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Effect of growth regulators on shoot proliferation and root development in micropropagation of pomegranate (*Punica granatum* L.)

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Abstract
In this study, the effect of growth regulators in different concentrations was tested for shoot and root development in micropropagation of pomegranate with nodal explants. The explants were collected in the morning, third node from the shoot tip. The explants were collected from healthy plants which were authenticated. For shoot development The MS media was supplemented with different concentrations of alpha-naphthalene acetic acid (NAA), indole-3-butyric-acid (IBA), 6-benzyl aminopurine (BAP) and kinetin and their combinations. For rot development MS media was supplemented with different concentrations of alpha-naphthalene acetic acid (NAA), indole-3-butyric-acid (IBA) was tested. It is observed that MS media when supplemented with ADS 15 mg/l + NAA 2.0 mg/l + BAP 2.0 mg/l had given highest maximum shoot proliferation, number of shoots, number of leaves along with highest number of nodes and intermodal length and survival per cent, followed by MS media supplemented with ADS 15 mg/l + NAA 1.0 mg/l + BAP 2.0 mg/l. MS media when supplemented with IBA 0.6 mg/l had given a maximum number of roots per shoot, highest number of shoots producing roots and more number of new leaves followed by MS media supplemented with 0.6 mg/l NAA.

Keywords: Sterilization, micropropagation, pomegranate, shoot proliferation, root growth

Introduction
Pomegranate (*Punica granatum* L.) is one of the most important commercial fruit crop grown all over the tropical and subtropical regions of the world for it's delicious edible fruits and pharmaceutical and ornamental usage (Jayesh and Kumar, 2004) [6]. To get true to type planting material, pomegranate is commercially propagated by stem cuttings (hard wood cutting) or by air layering. However, it has several limitations like low success rate, very slow procedure and new plants require at least one year for establishment and only limited number of plants can be produced through these methods. This results in non-availability of plantlets throughout the year.

In developed countries micropropagation is being commercially employed for mass production of disease free planting material for many fruit crops. Micropropagation offers the possibility of rapid clonal production of plantlets on commercial scale where, the demand is high and supply is low or for establishment of planting material for special uses in a compressed time frame *In-vitro* multiplication has been defined in three steps, initiating culture, increasing propagules and preparation of soil transfer (Murashige, 1974) [13]. These steps have been widely adapted by both research and commercial tissue culture laboratories because they are not only described procedural steps in the *in-vitro* propagation process but also usually represent points at which cultural environment need to be changed.

Two major developments, which made shoot culture feasible were the development of improved media for plant tissue culture (Murashige and Skoog, 1962) [12] and discovery of the cytokinins as a class of growth regulators with an ability to release lateral buds from dormancy.

Reliable and efficient regeneration *in vitro* through stimulation of axillary bud proliferation from nodal segment explant and apical buds or through organogenesis or embryogenesis directly from various explants or through callus had been already demonstrated in a number of woody species. Studies have been conducted on micropropagation of pomegranate trees over the past several years.

Protocols have been developed for regeneration of pomegranate plantlets *in vitro* through either organogenesis from callus derived from leaf segments, cotyledons (Murkute *et al*; 2002; Raj and Kanwar, 2012; Kanwar *et al*., 2010) [14, 9, 21], anthers (Soumendra Naik *et al*., 1999) [23] or through embryogenesis from various seedling explants, petals and immature zygotic embryos (Kanwar *et al*., 2010) [9].

In the past, several workers have studied different factors affecting *in vitro* plant regeneration of pomegranate and other crops. It is very difficult to produce plants *in vitro* in case of woody plants. Attempt is made to standardize best combination of growth regulators for shoot proliferation and root development.

Materials and Methods

The present work was done at COH, Venkataramannagudem and at Acharya Nagarjuna University, Guntur to establish the micropropagation protocol of pomegranate.

Selection of explant

The selection of an explant is very important in achieving eventual success in micropropagation. Murashige (1974) [13] described the different factors that influence the explants, which include, size and source of explants, season of collection, age of parent etc. Many workers have confirmed that, the quality of explant primarily determines the establishment of *in-vitro* cultures (John and Murray, 1981; Kim *et al*., 1981; Keathley, 1984) [7, 12, 11]. The type of explant used for the culture was nodal segment with axillary bud. The explants were collected in the morning, third node from the shoot tip. The explants were collected from healthy plants which were authenticated.

Sterilization of explants

Explants were washed and sterilized properly in order to remove dust and extraneous material and to eliminate the fungal and bacterial contamination.

Preparation of stock solutions and their storage

All the stock solutions required for the preparation of media were prepared by dissolving the required chemicals in double distilled water. Salts were dissolved by adding one compound at a time. Precipitation was usually avoided by preparing stock solutions of growth regulators such as auxins and cytokinins by dissolving in small volume of NaOH or HCl and then final volume was made by adding double distilled water in 1:1 ratio. All the stock solutions were kept in bottles with lids and stored at 4 °C.

Preparations of culture media and its sterilization

The media used for this investigation was Murashige and Skoog's (1962) [12] basal media. The MS media was supplemented with different concentrations of alpha-naphthalene acetic acid (NAA), indole-3-butyric-acid (IBA), 6-benzyl aminopurine (BAP) and kinetin and their combinations. The nutrient media consisted of inorganic nutrients, carbon source and vitamins. Growth regulators (Phytohormones), are not nutrients but they influence growth and development.

Generally, growth regulators are produced naturally in plants. But in cultures they are added selectively to culture media as sufficient quantities of growth regulators are not manufactured in cultures. As the phytohormones play a key role in tissue culture studies, efforts are required to determine optimum levels. The formation of adventitious shoots or roots was first determined by Skoog and Miller (1957) [22] through discovery of the regulation of organ formation i.e., shoots and roots by changing the ratio of cytokinin/auxin, when the ratio of cytokinin/auxin is high, it favours the formation of shoot but root formation is inhibited. The reverse favours the root formation, whereas the intermediate ratio induces unorganised growth of callus tissue. Regulation of plant growth and development including plant regulation from isolated cell and tissue is under the control of hormones. Among various hormones, auxins as IAA, IBA, NAA and 2, 4-D, cytokinins as 6-Benzyl amino purine (BAP), Kinetin, Zeatin, 2-iso pentenyl amino purine (2 ip), TDZ, gibberellins and ethylene are important. In this study for shoot proliferation MS medium with ADS 15 mg/l, NAA ranging from 0.0 to 2.0 mg/l and BAP ranging from 0.0 to 2.0 mg/l were tested. Whereas for root development MS medium with NAA ranging from 0.2 to 0.6 mg/l and IBA ranging from 0.2 to 0.6 mg/l were tested.

Preparation of stock solution of ADS

The stock solution of Adenine sulphate ($C_5H_7N_5O_4S$) was prepared by dissolving 30 mg in 100 ml of hot sterile distilled water. The required quantities were pipetted out to the medium as per the treatment.

Preparation of stock solution of auxin (NAA)

The stock solution of α-naphthalene acetic acid ($C_2H_{10}O_2$) was prepared by dissolving 10 mg in a small quantity of ethanol and the volume was made up to 200 ml with sterile distilled water. The required quantities were pipette out to the medium as per the treatment.

Preparation of stock solution of Cytokinin (BAP)

The stock solution of N-6-benzylaminopurine (BAP) was prepared by dissolving 10 mg in a small quantity of 0.1N NaOH and volume made to 200 ml by adding sterile distilled water. The required volume of stock solutions was added to the media as per the treatments.

Preparation of stock solution of IBA

The stock solution of Indole 3- butyric acid was prepared by dissolving 10 mg in few drops of ethanol/methanol and the volume made up to 200 ml by adding sterile distilled water. The required volume of stock solutions was added to the media as per the treatments.

Results and Discussion

Per cent shoot proliferation

The results revealed that the presence of NAA and BAP in combination resulted in good proliferation, (Fig-1) whereas, poor proliferation was recorded when they were used alone. Organogenesis depends upon the auxin/cytokinin ratio and not on their absolute concentrations (Murashiage & Skoog, 1962). Synergistic effects of BAP and NAA will lead to cell division, formation of nodular meristemoids and finally adventitious shoot bud regeneration. The results were in accordance with the findings of Murkute *et al.* (2004) [15], who reported more shoots per culture (1.9) with 1.0 B.A.P. + 0.5 NAA g/l, in pomegranate. Zimmerman and Swartz, (1994) suggested that NAA and BAP combinations were rewarding in many fruit species. Naik *et al*. (1999) [17] reported cytokinin

is effective for shoot regeneration when used in combination with an auxin.

Number of shoots developed per explants

The number of shoots developed per explant was highest in MS medium that used highest concentration of NAA and BAP are known to induce highest adventitious shoots (Murkute *et al.*, 2002) [14]. Naik and Chand (2003) [19] and Kanwar *et al.* (2010) [9] reported 57 per cent and 63 per cent shoot regeneration frequency in explants of pomegranate, whereas there was no shoot proliferation observed in MS medium without any growth regulator. Similar results reported by Drazeta and Naik *et al.* (1997) [4] in shoot tip explant and Naik *et al.* (1999 and 2000) [17, 18] in nodal explant in pomegranate. The addition of ADS (60 mg/l) along with other growth regulators was most effective in inducing shoot multiplication (Table-1) (Fig-2).

Shoot length

The highest shoot length was observed when MS medium supplemented with BAP and NAA and no shoot growth in case of MS medium without any growth regulators. Similar results were obtained by Murkute *et al.*, 2004 [15], as well as Gangamma and Arlikatta (2005) [5] in Jack fruit and Patil *et al.* (2011) [20] in pomegranate (Table-2) (Fig-3).

Number of leaves per shoot

It can be observed from trend concerning the maximum number of leaves on proliferation in the medium containing 0.4 mg/l BAP and 0.3 mg/l NAA (Fig-3) (Patil *et al.*, 2011) [20], while they reported that there was no leaf formation when MS media was not supplemented with growth regulators. Patil *et al.* (2011) [20] reported highest number of leaves was obtained when MS medium supplemented with BAP 2.5 mg/l.

Percent of microbial contamination

It is observed and concluded that MS media when supplemented with ADS 15 mg/l + NAA 2.0 mg/l + BAP 2.0 mg/l had given highest maximum shoot proliferation, number of shoots, number of leaves along with highest number of nodes and intermodal length and survival per cent, followed by MS media supplemented with ADS 15 mg/l + NAA 1.0 mg/l + BAP 2.0 mg/l.

Number of days taken for root initiation

The minimum number of days (15.33) were recorded in full strength MS medium supplemented with NAA 0.6 mg/l followed by MS medium supplemented with IBA 0.6 mg/l (17.66), the possible reason for early root initiation by NAA and IBA might be due to their involvement in ethylene biosynthesis (Arteca, 1990). These results were in conformity with the findings of Naik *et al.* (1999) [17] and Murkute *et al.* (2004) [15].

Number of roots developed

Addition of auxins to the medium induced rooting in the regenerated shoots. Similar results were reported by Naik *et al.* (1999) [17] that IBA was best rooting auxin. Same results were also reported by Chaugule *et al.* (2007) [3] in pomegranate, Yadav and singh (2011) in wood apple and Ashrafuzzaman *et al.* (2012) in jack fruit. No root development was observed when MS medium was not supplemented with any auxins (Table-3) (Fig-4).

Per cent of shoots giving roots

It was observed that per cent of shoots giving roots was highest in MS medium + IBA 0.6 mg/l (T_7) (96.66 per cent) followed by MS medium + IBA 0.6 mg/l (T_7) and MS medium + NAA 0.6 mg/l (T_4) (both 86.66 per cent). The results proved that IBA was found to be best auxin for root development. Similar results were reported by Murkute *et al.* (2004) [15] in pomegranate var. Ganesh as rooting in regenerated shoots from cotyledon derived callus was highest when half strength MS medium supplemented with IBA (Table-4) (Fig-5).

Length of roots

The root length was maximum in case of MS media supplemented with NAA 0.6 mg/l as NAA has been best auxin for root development, similar results was reported by Naik and Chand, 2003 [18]. Root development was not recorded in MS medium without any growth regulators.

Table 1: Number of shoots developed per explant (at 20th day, 40th day and 60th day)

S. No.	Treatments	Number of days			Mean
		20 days	40 days	60 days	
1	T_1- MS medium + ADS 15 mg/l + NAA 0.0 mg/l + BAP 0.0 mg/l	0.00 (0.70)	0.03 (0.72)	0.10 (0.77)	0.04 (0.73)
2	T_2- MS medium + ADS 15 mg/l + NAA 0.0 mg/l + BAP 1.0 mg/l	0.03 (0.72)	0.16 (0.81)	0.23 (0.85)	0.14 (0.79)
3	T_3- MS medium + ADS 15 mg/l + NAA 0.0 mg/l + BAP 2.0 mg/l	0.06 (0.75)	0.30 (0.89)	0.50 (0.99)	0.28 (0.87)
4	T_4- MS medium + ADS 15 mg/l + NAA 1.0 mg/l + BAP 0.0 mg/l	0.06 (0.75)	0.10 (0.77)	0.16 (0.81)	0.10 (0.77)
5	T_5- MS medium + ADS 15 mg/l + NAA 1.0 mg/l + BAP 1.0 mg/l	0.36 (0.92)	0.73 (1.10)	0.83 (1.15)	0.64 (1.05)
6	T_6- MS medium + ADS 15 mg/l + NAA 1.0 mg/l + BAP 2.0 mg/l	0.50 (0.99)	0.86 (1.16)	1.16 (1.28)	0.84 (1.14)
7	T_7- MS medium + ADS 15 mg/l + NAA 2.0 mg/l + BAP 0.0 mg/l	0.40 (0.94)	0.63 (1.06)	0.66 (1.07)	0.56 (1.02)
8	T_8-MS medium + ADS 15 mg/l + NAA 2.0 mg/l + BAP 1.0 mg/l	0.40 (0.94)	0.76 (1.12)	0.93 (1.19)	0.69 (1.08)
9	T_9- MS medium + ADS 15 mg/l + NAA 2.0 mg/l + BAP 2.0 mg/l	0.50 (0.99)	1.16 (1.29)	1.33 (1.35)	0.99 (1.21)
	Mean	0.25 (0.85)	0.52 (0.99)	0.65 (1.05)	
	S. Em. ±	0.04	0.03	0.03	
	CD (P = 0.05)	0.12	0.08	0.1	

Table 2: Length of shoots in cm (at 30th day and 60th day)

S. No.	Treatments	Number of days		Mean
		30 days	60 days	
1	T_1- MS medium + ADS 15 mg/l + NAA 0.0 mg/l + BAP 0.0 mg/l	0.00	0.00	0.00
2	T_2- MS medium + ADS 15 mg/l + NAA 0.0 mg/l + BAP 1.0 mg/l	0.08	0.15	0.11
3	T_3- MS medium + ADS 15 mg/l + NAA 0.0 mg/l + BAP 2.0 mg/l	0.15	0.26	0.20
4	T_4- MS medium + ADS 15 mg/l + NAA 1.0 mg/l + BAP 0.0 mg/l	0.11	0.27	0.19
5	T_5- MS medium + ADS 15 mg/l + NAA 1.0 mg/l + BAP 1.0 mg/l	0.78	1.4	1.09
6	T_6- MS medium + ADS 15 mg/l + NAA 1.0 mg/l + BAP 2.0 mg/l	0.72	1.26	0.99
7	T_7- MS medium + ADS 15 mg/l + NAA 2.0 mg/l + BAP 0.0 mg/l	0.65	1.18	0.91
8	T_8-MS medium + ADS 15 mg/l + NAA 2.0 mg/l + BAP 1.0 mg/ll	0.78	1.8	1.29
9	T_9- MS medium + ADS 15 mg/l + NAA 2.0 mg/l + BAP 2.0 mg/l	1.1	1.88	1.49
	Mean	0.49	0.91	
	S. Em. ±	0.15	0.27	
	CD (P = 0.05)	0.45	0.8	

Table 3: Number of roots developed per shoot

S. No.	Treatments	Days		Mean
		20 days	30 days	
1	T_1- MS medium	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
2	T_2- MS medium + NAA 0.2 mg/l	3.33 (6.14)	6.66 (12.28)	4.99 (9.21)
3	T_3- MS medium + NAA 0.4 mg/l	6.66 (12.28)	13.33 (21.14)	9.99 (16.71)
4	T_4- MS medium + NAA 0.6 mg/l	16.66 (23.85)	23.33 (28.78)	19.99 (26.31)
5	T_5- MS medium + IBA 0.2 mg/l	0.00 (0.00)	3.33 (6.14)	1.66 (3.07)
6	T_6- MS medium + IBA 0.4 mg/l	10.00 (15.00)	20.00 (26.56)	15.00 (20.78)
7	T_7- MS medium + IBA 0.6 mg/l	23.33 (28.78)	26.66 (30.99)	24.99 (29.88)
	Mean	8.57 (12.29)	13.33 (17.98)	
	S.Em.±	4.62	3.64	
	CD (P = 0.05)	14.02	11.04	

Table 4: Per cent of shoots giving roots

S. No.	Treatments	Days		Mean
		20 days	30 days	
1	T_1- MS medium	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
2	T_2- MS medium + NAA 0.2 mg/l	3.33 (6.14)	43.33 (35.85)	23.33 (20.99)
3	T_3- MS medium + NAA 0.4 mg/l	36.66 (31.92)	73.33 (59.21)	54.99 (45.56)
4	T_4- MS medium + NAA 0.6 mg/l	86.66 (68.85)	83.33 (66.14)	84.99 (67.49)
5	T_5- MS medium + IBA 0.2 mg/l	0.00 (0.00)	13.33 (13.07)	6.66 (6.53)
6	T_6- MS medium + IBA 0.4 mg/l	46.66 (38.06)	86.66 (68.85)	66.66 (53.45)
7	T_7- MS medium + IBA 0.6 mg/l	86.66 (72.78)	96.66 (83.85)	91.66 (78.31)
	Mean	37.13 (31.10)	56.66 (46.71)	
	S.Em.±	10.49	8.99	
	CD (P = 0.05)	31.82	27.27	

Table 5: Length of roots in cm

S. No.	Treatments	Days		Mean
		20 days	30 days	
1	T_1- MS medium	0.00	0.00	0.00
2	T_2- MS medium + NAA 0.2 mg/l	0.60	1.00	0.80
3	T_3- MS medium + NAA 0.4 mg/l	0.50	1.30	0.90
4	T_4- MS medium + NAA 0.6 mg/l	2.46	2.86	2.66
5	T_5- MS medium + IBA 0.2 mg/l	0.00	0.23	0.12
6	T_6- MS medium + IBA 0.4 mg/l	2.13	2.56	2.35
7	T_7- MS medium + IBA 0.6 mg/l	1.90	2.70	2.30
	Mean	1.08	1.52	
	S.Em.±	0.31	0.33	
	CD (P = 0.05)	0.95	0.99	

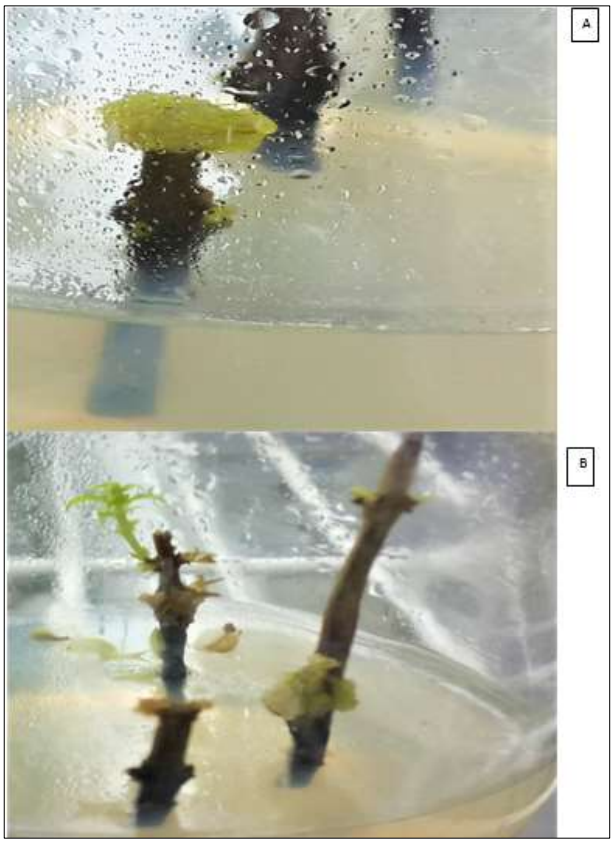

Fig 1: Callus formation and proliferation of pomegranate cv. Bhagwa shoots in A) MS medium + ADS 15 mg/l + NAA 1.0 mg/l + BAP 2.0 mg/l (T_6). B) MS medium + ADS 15 mg/l + NAA 2.0 mg/l + BAP 2.0 mg/l (T_9)

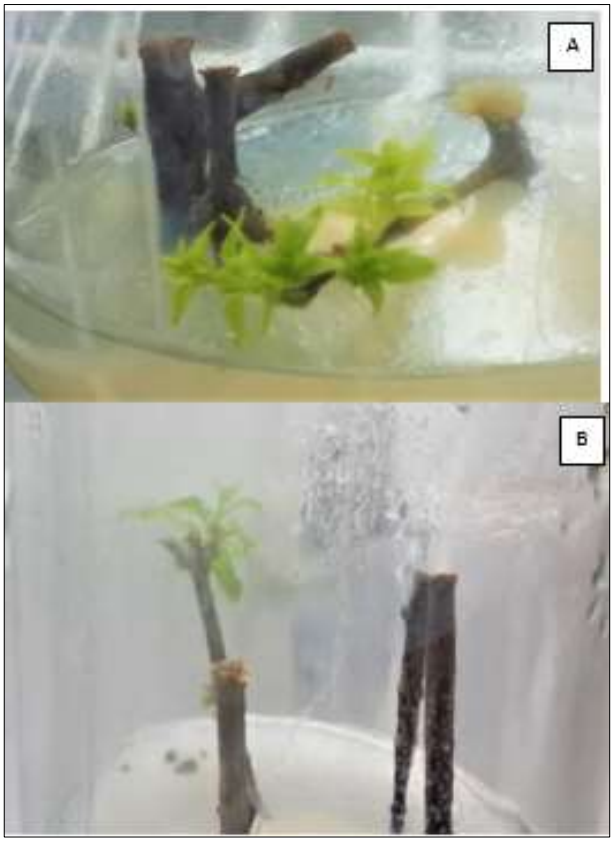

Fig 2: Proliferation of pomegranate cv. Bhagwa shoots A) MS medium + ADS 15 mg/l + NAA 2.0 mg/l + BAP 2.0 mg/l (T_9) B) MS medium + ADS 15 mg/l + NAA 1.0 mg/l + BAP 2.0 mg/l (T_6)

Fig 3: Multishoots ready for subculturing

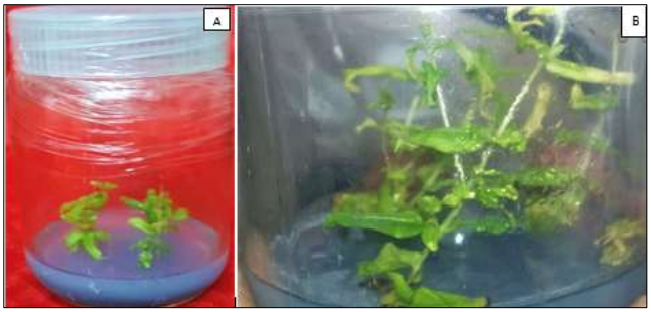

Fig 4: Elongation and multiplication of pomegranate cv. Bhagwa micro-shoots cultured on MS medium + ADS 15 mg/l + NAA 2.0 mg/l + BAP 2.0 mg/l (T_9) media A) Shoots at the time of transfer to elongation media B) After 25 days culture transfer in elongation media

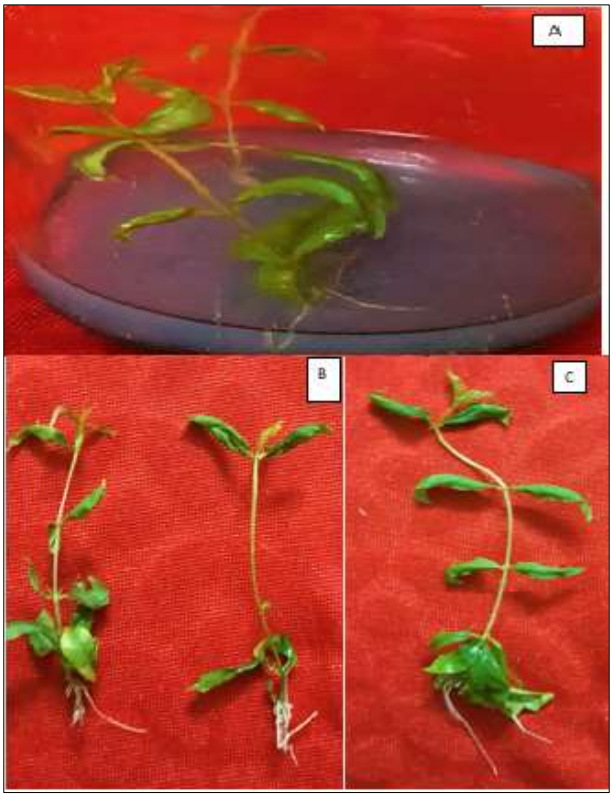

Fig 5: Rooted plantlets of pomegranate cv. Bhagwa in A) MS medium + NAA 0.6 mg/l (T_4) B) MS medium + IBA 0.4 mg/l (T_6) C) MS medium + IBA 0.6 mg/l (T_7)

Conclusions

The present investigation revealed that It is observed that MS media when supplemented with ADS 15 mg/l + NAA 2.0 mg/l + BAP 2.0 mg/l had given the highest maximum shoot proliferation, number of shoots, number of leaves along with a highest number of nodes and intermodal length and survival per cent, followed by MS media supplemented with ADS 15 mg/l + NAA 1.0 mg/l + BAP 2.0 mg/l.

MS media when supplemented with IBA 0.6 mg/l had given a maximum number of roots per shoot, the highest number of shoots producing roots and more number of new leaves followed by MS media supplemented with 0.6 mg/l NAA.

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