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Establishment of axenic cultures in Pomegranate cultivars Bhagwa and Super Bhagwa

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Abstract
Success of a commercially viable protocol for mass multiplication of plants using tissue culture begins with effective elimination of microbial contamination by pre-treatment and surface sterilization methods. In the present study, nodal explants of pomegranate cv. Bhagwa and Super Bhagwa were established using different durations and concentrations of anti-microbial agents followed by inoculation in the culture media for initiation. Among different treatments tested, it was observed that in both the cultivars explants pre-treated with Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200ppm for 1 h followed by surface sterilization in laminar airflow chamber with Mercuric chloride ($HgCl_2$)@ 0.1% for 6 min. was proved to be effective method in culture establishment, with maximum survival percentage (57.13% and 67.41% respectively). Among the cultivars Bhagwa showed the maximum survival percentage (33.67% and 36.43%, respectively) when treated with Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200ppm for 1 h followed by surface sterilization with Mercuric chloride ($HgCl_2$) @ 0.1% for 6 min with minimum mortality of the explants.

Keywords: Pre-treatment, Surface sterilization, Bhagwa, Super Bhagwa

Introduction
Pomegranate (*Punica granatum* L.) is an economically important fruit crop of the tropical and subtropical regions of the world. The name Pomegranate is derived from two Latin words 'pomum' meaning 'apple' and 'granatus' meaning 'full of seeds'. It belongs to the family Punicaceae. It comprises only one genus (*Punica*) and two species *viz*., *P. granatum* (2n=16) and *P. protopunica* (2n=18). It is native of Iran and is spread throughout the Mediterranean regions of Asia, Africa and Europe (Kumar *et al.*, 2017) [8]. Pomegranate is in great demand for both domestic and export market. It is commercially cultivated for consumption of fresh fruits which is highly nutritive and is rich in proteins, fats, fiber, carbohydrates, minerals like Fe, Ca, and antioxidant component like phenol, pigments and tannins (Kumar and Neeraj, 2018) [9]. Apart from its demand for fresh fruits and juice, the processed products like pomegranate wine, pomegranate tea and candy are also gaining more importance in the world trade (Hmid *et al*., 2017) [5]. Different part of the plant like, bark, leaves, fruit, fruit extract or juice and fruit rind have been reported to show various medicinal activities *viz*., antimicrobial, anti-cancer, cardio-protective, and anti-inflammatory activity. Also, the plant could be used for the treatment of diabetes mellitus and obesity, and can also improve sperm quality (Lepionka *et al*., 2019; Mohamad Sukri *et al*., 2019) [10, 13]. Pomegranate has the ability to withstand harsh and adverse climatic conditions. It's versatile adaptability, hardy nature, low maintenance cost, steady but high yields, better keeping quality, fine table and therapeutic values has led to a steady increase in area and production of pomegranate.

Although the conventional methods of vegetative propagation has reached commercial acceptability, there are several limitations like low multiplication rate, lack of large quantity of required quality planting material, easily prone to pest and diseases results in non- availability of plantlets through-out the year. Therefore, for desire of better alternative, tissue culture technique has been exploited in pomegranate for mass multiplication of elite, healthy, robust plantlets in a short time and year round production. Apart from this, in recent years, bacterial blight has become the one of the most devastating disease in pomegranate. It affects the fruit yield around the range of 60 to 70 per cent. The success of commercial multiplication by using *in vitro* techniques are generally depends on four main stages. Among them, establishment of an aseptic culture is the first and fore most important one.

Hence, the present work is aimed to study the effect of different pre-treatment and surface sterilization methods on culture establishment in pomegranate cv. Bhagwa and Super Bhagwa.

Materials and Methods

The present investigation, was undertaken in the Department of Fruit Science, College of Horticulture, during the year 2018-2022 at Tissue Culture Laboratory, Horticultural Research Station, Kovvur, Dr. YSRHU, West Godavari District of Andhra Pradesh.

For *in vitro* propagation, apparently healthy mother plants were selected for excising the explants. Pomegranate cultivars of Bhagwa and Super Bhagwa were sourced from NRC Pomegranate, Sholapur and established mother gardens at Horticulture Research Station, Kovvur. Nodal explants were collected from the 1-2 year old mother plants. Actively growing vigorous shoots were selected separated from mother plants with the help of secateurs. All the leaves were removed using a sharp scissor and size of the explants was reduced to 2–3 cm. Excised nodal segments were thoroughly washed under running water for 10-15 minutes. Later on, the explants were transferred to glass bottle having double distilled water with 2 -3 drops of 0.1% Tween-20 solution with a gentle shake for 20 min. Afterwards, the following pre-treatment and surface sterilization treatments were employed for better axenic culture establishment.

Preparation of culture media

Murashige and Skoog (1962) medium was used as the basal medium throughout the experiment as it was the most favorable medium particularly for plant regeneration. The culture medium was supplemented with (1 mg/l) BAP + (0.5 mg/l) NAA for culture establishment as per the earlier standardized protocol. The carbon source employed was sucrose and the gelling agent was agar (Hi- Media, TC grade). For the preparation of media, stock solutions were prepared at the beginning and stored at 4±1 ^{0}C temperature. The respective media were prepared from stock solutions.

600 ml double distilled water (DDW) was taken in one liter beaker and required quantities of MS stock solutions were dissolved in DDW using magnetic stirrer. The pH of the media was adjusted to 5.80 by drop wise addition of 1N HCl/NaOH, as per the requirement. MS media consisting of sucrose and agar-agar was used as control. The prepared media solutions were kept for boiling on hot plate and required amount of agar-agar (8 g l^{-1}) was added slowly with continuous stirring to the slightly boiled media. Completely boiled media was then dispensed into the jar bottles (25 ml/bottle) and tightly plugged with autoclavable polypropylene caps and labeled to indicate the specific treatment. The media was autoclaved at 121 ^{0}C temperature and 15 lbs pressure for 18 minutes for sterilization. Then the media was allowed to cool for solidification and kept in media storage room 25 ± 2 ^{0}C for about a week to check contamination for future use.

After each treatment, observations on nature of microbial contamination, percent fungal contamination, percent bacterial contamination, percent survival, mortality of explants due to chemical toxicity were recorded replication wise after 30 days of culture initiation.

Statistical Analysis

Completely randomized design was followed for the present experiment. The data was analyzed using computer software programmed by the method of variance outlined by Panse and Sukhatme (1997) [14]. Statistical significance was tested by F value at 5 per cent level of significance. Critical difference at 0.05 level was worked out for the effects which were significant.

Results and Discussions

Standardization of different pre-treatment methods in pomegranate cultivars

Naturally growing plants have a diverse group of micro-organisms on their surface which is the major source of *in vitro* contaminants observed during the development of micro-propagation protocol. The duration of the pretreatment agents have a significant effect on explants. In this study, various anti-microbial agents were tried in different combinations and durations to eliminate the microbial contamination from the nodal explants. Among different treatments tested, explants treated with Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200ppm for 2 h (T_6) recorded significantly minimum fungal contamination (6.24%) bacterial (13.54%) and total microbial contamination (19.78%) followed by Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200ppm for 1 h (T_5) (8.23%, 17.60% and 25.83% respectively). Maximum fungal, bacterial and total microbial contamination percentage (64.88%, 35.12% and 100.00%) was recorded in T_0 (control). In case of cultivars lowest fungal, bacterial and total microbial contamination (26.39%, 21.78% and 48.17%) was recorded under Bhagwa explants (C_1) over Super bhagwa (C_2), interaction effect of pre-treatments and cultivars also showed significant difference which was presented in table 1.

From the results of table 2 it was revealed that mortality of the explants other than microbial contamination were found significantly reduced (0%) in control (T_0) where as an increase (32.93%) in explants mortality other than microbial contamination was observed in explants pre-treated with Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200 ppm – 2 h (T_6). Among the different treatments tested, lowest total mortality and maximum survival percentage (42.86% and 57.13%) was observed in explants treated with Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200ppm – 1 h (T_5), where as, complete mortality was recorded in treatment T_0 (Control). Among the cultivars, lowest mortality of the explants other than microbial contamination, total mortality percentage and highest survival percentage (16.06%, 64.23% and 33.67%) was recorded in nodal explants of Bhagwa (C_1) over the cultivar Super bhagwa (C_2). Interaction effect showed significant difference with respect to explants other than microbial contamination, total mortality percentage and survival percentage of explants. The results of present investigation were similar to the findings of Kumar *et al.*, 2017 [8] in marigold, Sindhu *et al.*, 2015 in Lillum and Kalaivani *et al.*, 2019 in crossandra with higher survival (66.67%, 80% and 78.21%, respectively) in treatment combination containing Carbendazim 0.2% and 8 HQ 200 ppm.

Table 1: Effect of Pre- treatments on percent fungal, bacterial and total microbial contamination in pomegranate cv. Bhagwa (C_1) and Super bhagwa (C_2)

Treatments	Pre-treatments details	Fungal contamination percent		Mean	Bacterial contamination percent		Mean	Total microbial contamination percent		Mean
		Cultivars			Cultivars			Cultivars		
		C_1	C_2		C_1	C_2		C_1	C_2	
T_0	Control (Distilled water wash)	63.79 (52.98)	65.96 (54.31)	64.88 (53.65)	36.20 (36.97)	34.03 (35.64)	35.12 (36.31)	100.00	100.00	100.00
T_1	Carbendazim 0.1% + (Metalaxyl-M + Mancozeb) 0.1% + 8 HQ 200ppm – 30 mins	36.52 (37.15)	42.52 (40.68)	39.52 (38.91)	26.96 (31.27)	29.17 (32.67)	28.07 (31.97)	63.48	71.69	67.59
T_2	Carbendazim 0.1% + (Metalaxyl-M + Mancozeb) 0.1% + 8 HQ 200ppm – 1 h	26.71 (31.10)	35.02 (36.26)	30.87 (33.68)	23.04 (28.65)	31.37 (34.04)	27.20 (31.34)	49.76	66.39	58.07
T_3	Carbendazim 0.1% + (Metalaxyl-M + Mancozeb) 0.1% + 8 HQ 200ppm – 2 h	27.16 (31.39)	23.31 (28.85)	25.24 (30.12)	17.02 (24.35)	21.81 (27.79)	19.42 (26.07)	44.19	45.13	44.66
T_4	Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200ppm – 30 mins	17.14 (24.43)	21.81 (27.82)	19.48 (26.13)	19.48 (26.17)	27.25 (31.44)	23.37 (28.81)	36.63	49.07	42.85
T_5	Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200ppm – 1 h	7.51 (15.86)	8.94 (17.38)	8.23 (16.62)	16.78 (24.17)	18.41 (25.39)	17.60 (24.78)	24.29	27.36	25.83
T_6	Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200ppm – 2 h	5.91 (14.06)	6.58 (14.83)	6.24 (14.45)	12.95 (21.07)	14.12 (22.05)	13.54 (21.56)	18.87	20.70	19.78
Mean		26.39 (29.57)	29.16 (31.45)		21.78 (27.52)	25.17 (29.86)		48.17	54.33	
		C	T	C x T	C	T	C x T	C	T	C x T
SE(m) ±		0.274	0.512	0.725	0.293	0.547	0.774	0.482	0.902	1.275
CD at 5%		0.797	1.492	2.110	0.852	1.594	2.254	1.403	2.625	3.713

Note: 1. Figures in parenthesis indicates angular transformation transformed values)
2. Streptomycin 200mg L-1 was added to all the treatment except control

Table 2: Effect of Pre-treatments on percent mortality other than microbial contamination, total mortality and survival percentage in pomegranate cv. Bhagwa (C_1) and Super Bhagwa (C_2)

Treatments	Pre treatments details	Mortality other than microbial contamination		Mean	Total mortality (%)		Mean	Survival percentage (%)		Mean
		Cultivars			Cultivars			Cultivars		
		C_1	C_2		C_1	C_2		C_1	C_2	
T_0	Control (Distilled water wash)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	100.00	100.00	100.00	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T_1	Carbendazim 0.1% + (Metalaxyl-M + Mancozeb) 0.1% + 8 HQ 200ppm – 30 mins	12.82 (20.97)	14.02 (21.98)	13.42 (21.47)	76.31	85.72	81.01	23.69 (29.10)	14.27 (22.14)	18.98 (25.62)
T_2	Carbendazim 0.1% + (Metalaxyl-M + Mancozeb) 0.1% + 8 HQ 200ppm – 1 h	16.10 (23.64)	18.39 (25.37)	17.24 (24.51)	65.86	84.78	75.32	25.66 (30.42)	15.21 (22.87)	20.44 (26.65)
T_3	Carbendazim 0.1% + (Metalaxyl-M + Mancozeb) 0.1% + 8 HQ 200ppm – 2 h	22.76 (28.46)	28.59 (32.31)	25.68 (30.38)	66.95	73.72	70.34	33.04 (35.06)	26.27 (30.74)	29.65 (32.90)
T_4	Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200ppm – 30 mins	12.30 (20.52)	13.13 (21.18)	12.71 (20.85)	48.93	62.20	55.56	44.90 (42.05)	37.79 (37.91)	41.35 (39.98)
T_5	Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200ppm – 1 h	16.31 (23.80)	17.76 (24.91)	17.03 (24.36)	40.60	45.13	42.86	59.39 (50.39)	54.87 (47.77)	57.13 (49.08)
T_6	Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200ppm – 2 h	32.13 (34.51)	33.72 (35.47)	32.93 (34.99)	51.00	54.43	52.71	49.00 (44.40)	45.57 (42.43)	47.28 (43.42)
Mean		16.06 (21.70)	17.94 (23.03)		64.23	72.28		33.67 (33.06)	27.71 (29.12)	
		C	T	C x T	C	T	C x T	C	C	T
SE(m) ±		0.202	0.377	0.533	0.565	1.057	1.496	0.331	0.618	0.875
CD at 5%		0.587	1.098	1.553	1.646	3.079	4.355	0.962	1.801	2.547

Note: 1. Figures in parenthesis indicates angular transformation transformed values)
2. Streptomycin 200mg L-1 was added to all the treatment except control

Data presented in table 1 & 2 revealed that, all pre-treatments showed significantly better results when compared to the control. Among the different pre-treatments tested it was observed that Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200 ppm – 2 h (T_6) showed mean lowest total microbial contamination (19.78%) followed by Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200 ppm – 1 h (T_5) (25.83%). However, the highest survival percentage (57.13%) was observed under explants treated with Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200ppm – 1 h (T_5) compared to Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200ppm – 2 h (T_6) (47.28%), this might be due to high mortality of the explants, as higher concentrations of these disinfectants and prolonged durations of treatment became toxic and were responsible for poor growth and low establishment of

cultures. Fungicide dosage and treatment duration depend on the type and tenderness of explant. Among the cultivars, nodal explants of Bhagwa was proved to be best for most of the parameters, this might be due to variation in their genotype.

Standardization of the different surface-sterilization methods in pomegranate cultivars

It is clearly evident from the Table 3 that in case of surface sterilizing agents both the cultivars recorded significantly minimum mean fungal, bacterial and total microbial contamination percentage (9.43%, 10.81% and 20.24% receptively) when treated with Mercuric chloride ($HgCl_2$) @ 0.1% for 8 min (T_4), which was on par with Mercuric chloride ($HgCl_2$) @ 0.1% for 6 min (T_3) (9.74%, 10.93% and 20.67% receptively) while the maximum (73.84%, 25.13% and 98.97%) was recorded in control (T_0). Among the cultivars the nodal explants of Bhagwa cultivar (C_1) recorded lowest mean fungal, bacterial and total microbial contamination percentage (35.66%, 16.46% and 51.51%) over Super bhagwa (C_2). However, Table 4 showed that maximum explants mortality other than microbial contamination (27.96%) was observed in T_4 (Mercuric chloride ($HgCl_2$) @ 0.1% for 8 min).

Table 3: Effect of surface sterilization treatments on percent fungal, bacterial and total microbial contamination in pomegranate cv. Bhagwa (C_1) and Super Bhagwa (C_2)

Treatments	Surface sterilization treatments details	Fungal contamination percent		Mean	Bacterial contamination percent		Mean	Total microbial contamination percent		Mean
		Cultivars			Cultivars			Cultivars		
		C_1	C_2		C_1	C_2		C_1	C_2	
T_0	Control (Distilled water wash)	71.40 (57.65)	76.27 (60.83)	73.84 (59.24)	26.94 (31.24)	23.32 (28.85)	25.13 (30.05)	98.34 (82.71)	99.60 (86.88)	98.97 (84.79)
T_1	Mercuric chloride ($HgCl_2$)@ 0.1% for 2 min	37.18 (37.55)	45.27 (42.23)	41.20 (39.89)	21.77 (27.77)	24.84 (29.88)	23.31 (28.82)	58.96 (50.14)	70.06 (56.80)	64.51 (53.47)
T_2	Mercuric chloride ($HgCl_2$)@ 0.1% for 4 min	11.18 (19.53)	17.63 (24.81)	14.40 (22.17)	12.02 (20.25)	14.64 (22.46)	13.33 (21.36)	23.21 (28.78)	32.27 (34.59)	27.74 (31.69)
T_3	Mercuric chloride ($HgCl_2$)@ 0.1% for 6 min	9.34 (17.78)	10.14 (18.56)	9.74 (18.17)	9.68 (18.12)	12.18 (20.40)	10.93 (19.26)	19.02 (25.84)	22.33 (28.18)	20.67 (27.01)
T_4	Mercuric chloride ($HgCl_2$)@ 0.1% for 8 min	8.85 (17.29)	10.07 (18.43)	9.43 (17.86)	8.84 (17.28)	12.78 (20.88)	10.81 (19.08)	17.69 (24.86)	22.80 (28.48)	20.24 (26.67)
T_5	Sodium hypochloride (NaOCl) @ 10% for 10 min	59.15 (50.25)	66.88 (54.84)	63.01 (52.55)	22.45 (28.25)	25.42 (30.24)	23.93 (29.24)	81.60 (64.58)	92.30 (74.17)	86.95 (69.38)
T_6	Sodium hypochloride (NaOCl) @ 10% for 15 min	52.56 (46.45)	56.93 (48.96)	54.47 (47.70)	13.51 (21.50)	16.56 (23.95)	15.03 (22.72)	66.07 (54.37)	73.49 (59.01)	69.78 (56.69)
Mean		35.66 (35.21)	40.44 (38.38)		16.46 (23.49)	18.53 (25.24)		52.13 (47.32)	58.98 (52.59)	
		C	T	C x T	C	T	C x T	C	T	C x T
SE(m) ±		0.130	0.243	0.343	0.336	0.628	0.888	0.387	0.724	1.024
CD at 5%		0.378	0.707	1.000	0.977	1.828	NS	1.128	2.109	2.983

Note: 1. Figures in parenthesis indicates angular transformation transformed values)

Table 4: Effect of surface sterilization treatments on percent mortality other than microbial contamination, total mortality and survival percentage in pomegranate cv. Bhagwa (C_1) and Super Bhagwa (C_2)

Treatments	Surface sterilization treatments details	Mortality other than microbial contamination		Mean	Total mortality (%)		Mean	Survival percentage (%)		Mean
		Cultivars			Cultivars			Cultivars		
		C_1	C_2		C_1	C_2		C_1	C_2	
T_0	Control (Distilled water wash)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	98.34 (82.71)	99.60 (86.88)	98.97 (84.79)	1.65 (7.25)	0.40 (3.07)	1.02 (5.16)
T_1	Mercuric chloride ($HgCl_2$)@ 0.1% for 2 min	10.48 (18.88)	5.44 (13.48)	7.96 (16.18)	69.44 (56.42)	75.50 (60.31)	72.47 (58.37)	30.56 (33.53)	24.49 (29.64)	27.52 (31.59)
T_2	Mercuric chloride ($HgCl_2$)@ 0.1% for 4 min	10.38 (18.77)	8.49 (16.93)	9.43 (17.85)	33.59 (35.40)	40.76 (39.65)	37.17 (37.53)	66.40 (54.56)	59.23 (50.30)	62.82 (52.43)
T_3	Mercuric chloride ($HgCl_2$)@ 0.1% for 6 min	13.28 (21.36)	10.53 (18.92)	11.90 (20.14)	32.31 (34.62)	32.86 (34.95)	32.58 (34.79)	67.68 (55.33)	67.14 (55.00)	67.41 (55.17)
T_4	Mercuric chloride ($HgCl_2$)@ 0.1% for 8 min	28.63 (32.33)	27.30 (31.47)	27.96 (31.90)	46.32 (42.87)	50.10 (45.03)	48.21 (43.95)	53.67 (47.09)	49.90 (44.92)	51.78 (46.00)
T_5	Sodium hypochloride (NaOCl) @ 10% for 10 min	7.18 (15.50)	5.05 (12.86)	6.11 (14.18)	88.78 (70.42)	97.35 (81.18)	93.07 (75.80)	11.21 (19.54)	2.64 (8.77)	6.93 (14.16)
T_6	Sodium hypochloride (NaOCl) @ 10% for 15 min	10.07 (18.49)	7.31 (15.63)	8.69 (17.06)	76.15 (60.77)	80.80 (64.02)	78.48 (62.39)	23.84 (29.19)	19.19 (25.94)	21.52 (27.56)
Mean		11.43 (17.90)	9.16 (15.61)		63.56 (54.74)	68.14 (58.86)		36.43 (35.21)	31.85 (31.09)	
		C	T	C x T	C	T	C x T	C	C	T
SE(m) ±		0.808	0.432	1.142	0.387	0.724	1.024	0.387	0.724	1.024
CD at 5%		0.214	0.400	0.566	1.127	2.109	2.983	1.127	2.109	2.983

Note: 1. Figures in parenthesis indicates angular transformation transformed values)

Among the treatments, lowest total mortality (32.58%) and highest survival percentage (67.41%) was observed under when auxiliary buds were treated with Mercuric chloride ($HgCl_2$) @ 0.1% for 6 min (T_3) while, highest total mortality (98.97%) and lowest survival percentage (1.02%) was observed under treatment Control (T_0). In case of cultivars lowest total mortality of the explants and highest survival percentage (36.43%) was reported in nodal explants of Bhagwa over Super bhagwa (Table 4). Interaction effect of cultivars and surface sterilization treatment showed significant difference except for bacterial contamination percentage.

From the above parameters, it was revealed that there was a reduction in contamination percent with increase in the duration of surface sterilization time. Among the two surface sterilizing agents, mercuric chloride ($HgCl_2$) was found to be most effective over Sodium hypochloride (NaOCl).This might be due to NaOCl, act as weak/or mild sterilizing agent, should be used in higher percentage to control contamination, however, Mercuric chloride ($HgCl_2$) is stronger than sodium hypochloride (NaOCl), which has bactericidal action, was more effective and showed better decontamination percentages (Mahmoud and Al-Ani, 2016) [11]. However, highest mortality of explants other than microbial contamination was observed under Mercuric chloride ($HgCl_2$) @ 0.1% for 8 min (T_4) which might be due to prolonged exposure to $HgCl_2$ may have negative effects on the explants as the cutting ends are the entry points of the active compounds, so long period of exposure to $HgCl_2$ leads to browning and death of the explants (Emoghene *et al.*, 2020). Therefore, explants treated with Mercuric chloride ($HgCl_2$) @ 0.1% for 6 min (T_3) was found to be best surface sterilization treatment with maximum survival percentage (67.41%) that may be due to lesser exposure of them with optimum duration of $HgCl_2$ which might have led to less bleaching activity of chlorine and also attributed due to hardy nature of nodal segment made them survive better (Singh *et al.* 2011) [17]. Among the cultivars, nodal explants of Bhagwa was proved to be best for most of the parameters, this might be due to variation in the genotype. The above findings are similar to the reports of Gorad *et al.* (2018), Gondhali *et al.*(2016), Prabhuling and Huchesh, 2018, Suhasini *et al.* (2017), Singh *et al.* (2014), Kalalbandi *et al.*(2014) [4, 3, 15, 19, 18, 7] in pomegranate, El-Raflea *et al.* (2017) [2] in guava and Kajla, *et al*. (2018) [6] in rose.

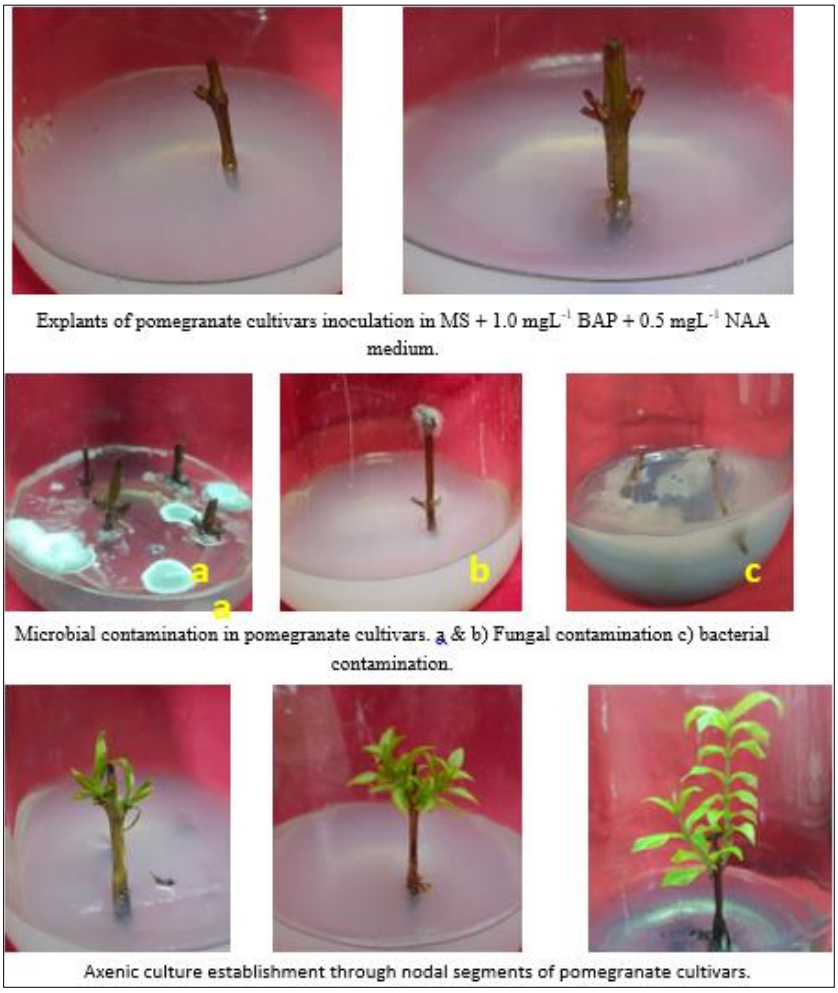

Plate 1: Effect of pre-treatments and surface sterilization treatments on axenic culture establishment in pomegranate cultivars Bhagwa and Super Bhagwa

Conclusion

The present study advocated the use of anti-microbial agents particularly nodal cuttings of pomegranate explants pre-treated with Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200ppm for 1 h followed by surface sterilization in laminar airflow chamber with Mercuric chloride ($HgCl_2$) @ 0.1% for 6 min was proved to be effective method for mass multiplication of pomegranate cultivars *viz.*, Bhagwa and

Super bhagwa in *in vitro* with maximum explants survival percentage in culture establishment stage

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