentration of BAP increased up to 22.2 M and decrease at higher concentrations of BAP (33 - 44.4 M). In pitaya, medium with low concentrations of BAP (0–2 µM) encouraged the growth of healthy shoots free of apical necrosis and encouraged the production of several shoots per areole (Vinas *et al.,* 2012). According to Lemos and Blake (1996) high cytokinin concentrations typically encourage the development of plenty of cuttings. Therefore, it has been demonstrated that the use of BAP (2 and 4 mg/L) considerably increases the quantity of cuttings produced from *A. muricata* explants. MS medium with BAP (2.5 mg/L) had the maximum number of shoots (6-8) from nodal segments of custard apple which was found by Krishna *et al.* (2011).

The most well-known banana shoot tip culture technique was created by adding benzyl aminopurine (BAP) to basal media, which is generally known to diminish apical dominance and encourage both axillary and adventitious shoot growth from meristematic explants (Jafari *et al.,* 2011). Ba *et al.* (2022) found Axillary, cotyledonary and apical nodes of *Annona senegalensis* in MS medium with BAP (2.0 mg/L) found an average shoot length of 5.01 cm, 4.7 cm and 6.57 cm respectively. MS medium with BAP (0.5 mg/L) recorded 1.5 shoots per nodal segment and 2.12 cm average length of the shoot in *Annona glabra* nodal segments (Deccetti *et al.,* 2005).

CONCLUSION

From the above experiment T_5 (0.75 mg/L), T_6 (1.0 mg/L) and T_7 (1.50 mg/L) were selected for further experiment as it showed higher response with minimum days for shoot initiation, higher shoot induction per cent, the maximum number of shoots and higher length compared with other treatments for green nodal explants and same treatments were selected for leaf as it possesses least contamination, good health better survival of explants.

Table 1: Initiation of response and days taken for shoot induction of *Annona muricata* green nodal explant cultivated *in vitro* in MS medium supplemented with different concentration of BAP

Treatment	BAP (mg/L)	Initiation of response (%)		Days taken for shoot induction
		15 Days	30 Days	
T_1	0	36.67 (37.26)^{e}	53.33 (46.91)^{f}	21.67 (4.76)^{a}
T_2	0.1	53.33 (46.91)^{d}	70.00 (56.79)^{de}	19.00 (4.47)^{ab}
T_3	0.25	70.00 (56.83)^{c}	80.00 (63.43)^{cd}	18.67 (4.43)^{ab}
T_4	0.50	70.00 (56.83)^{c}	80.00 (63.43)^{cd}	18.00 (4.36)^{b}
T_5	0.75	85.00 (67.21)^{b}	85.00 (67.21)^{bc}	15.33 (4.04)^{c}

T_6	1.00	90.00 (71.57)^{ab}	90.00 (71.57)^{ab}	15.00 (4.00)^{c}
T_7	1.50	95.00 (77.08)^{a}	95.00 (77.08)^{a}	15.00 (4.00)^{c}
T_8	2.00	70.00 (56.83)^{c}	80.00 (63.43)^{cd}	18.67 (4.43)^{ab}
T_9	2.50	70.00 (56.83)^{c}	75.00 (60.07)^{cde}	20.00 (4.58)^{ab}
T_{10}	3.00	53.33 (47.00)^{d}	73.33 (58.97)^{cde}	20.67 (4.65)^{ab}
T_{11}	3.50	50.00 (45.03)^{d}	63.33 (53.18)^{ef}	21.00 (4.67)^{ab}
S.Em±		1.67	1.85	0.08
LSD at 0.01		6.72	7.46	0.32
CV (%)		5.13	5.18	3.09

Figures above paranthesis indicate the actual values and figures in paranthesis are arc sine transformed values for initiation of response and square root transformed values for days taken for shoot induction. Values with the same letter are satistically non-significant at LSD *($p \leq 0.01$).*

Table 2: Bacterial and fungal contamination of *Annona muricata* green nodal explant cultivated *in vitro* in MS medium supplemented with different concentration of BAP

Treatment	BAP (mg/L)	Bacterial contamination (%)		Fungal contamination (%)	
		15 Days	30 Days	15 Days	30 Days
T_1	0	25.00 (30.00)^{a}	25.00 (29.99)^{d}	0.00 (0.29)^{a}	0.00 (0.29)^{c}
T_2	0.1	0.00 (0.29)^{c}	30.00 (33.21)^{c}	0.00 (0.29)^{d}	00.00 (0.29)^{c}
T_3	0.25	0.00 (0.29)^{c}	40.00 (39.23)^{b}	0.00 (0.29)^{d}	0.00 (0.29)^{c}
T_4	0.50	0.00 (0.29)^{c}	0.00 (0.29)^{f}	40.00 (39.23)^{b}	50.00 (45.00)^{a}
T_5	0.75	0.00 (0.29)^{c}	0.00 (0.29)^{f}	30.00 (33.2)^{c}	50.00 (45.00)^{a}
T_6	1.00	0.00 (0.29)^{c}	20.00 (26.56)^{e}	0.00 (0.29)^{d}	0.00 (0.29)^{c}
T_7	1.50	20.00 (26.56)^{b}	30.00 (33.21)^{c}	0.00 (0.29)^{d}	0.00 (0.29)^{c}
T_8	2.00	0.00 (0.29)^{c}	20.00 (26.56)^{e}	0.00 (0.29)^{d}	0.00 (0.29)^{c}
T_9	2.50	0.00 (0.28)^{c}	50.00 (45.00)^{a}	30.00 (33.20)^{c}	40.00 (39.23)^{b}
T_{10}	3.00	0.00 (0.28)^{c}	0.00 (0.29)^{f}	30.00 (33.20)^{c}	40.00 (39.23)^{b}
T_{11}	3.50	0.00 (0.28)^{c}	20.00 (26.53)^{e}	0.00 (0.28)^{d}	0.00 (0.28)^{c}
S.Em±		0.09	0.66	0.36	0.48
LSD at 0.01		0.37	2.64	1.47	1.94
CV (%)		2.93	4.78	3.02	5.38

Figures above paranthesis indicate the actual values and figures in paranthesis are arc sine transformed values. Values with the same letter are satistically non-significant at LSD *($p \leq 0.01$).*

Table 3: Health and shoot induction of *Annona muricata* green nodal explant cultivated *in vitro* in MS medium supplemented with different concentration of BAP

Treatment	BAP (mg/L)	Health		Shoot induction (%)	
		15 Days	30 Days	15 Days	30 Days
T_1	0	+++	+++	0.00 (0.29)^{e}	0.00 (0.29)^{f}
T_2	0.1	+++	+++	0.00 (0.29)^{e}	36.67 (37.26)^{e}
T_3	0.25	+++	+++	0.00 (0.29)^{e}	53.33 (46.91)^{cd}
T_4	0.50	+++	+++	30.00 (33.21)^{c}	63.33 (52.75)^{c}

T_5	0.75	+++	+++	30.00 (33.21)^{c}	90.00 (72.15)^{b}
T_6	1.00	+++	+++	40.00 (39.23)^{b}	93.33 (77.08)^{ab}
T_7	1.50	+++	+++	50.00 (45.00)^{a}	96.67 (80.33)^{a}
T_8	2.00	+++	+++	20.00 (26.56)^{d}	40.00 (39.23)^{de}
T_9	2.50	+++	+++	0.00 (0.28)^{e}	36.67 (37.09)^{e}
T_{10}	3.00	+++	+++	0.00 (0.28)^{e}	33.33 (35.06)^{e}
T_{11}	3.50	+++	++	0.00 (0.28)^{e}	26.67 (30.86)^{e}
S.Em±				0.40	1.93
LSD at 0.01				1.62	7.77
CV (%)				4.29	7.23

Figures above paranthesis indicate the actual values and figures in paranthesis are arc sine transformed values for explant show shoot induction. Values with the same letter are satistically non-significant at LSD *($p \leq 0.01$).*

Table 4: Health, bacterial and fungal contamination of *Annona muricata* leaf explant cultivated *in vitro* in MS medium supplemented with different concentration of BAP

Treatment		Health		Bacterial Contamination (%)		Fungal Contamination (%)	
	BAP (mg/L)	15 days	30 days	15 days	30 days	15 days	30 days
T_1	0	++	+	0.00 (1.00)	0.00 (0.29)^{d}	0.00 (0.29)^{d}	0.00 (0.29)^{d}
T_2	0.1	+++	+	0.00 (1.00)	50.00 (45.00)^{a}	0.00 (0.29)^{d}	0.00 (0.29)^{d}
T_3	0.25	+++	+	0.00 (1.00)	0.00 (0.29)^{d}	30.00 (33.20)^{c}	30.00 (33.20)^{c}
T_4	0.50	+++	++	0.00 (1.00)	0.00 (0.29)^{d}	50.00 (45.00)^{a}	50.00 (45.00)^{a}
T_5	0.75	+++	++	0.00 (1.00)	0.00 (0.29)^{d}	0.00 (0.29)^{d}	0.00 (0.29)^{d}
T_6	1.00	+++	++	0.00 (1.00)	0.00 (0.29)^{d}	0.00 (0.29)^{d}	0.00 (0.29)^{d}
T_7	1.50	+++	++	0.00 (1.00)	20.00 (26.56)^{c}	0.00 (0.29)^{d}	0.00 (0.29)^{d}
T_8	2.00	+++	+	0.00 (1.00)	30.00 (33.20)^{b}	0.00 (0.29)^{d}	0.00 (0.29)^{d}
T_9	2.50	+++	+	0.00 (1.00)	0.00 (0.28)^{d}	40.00 (39.23)^{b}	40.00 (39.23)^{b}
T_{10}	3.00	++	+	0.00 (1.00)	30.00 (33.21)^{b}	0.00 (0.29)^{d}	0.00 (0.29)^{d}
T_{11}	3.50	++	+	0.00 (1.00)	0.00 (0.28)^{d}	50.00 (45.00)^{a}	50.00 (45.00)^{a}
S.Em±				0.00	0.35	0.62	0.41
LSD at 0.01				NS	1.42	2.51	1.64
CV (%)				0.01	4.81	7.23	4.72

Figures above paranthesis indicate the actual values and figures in paranthesis are and arc sine transformed values for contamination. Values with the same letter are satistically non-significant at LSD *($p \leq 0.01$).*

Table 5: Number of shoots per explant and shoot length of *Annona muricata* green nodal explant cultivated *in vitro* in MS medium supplemented with different concentration of BAP

Treatment	BAP (mg/L)	Number of shoots per explant		Shoot length (cm)	
		15 Days	30 Days	15 Days	30 Days
T_1	0	0.00 (1.00)^{c}	0.00 (1.00)^{d}	0.00 (1.00)^{f}	0.00 (1.00)^{g}
T_2	0.1	0.00 (1.00)^{c}	0.67 (1.29)^{c}	0.00 (1.00)^{e}	0.50 (1.22)^{f}
T_3	0.25	0.00 (1.00)^{c}	0.67 (1.29)^{c}	0.00 (1.00)^{e}	0.83 (1.35)^{de}

T_4	0.50	0.33 (1.15)^{b}	1.08 (1.44)^{b}	0.17 (1.08)^{d}	0.92 (1.38)^{cd}
T_5	0.75	1.00 (1.41)^{a}	1.50 (1.58)^{a}	0.92 (1.38)^{b}	1.67 (1.63)^{b}
T_6	1.00	1.00 (1.41)^{a}	1.58 (1.61)^{a}	1.00 (1.41)^{b}	1.83 (1.68)^{b}
T_7	1.50	1.00 (1.41)^{a}	1.67 (1.63)^{a}	1.33 (1.53)^{a}	2.08 (1.76)^{a}
T_8	2.00	0.33 (1.15)^{b}	1.00 (1.41)^{b}	0.67 (1.29)^{c}	1.00 (1.41)^{c}
T_9	2.50	0.00 (1.00)^{c}	0.77 (1.33)^{c}	0.00 (1.00)^{d}	0.83 (1.35)^{de}
T_{10}	3.00	0.00 (1.00)^{c}	0.67 (1.29)^{c}	0.00 (1.00)^{e}	0.83 (1.35)^{de}
T_{11}	3.50	0.00 (1.00)^{c}	1.00 (1.41)^{b}	0.00 (1.00)^{e}	0.67 (1.29)^{e}
S.Em±		0.01	0.01	0.01	0.01
LSD at 0.01		0.02	0.05	0.03	0.06
CV (%)		0.92	1.54	0.94	1.78

Figures above paranthesis indicate the actual values and figures in paranthesis are square root transformed values. Values with the same letter are satistically non-significant at LSD *($p \leq 0.01$)*.

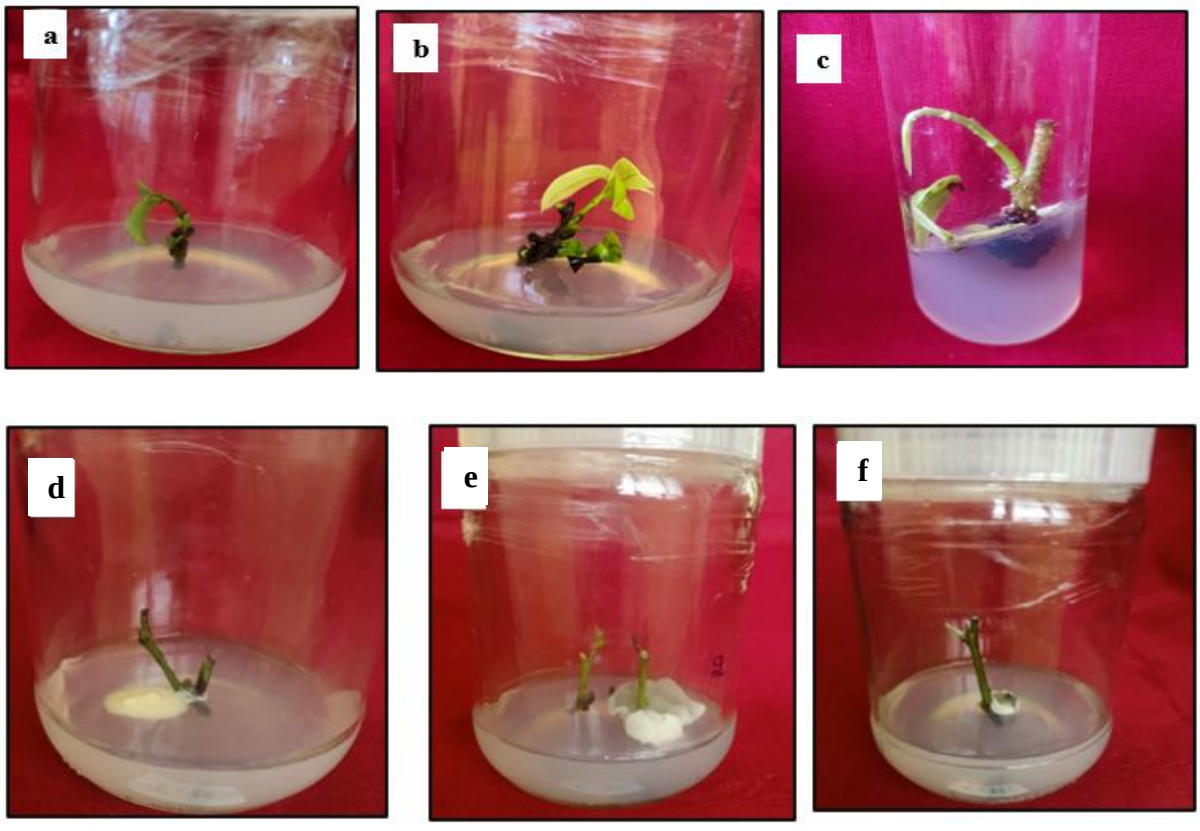
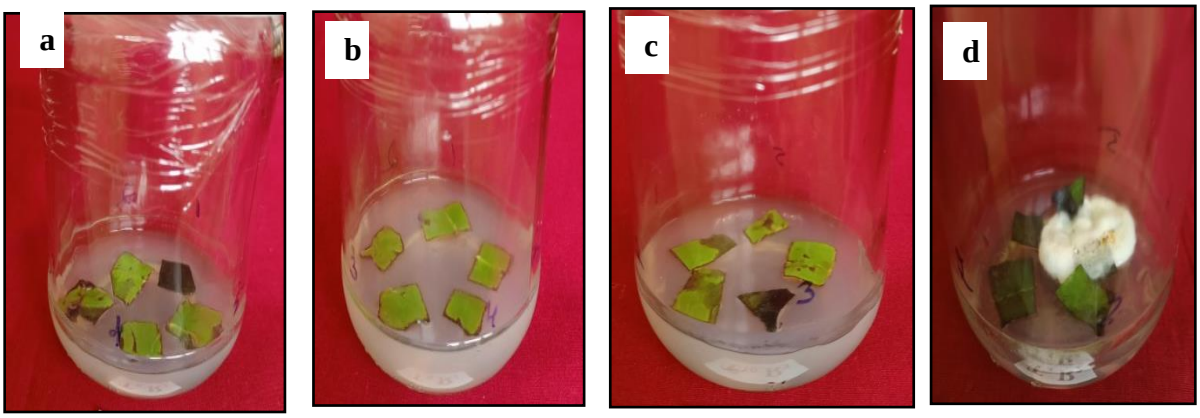


Plate 2: Effect of different concentrations of BAP (6-Benzylaminopurine) on shoot multiplication of *Annona muricata* in leaves explants; (a), (b), (c) - T_5 (0.75 mg/L), T_6 (1.0 mg/L) and T_7 (1.50 mg/L) respectively, fungal contamination in (d) – T_1 (control) after 30 days of initiation



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