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Optimizing *in vitro* axenic culture establishment in pomegranate cultivars Bhagwa and Super Bhagwa

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Abstract
An investigation was undertaken to standardise the *in vitro* culture establishment in pomegranate cultivars Bhagwa and Super Bhagwa by using different combinations and concentrations of growth regulators. Among different treatments tested, it was observed that The explants of pomegranate cv. Bhagwa inoculated on MS media supplemented with different combinations and concentrations of plant growth regulators had performed well over the explants cv. Super Bhagwa in terms of number of sprouts explant^{-1} (2.34), shoot length (2.41cm), percent callus induction (1.81%) and culture establishment index (140.42). The culturing of nodal explants of pomegranate cultivars on MS media supplemented with 1mgL^{-1} of BAP and 0.5 mgL^{-1} of NAA (T_2) had recorded the less number of days for bud initiation (10.45d), maximum percent (86.83%) of culture establishment, number of sprouts (4.49), leaves (7.83), shoot length (4.05cm) explant^{-1} and culture establishment index (390.52) over other treatments and the control treatment showed no culture establishment.

Keywords: *in vitro*, Bhagwa, super Bhagwa, BAP, TDZ, NAA

Introduction
The pomegranate (*Punica granatum* L.) is one of the oldest known edible fruits that belongs to family Lythraceae. It is an economically important crop of the semi-arid tropics of the world due to its highly nutritious value with high returns and great export demand, versatile adaptability, low irrigation water requirement and pharmaceutical value (Bhandari, 2012; Singh *et al.*, 2012; Singh *et al.*, 2016) [1, 2, 3]. The fruit is a good source of sugars and Vitamin C, whereas seeds with arils are sun-dried and commercially marketed as condiment or spice, food flavoring agents, besides, having huge market potential as pomegranate seed oil owing to its immense medicinal properties (Parmar and Kaushal 1982; Pal and Singh, 2017.) [4, 7]. The plant is well adapted to drought with high distribution extending from Iran to Himalaya in Northern India and has been cultivated throughout the Mediterranean region (Soloklui *et al*., 2012; Singh *et al.*, 2015) [6, 7]. Pomegranate is conventionally propagated through air layering, hard wood and soft wood cuttings (Chandra *et al.*, 2014) [8]. But these traditional propagation methods do not ensure disease free and healthy plants.
Mass multiplication through tissue culture is necessary to meet the rapidly increasing demand for elite pomegranate planting material. Hence, the present work is proposed to study the effect of different treatments in culture establishment in pomegranate cultivars.

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 were pre-treatmented with the solution of Carbendazim 0.2% + (Metalaxyl-M + Mancozeb) 0.2% + 8 HQ 200 ppm for 1 h followed by washings with autoclaved double distilled water under Laminar Air Flow and surface sterilization with $HgCl_2$ (0.1% for 6 min) followed by four washings with autoclaved double distilled water resulted in maximum percentage of axenic culture.

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 combinations and concentrations of BAP and NAA for culture establishment as per the treatments tested for culture intiation. 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 ^{O}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 ˚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.

Inoculation of explants

The surface of the explants was sterilized in laminar flow chamber. Later the explants were given a fresh cut under laminar air flow and were inoculated on MS media enriched with different combinations of growth regulators as per initiation experiment. During the inoculation, the cap of the culture bottle was removed and mouth of the bottles was flamed over the spirit lamp in the chamber. The sterilized and trimmed explants were quickly transferred to the culture medium by gently pressing with the sterilized forceps to secure firm contact with the media and again the rim of the bottles was flamed and was capped tightly.

After each treatment, observations on culture establishment (%), number of days required for bud initiation, number of sprouts produced explants^{-1}, shoot length, number of leaves formed shoot^{-1}, percent callus induction and culture establishment index were recorded replication wise after 25 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) [20]. 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 growth regulators for culture establishment in pomegranate cultivars.

Different BAP (0.5, 1.0, 2.0 and 3.0 mg/l) and TDZ (0.01, 0.05, 0.1, 0.5 mg/l) concentrations were tried along with NAA (0.5 mg/l) for culture establishment. Under our experimental conditions, among the different combinations and concentrations of growth regulators tested, The mean percent culture establishment was found significantly high (86.83%) in nodal cuttings inoculated on MS medium supplemented with BAP @ 1mgL^{-1} + NAA @ 0.5mgL^{-1} (T_2) followed by MS medium supplemented with BAP @ 2 mgL^{-1} + NAA @ 0.5 mgL^{-1} (T_3) (83.83%). The percent culture establishment was failed (0%) by inoculation of nodal cuttings on MS media devoid of growth regulators (Control treatment-T_0). The culture establishment percent Amon cultivars was also found significant with highest mean value (44.51%) in explants of pomegranate cv. Super Bhagwa (C_2) than cv. Bhagwa (C_1) (43.59%). The interaction between treatment and cultivars was also significant.

Early (10.45d) bud induction was observed on MS medium supplemented with BAP @ 1 mgL^{-1} + NAA @ 0.5mgL^{-1} (T_2) followed by BAP @ 2 mgL^{-1} + NAA @ 0.5 mgL^{-1} (T_3) (14.52d). which were stastically significant with other treatments. The number of days required for bud initiation was found maximum (41.56d) in auxiliary buds cultured on MS medium supplemented with TDZ @ 0.01 mgL^{-1} + NAA @ 0.5 mgL^{-1} (T_5). Among the cultivars, significantly earlier axillary bud sprouting (20.70d) was noticed in pomegranate cv. Bhagwa (C_1) over Super Bhagwa (C_2) (20.35d). The interaction between treatment and cultivars was also significant (table -1).

Table 1 and 2 also revealed that, maximum number of micro-shoots per explant (4.49) and maximum shoot length (4.05cm) was recorded in BAP @ 1 mgL^{-1} + NAA @ 0.5mgL^{-1} (T_2), which was statistically superior with all other treatments for both the parameters. The cultivar response to different combinations and concentrations of growth regulators also showed significant difference with high mean value (2.34 and 2.41 cm respectively) in explants developed from nodal cuttings of pomegranate cv. Bhagwa (C_1) than cv. Super Bhagwa (C_2) (2.12 and 2.25cm, respectively) for number of micro-shoots and longest shoot length. However the interaction effect was found non significant.

Table 1: Effect of different concentrations of growth regulator combinations on culture establishment (%), number of days for bud initiation (No.) and number of sprouts explants^{-1} (No.) in Pomegranate cultivars

Treatments	Growth regulator details	Culture establishment (%)		Mean	Number of days for bud initiation (No.)		Mean	Number of sprouts explants^{-1} (No.)		Mean
		Cultivars			Cultivars			Cultivars		
		C_1	C_2		C_1	C_2		C_1	C_2	
T_0	Control (MS media devoid of	0.00	0.00	0.00	0.00 (1.00)	0.00 (1.00)	0.00	0.00 (1.00)	0.00 (1.00)	0.00

	any growth regulators)	(0.00)	(0.00)	(0.00)			(1.00)			(1.00)
T_1	MS + 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	51.66 (45.93)	57.33 (49.19)	54.50 (47.56)	19.68 (4.54)	18.84 (4.45)	19.26 (4.50)	1.83 (1.68)	1.62 (1.61)	1.72 (1.64)
T_2	MS + 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	88.66 (70.30)	85.00 (67.21)	86.83 (68.75)	11.08 (3.47)	9.81 (3.28)	10.45 (3.38)	4.65 (2.37)	4.34 (2.31)	4.49 (2.34)
T_3	MS + 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	87.00 (68.84)	80.66 (63.90)	83.83 (66.37)	13.96 (3.86)	15.08 (4.00)	14.52 (3.93)	3.55 (2.13)	3.09 (2.02)	3.32 (2.07)
T_4	MS + 3.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	80.00 (63.41)	75.66 (60.44)	77.83 (61.93)	15.43 (4.05)	16.32 (4.16)	15.87 (4.10)	2.99 (1.99)	2.59 (1.89)	2.79 (1.94)
T_5	MS + 0.01 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA	0.00 (0.00)	9.66 (18.07)	4.83 (9.03)	42.73 (6.61)	40.40 (6.43)	41.56 (6.52)	1.06 (1.43)	1.02 (1.42)	1.04 (1.43)
T_6	MS + 0.05 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA	20.00 (26.55)	22.33 (28.17)	21.16 (27.36)	39.07 (6.33)	37.77 (6.22)	38.42 (6.27)	1.60 (1.61)	1.29 (1.51)	1.44 (1.56)
T_7	MS + 0.1 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA	38.66 (38.43)	41.33 (39.99)	40.00 (39.21)	19.03 (4.47)	20.76 (4.66)	19.90 (4.57)	2.73 (1.93)	2.65 (1.91)	2.69 (1.92)
T_8	MS + 0.5 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA	26.33 (30.85)	28.66 (32.35)	27.50 (31.60)	25.30 (5.12)	24.20 (5.02)	24.75 (5.07)	2.67 (1.91)	2.49 (1.86)	2.58 (1.89)
Mean		43.59 (38.26)	44.51 (39.92)		20.70 (4.38)	20.35 (4.36)		2.34 (1.78)	2.12 (1.72)	
		C	T	C x T	C	T	C x T	C	T	C x T
SE(m) ±		0.203	0.432	0.610	0.021	0.044	0.062	0.028	0.013	0.040
CD at 5%		0.586	1.243	1.758	N/A	0.125	0.177	0.038	0.082	N/A
NOTE: 1. Figures in parenthesis indicates transformation transformed values)										

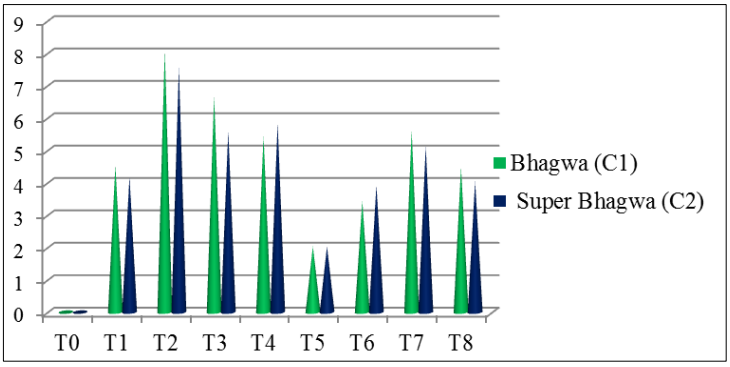

Fig 1: Effect of different combinations and concentrations of growth regulators on number of leaves per shoot in Pomegranate cultivars.

Under our experimental conditions (Figure-1) the variation in number of leaves produced shoot^{-1} was found non-significant between the two pomegranate cultivars and their interaction between different concentrations and combinations of growth regulators. Significantly, the maximum number of leaves (7.83) were produced shoot^{-1} by inoculation of nodal cuttings of pomegranate cultivars on MS media supplemented with BAP @ 1 mgL^{-1} + NAA @ 0.5 mgL^{-1} (T_2) when compared to other treatments.

The percent of callus induction explant^{-1} was not found in auxiliary buds cultured on MS media supplemented with different combinations and concentrations of BAP + NAA. The percent of callus induction explant^{-1} was found significantly high (5.41%) in auxiliary buds cultured on MS medium supplemented with TDZ @ 0.01 mgL^{-1} + NAA @ 0.5 mgL^{-1} (T_5) followed by (3.53%). In case of cultivars callus induction explant^{-1} was found significantly high (1.81%) in nodal cuttings of pomegranate cv. Bhagwa (C_1) in contrast to the explants pomegranate cv. Super Bhagwa (C_2) (0.65%). Interaction of different combinations and concentrations of growth regulators and cultivars was found non-significant (table-2).

The data presented in table-2 on influence of pomegranate cultivars, different combinations and concentrations of plant growth regulators and their interaction effect on culture establishment index was found significant. The highest mean culture establishment (140.42) index was recorded with explants of pomegranate cv. Bhagwa (C_1) than the explants of pomegranate cv. Super Bhagwa (C_2) (124.75). Among treatments, BAP @ 1 mgL^{-1} + NAA @ 0.5 mgL^{-1} (T_2) recorded highest culture establishment index (390.52) followed by (279.39) MS media along with BAP @ 2mgL^{-1} + NAA @ 0.5 mgL^{-1} (T_3) treatment. However, no culture establishment was reported in explants produced from nodal cuttings inoculated on MS media supplemented without plant growth regulators (T_0).

From the present experiment we can interfere that nodal cuttings inoculated on MS media supplemented with BAP than MS media supplemented with TDZ. It might be due to the ability of plant tissues to metabolize BAP more readily than other synthetic growth regulators or the ability of BAP to induce the production of natural hormones such as zeatin within the tissue (Prabhuling and Huchesh, 2018) [13]. This study also showed with increase in concentration of BAP above said paramaters mean value was increased upto certain level *i.e.*, BAP @ 1mgL^{-1}, and showed a positive correlation between the concentration of BAP upto 1mgL^{-1} of BAP for culture establishment (%), number of days required for bud initiation, number of sprouts produced explants^{-1}, shoot length, number of leaves formed shoot^{-1}, percent callus induction and culture establishment index. Further increase in BAP concentration above 1mgL^{-1} had showed negative correlation effect. Therefore, apparently a certain level of BAP is required to obtain the best effect (Arad *et al.*, 2014)

[10]. The possible reason may be higher concentration of exogenous hormone could compete with endogenous hormone for the active sites or cause the conformational change in receptor protein and reduce the total number of functioning complex that stimulate the endogenous hormone level. The ratio between the exogenous and endogenous hormones and the natural turnover of functional complex determined the shoot formation pattern. The exogenous application of different concentrations of the hormone may affect the nutrient uptake from medium, which in turn reduces the nutrient supply to the actively dividing meristamatic tissue thereby reduced the shoot bud proliferation (Hamad and Taha, 2008).

Among the cultivars superior performance of Bhagwa over Super Bhagwa might be attributed to genotypic variation in explant growth response to the endogenous growth regulators itself. Some explants had exhibit genetically slow growth response and other explants had exhibit rapid growth response leads to produce more shoot proliferation, shoot growth by increasing cell division and cell elongation (Samanhudi, 2007) [9]. The present results are in line with the findings of Parmar *et al.*, 2015 [17], Singh and Patel (2014) [15], Desai *et al.*, 2018 [16], Sonone and Kshirsagar, 2016 [18], Mulaei *et al.*, 2019 [11] in Pomegranate, Sen and Patel, 2018 [16] in Fig, Aghaye (2012) [19] in Almond and Kanwar *et al.*, 2016 [14] in Sour orange.

Table 2: Effect of different concentrations of growth regulator combinations on shoot length (cm), callus induction percentage (%) and culture establishment index in Pomegranate cultivars

Treatments	Growth regulator details	Shoot length (cm)		Mean	Callus induction percentage (%)		Mean	Culture establishment index		Mean
		Cultivars			Cultivars			Cultivars		
		C_1	C_2		C_1	C_2		C_1	C_2	
T_0	Control (MS media)	0.00	0.00	0.00	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00	0.00	0.00
T_1	MS + 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	3.55	3.29	3.42	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	94.61	93.80	94.20
T_2	MS + 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	4.30	3.80	4.05	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	412.44	368.59	390.52
T_3	MS + 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	3.76	3.61	3.68	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	309.28	249.50	279.39
T_4	MS + 3.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	3.44	3.24	3.34	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	239.75	196.05	217.90
T_5	MS + 0.01 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA	0.96	0.85	0.90	7.00 (15.09)	3.83 (11.22)	5.41 (13.16)	0.00	4.44	2.22
T_6	MS + 0.05 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA	1.44	1.31	1.37	5.00 (12.54)	2.06 (6.64)	3.53 (9.59)	32.14	28.96	30.55
T_7	MS + 0.1 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA	2.18	2.11	2.15	1.66 (6.03)	0.00 (0.00)	0.83 (3.01)	105.36	109.84	107.60
T_8	MS + 0.5 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA	2.04	2.03	2.04	2.66 (9.08)	0.00 (0.00)	1.33 (4.54)	70.19	71.58	70.88
Mean		2.41	2.25		1.81 (4.75)	0.65 (1.98)		140.42	124.75	
		C	T	C x T	C	T	C x T	C	T	C x T
SE(m) ±		0.032	0.069	0.097	0.460	0.976	1.381	3.692	7.832	11.076
CD at 5%		0.093	0.197	N/A	1.325	2.811	3.976	10.633	22.555	31.898

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

T_1: MS + 0.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA

T_2: MS + 1.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA

T_3: MS + 2.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA

T_4: MS + 3.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA

T_5: MS + 0.01 mgL^{-1} TDZ + 0.5 mgL^{-1} NAA

T_7: MS + 0.1 mgL^{-1} TDZ + 0.5 mgL^{-1} NAA

T_8: MS + 0.5 mgL^{-1} TDZ + 0.5 mgL^{-1} NAA

Fig 2: Shoot bud induction in Bhagwa as influenced by composition of media at 25 DAI

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