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In vitro propagation of pomegranate

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ABSTRACT

An investigation on "Micropropagation of pomegranate (PunicagranatumL.) Var. Bhagwan" In the present investigation total five sub-experiments was carried out. In which CRD was applied for a laboratory experiment and RBD for field experiment to find out best explants, surface sterilants, shooting media, rooting media, and hardening mixtures. Regarding the suitability of explants, shoot tip was best for culture establishment. The study on surface sterilization revealed that explants treated with 0.1 percent mercuric chloride for 10 minutes showed the maximum survival percentage (90.58%) and less contamination percentage (9.42) in shoot tip explants. Shoot differentiation study showed that maximum number of shots per shoot tip explant (1.73) was recorded in treatment containing MS + BAP 2.0 mg/l, maximum shoot length (2.80 cm) was observed on medium containing MS + BAP 2.5 mg/l and maximum number of leaves (7.73) was recorded on medium containing MS +BAP 2.5mg/among the auxins used in the rooting experiment the maximum number of roots(9.66) per shoot tip explant was recorded in treatment containing ½ MS + NAA 6 mg/l. Maximum root length (3.20 cm) was found on medium containing ½ MS + IBA mg/l. While hardening in different hardening media, it was observed that maximum (71.87%) survival of plantlets from shoot tip explants was found on medium containing soil + sand (1:1v/v).

Keywords: *Micro propagation, In vitro, Pomegranate, Bhagwa*

1. INTRODUCTION

Pomegranate (Punicagranatum L.) Var. Bhagwa belonging to the family Punicaceae is one of the favorite table fruit of tropical and subtropical countries. Pomegranate is native of Iran and is extensively cultivated in Mediterranean countries like Spain, Morocco, and also in Afghanistan and Baluchistan. India is the world's leading producing country of pomegranate. The estimated area under pomegranate was 1.25 lakh ha with total production 8.20 lakh mt and productivity of 6.6 mt/ha. In India, through a number of states cultivate pomegranate, the main state which has the maximum area in Maharashtra followed by Karnataka.

The availability of planting material from the existing plantation is insufficient to meet the ever-increasing demand. The need of the hour is to produce large quantum and disease-free planting material from existing plant material economically. The exploitation of the concept of totipotency (Haberlandt, 1902) has progressed from remote possibility to a rapidly expanding reality as evident from the number of species that are now being successfully propagated through tissue culture. Besides this *in vitro* culture technique is becoming increasingly popular as an alternative and feasible means of vegetative propagation in some commercially important plants (Murashige and Skoog 1962).In India, very few attempts have been made on *in vitro* propagation studies in pomegranate to exploit the possibility of micropropagation technique for rapid multiplication. By using shoot tip culture, nodal segments, cotyledons, petals and leaf margins the *in vitro* multiplication of elite and popular varieties of pomegranate were carried by Chaugule 2002 in Mridula, Murkute et al., 2004 in variety Ganesh (Natraja et al.,1995) in Jyoti are being reported.

Keeping these aspects in view the present investigation entitled "Micropropagation of pomegranate (Punicagranatum L.) Var. Bhagwa" was undertaken at Tissue Culture Laboratory, Marathwada Krishi Vidhyapeeth, Parbhani to develop a dependable protocol for large-scale clonal multiplication of pomegranate under *in vitro* conditions.

2. METHODOLOGY

2.1 Collection of explants

The different explants used in this study were a nodal segment, leaf segment, shoot tip was collected from pomegranate trees Var. Bhagwa maintained in the Central Nursery Scheme of M.K.V, Parbhani.

2.2 Excision and sterilization of explants

After taking the explants of optimum size from a plant source, they were washed with detergent (Tween 20) solution for 10 minutes and under running tap water 4-5 washing was given to remove traces of detergent with the 3-4 washing of Double distilled water given. All further operations were carried out in

aseptic conditions in laminar airflow cabinet. All the explants were surface sterilized with 0.1% HgCl2 or NaOCl2 (0.5 %) solution at different times and immediately rinsed with sterile distilled water 3-4 times to remove all the traces of HgCl2 or NaOCl2.

The explants were cut aseptically in laminar airflow cabinet to get explants of appropriate size and shape. The shoot tip of 1.0-1.5cm nodal segments of 1.0-1.5 cm with at least one auxiliary bud and leaf segments of 3mm x 5mm size were cut for inoculation.

2.3 Inoculation of explants

The surface sterilized and aseptically cut explants were finally placed on media by working in a laminar airflow cabinet. The test tubes containing medium prepared as per various treatments were unplugged by holding them over a spirit lamp and inoculations were performed by placing explants on the surface of the medium with the help of flame sterilized long forceps and replacing the plug of the test tube. During inoculation, the explants were properly positioned in the media and were gently pressed with forceps to secure their firm contact with the media.

2.4 Incubation of culture

The culture test tubes after inoculation were kept in a culture room at 25± 10C temperature with a relative humidity of 65± 5 percent and were exposed to 16 hours photoperiod and 8hour dark period with 2500-3000 Lux photoperiods.

3. EXPERIMENTAL DETAILS

3.1 Experiment- I: Effect of type of explants on culture establishment.

Treatment details:

T1: Shoot tip, **T2:** Nodal segments, **T3:** Leaf segments

Design: CRDReplications: 7

All explants were inoculated on MS- media containing 3 percent sucrose and 0.8 percent agar.

3.2 Experiment-II: In order to avoided browning and infection from fungus and bacteria. The sterilization of explants is necessary. Washing of explants response to sterilization procedures by using $NaOCl_2$ and $HgCl_2$ is given as under below trail.

Standardization of sterilization procedure for explants

Treatment detail:

T1: $NaOCl_2$ (0.5%) 5 minutes, **T2:** $NaOCl_2$ (0.5%) 10 minutes

T3: $NaOCl_2$ (0.5%) 15 minutes, **T4:** $HgCl_2$ (0.1%) 5 minutes

T5: $HgCl_2$ (0.1%) 10 minutes, **T6:** $HgCl_2$ (0.1%) 12 minutes

Design : CRD**Replication** : 4

3.3 Experiment-III: Effect of MS media and different concentration of cytokinins on shoot proliferation

Treatment details:

T1: MS + BAP 1.0 mg/l, **T2:** MS+ BAP 1.5 mg/l

T3: MS+ BAP 2.0 mg/l, **T4:** MS+ BAP 2.5 mg/l

T5: MS+ Kn 0.4 mg/l, **T6:** MS+ Kn 0.6 mg/l

T7: MS+ Kn 0.8 mg/l, **T8:** MS+ Kn 1.0 mg/l

T9: MS+ (Control)

Design : CRD**Replication**: 3.

3.4 Experiment-IV

Effect of source and concentration of auxins on *in vitro* rooting

Treatment Details:

T1: ½ MS + NAA 2 mg/l, **T2:** ½ MS+ NAA 4 mg/l

T3: ½ MS+ NAA 6 mg/l, **T4:** ½ MS+ NAA 8 mg/l **T5:** ½ MS+ IBA 2 mg/l, **T6**: ½ MS+ IBA 4 mg/l

T7: ½ MS+ IBA 6 mg/l, **T8**: ½ MS+ IBA 8 mg/l**T9**: 1/2MS + control

Design : CRD**Replication**: 3

3.5 Experiment-V

Effect of different proportion of hardening media on seedling

In order to acclimatize *in vitro* produced pomegranate Var. Bhagwa plantlets to natural environment following experiment was carried out. After 15-20 days culture of micro shoots on rooting medium which was resulted in the sufficient rooting of shoots, the uniform plantlets were transplanted to polythene bag of different proportion of hardening media and percent survival was calculated.

T1: Sand, **T2:** Soil + Sand (1:1 v/v)

T3: Soil + Sand + Vermicompost + (1:1: v/v)

Design : RBD**Replication**: 8

Observation to be recorded

Mean number of shoots produced per explants

Mean shoot length (cm)

The mean length (cm) of each shootlet was recorded at transferring them to media for root differentiation.

Mean number of leaves per shoot

The mean numbers of leaves from each shoot of the nodal segment and shoot tips were recorded under culture.

Percent survival of explants

Observation of percent survival explants was recorded after inoculation and percent survival was calculated.

Percent contamination

Percent contamination of initiation culture was recorded by any fungal, bacterial contamination at the time of surface sterilization

Mean number of roots per shoots

The number of roots per shoot produced by the plantlets was counted.

Mean root length (cm)

The root length produced by the plantlet was measured with help of iron scale in centimeter.

Number of days taken for rooting

The number of days taken for root initiation were counted treatment and represented as numbers of days for root induction.

Percent survival of plantlets after hardening

The number of plantlets survived out of total plantlets subjected to hardening was counted at 5 weeks after transplanting in different hardening media and the percentage was calculated

Statistical analysis

The completely randomized design was employed to analyze the "*Micropropagation* of pomegranate Var. Bhagwa" in Laboratory condition and randomized block design in greenhouse condition. Ten aliquots having one explant in each treatment were used for recording different observation and mean values of five aliquots were used for statistical analysis. The data in percentage were transformed to arc sin values for statistical analysis. The data were subjected to ANOVA as suggested by Panse and Sukhatme (1967). Critical difference

values were tabulated at 1 percent probability for a laboratory experiment and at 5 percent probability for field experiment where 'F' test was significant.

4. RESULT

4.1 Effect of type of explants on culture establishment

4.1.1 Mean number of shoots produced per explants

Data pertaining to the mean a number of shoots produced per explants are presented in Table 1. The results are shown in the plate. A perusal of data in Table 1 indicated that there were significant differences among the treatments with respect to a number of shoots per explants. A mean number of shoot per explants ranged from 0.00 to 2.40 under a different type of explants. The highest number of the shoot (2.40) was observed on the nodal segment followed in the treatment of shoot tip (1.48). Leaf segment did not show any shoot proliferation.

Treatment detail.

Number of shoot per explants

Table 1: Effect of type of explants on the mean number of shoots produced per explants

T1	Shoot tip	1.48
T2	Nodal segments	2.40
T3	Leaf segments	0.00
S.E		0.15
C.D at 1%		0.47

4.1.2 Mean number of leaves per explants

Data pertaining to mean number leaves produced per shoot of explants are presented in Table 2. The result respects are shown in Plate. A perusal of data in Table 2 indicates that there were significant differences among the treatment with respect to a number of leaves per explants. A mean number of leaves per explants ranged from 0.00 to 7.28 under a different type of explants. A maximum number of leaves (7.28) was observed on the nodal segment followed by shoot tip (4.68). Leaf segments showed no leaves production.

Treatment-Treatment detail

Number of leaves per shoots

Table 2. Effect of type of explants on the mean number of leaves per explants

T1	Shoot tip	4.68
T2	Nodal segments	7.28
T3	Leaf segments	0.00
S.E		0.13
C.D at 1%		0.41

4.1.3 Mean shoot length of explants (cm)

Data pertaining to mean shoot length (cm) produced by different explants are presented in Table 3.the result was shown in Plate.

It was evident from the data presented in Table 3 that the shoot length (cm) produced on different explants were statistically significant. The length of shoot ranged from 0.00 cm to 2.53cm in different explants with a general mean of 1.65cm. Significantly maximum length of shoot (2.53cm) was recorded in the nodal segment, except shoot tip (2.42cm) which were statistically at par. Leaf segment did not show shoot length (cm).

Treatment -Treatment detail

Shoot length (cm)

Table 3: Effect of the type of explants on mean shoot length (cm)

T1	Shoot tip	2.42
T2	Nodal segments	2.53
T3	Leaf segments	0.00
S.E		0.17
C.D at 1%		0.51

4.1.4 Percent contamination of explants after initiation

Data pertaining to the effect of type of explants on percent contamination of explants are presented in Table 4.The data presented in Table 4 indicated that the type of explants had been contaminated in different treatments under study. The highest percent of contamination was observed with (20 %) in the nodal segment and the minimum percentage of contamination (14.28%) was observed in the shoot tip. Leaf segment was observed dried.

Treatment -Treatment detail

Percent contamination

Table 4: Effect of the type of explants on percent contamination after initiation

T1	Shoot tip	14.28(8.21)*
T2	Nodal segments	20.00(11.53)*
T3	Leaf segments	0.00
S.E		0.53
C.D at 1%		1.57

(*) fig denotes arc sign value

4.1.5 Percent survival of explants after initiation

Data pertaining to percent survival are presented in Table 5. A perusal of data in Table 5 resulted that there was a significant difference observed among the different treatments mean survival percentage ranged from 0.00 to 44.25 percent under different treatment. Significantly the maximum survival percentage of explants was observed in the shoot tip (44.25%) and minimum survival percentage (35.65%) was noticed in the nodal segment. There was no survival percentage of explants in the leaf segment.

Treatment -Treatment detail

Percent survival of explants

Table 5: Effect of type of explants on percent survival of explants after initiation

T1	Shoot tip	44.25(26.29)*
T2	Nodal segments	35.65(20.89)*
T3	Leaf segments	0.00
S.E		1.02
C.D at 1%		3.04

(*) fig denotes arc sign value

4.1.6 Effect of surface sterilant and duration of time on shoot tips of explants

Shoot tip explants showed better survival percent and less contamination as compared to rest of explants under study. Hence shoot tip explants were cultured on the shoot and root media to study shoot proliferation, root proliferation and further hardening of plantlets.

4.1.7 Contamination percentage of shoot tip explants after Sterilization

Data pertaining to the contamination percentage are presented in Table 6. It is evident from the data presented in Table 6 that the contamination percent differed significantly under different treatment lowest contamination percent (9.42%) was recorded in treatment T5 i.e. ($HgCl_2$ 0.1% for 10 minutes)

which was significant over the other treatment under study. Highest contamination of shoot tip of explants was recorded in treatment T1, except T3 and T6, which were statistically at par with each other. The treatment T2 recorded next more contamination (78.07%)

4.1.8 Survival percentage of shoot tip explants after sterilization

The data pertaining to of surface sterilant and duration of treatment on sterilization procedure for shoot tip of explants are presented in Table 6. The data revealed that the survival percent differed significantly and ranged from (0.00 to 90.58 %) different treatment under study.

Highest survival percent was recorded (90.58%) in treatment T5 ($HgCl_2$ 0.1%) for 10 minutes which was significant over the rest of the treatments under study. It was found that the explants were killed when the treatment duration was increased beyond 10 minutes for both surface sterilants ($NaOCl_2$ 0.5%) and $HgCl_2$ 0.1%) resulting into 0.00 percent survival of shoot tip explants.

Treatment-Treatment detail (min)

Table 6: Effect of surface sterilant and duration of time on shoot tip of explants

		Survival percent	**Contamination percent**
T1	NaOCl2 (0.5%) 5min	0.00(0.00)*	100.00(0.00)*
T2	NaOCl2 (0.5%)10	21.91(78.09)*	78.07(21.93)*
T3	NaOCl2 (0.5%) 15	0.00(0.00)*	100.0(0.00)*
T4	HgCl2(0.1%) 5	57.55(42.45)*	42.45(57.55)*
T5	HgCl2(0.1%) 10	90.58(9.42)*	9.42(90.58)*
T6	HgCl2(0.1%)12	0.00(0.00)*	100.0(0.00)*
S.E		1.30	6.03
C.D at 1%		3.87	17.90

(*) fig denotes arc sign value

4.2 Standardization of the growth regulators for shoot growth

4.2.1 Mean a number of shoots produced on shoot tip explants.

Data pertaining to the mean a number of shoots produced in different treatment are presented in Table 7. The results are shown in Plate. Data presented in Table 7 revealed that a number of shoots produced on shoot tip explants under different growth regulators differed significantly. The highest number of shoot per shoot tip of explants was recorded in treatment T3 (1.73) which was at par with T1, T2, and T4. The lowest number of the shoot (0.93) was recorded in treatment T7 and T8 which was at par with T5 and T6. No shoots per shoot tip explants were recorded in Treatment T9.

Treatment -Treatment detail

Number of shoots

Table 7: Effect of growth regulators on a mean number of shoots produced on shoot tip explants.

T1	MS + BAP 1.0 mg/l	1.33
T2	MS + BAP 1.5 mg/l	1.53
T3	MS + BAP 2.0 mg/l	1.73
T4	MS + BAP 2.5 mg/l	1.60
T5	MS + Kn 0.4 mg/l	1.13
T6	MS+ Kn 0.6 mg/l	1.00
T7	MS+ Kn 0.8 mg/l	0.93
T8	MS + Kn 1.0 mg/l	0.93
T9	MS + control	0.00
S.E		0.17
C.D at 1%		0.52

4.2.2 Mean number of leaves per shoots of shoot tip explants

The data regarding the effect of a growth regulator on a mean number of leaves per shoot of shoot tip explants in different treatment is presented in Table 8. The results effect is shown in Plate. Data from Table 8 revealed that significant a number of leaves per shoot tips of explants (7.73) was recorded in treatment T4 which was at par with T2, T3, and T5. The less number of leaves (5.60) was recorded in treatment T6 which was at par with T1, T7, and T8. No leaves per shoot tip of explants were recorded in treatment T9.

Treatment-Treatment detail

Number of leaves

Table 8: Effect of growth regulators on a mean number of leaves per shoots of shoot tip explants

T1	MS + BAP 1.0 mg/l	5.66
T2	MS + BAP 1.5 mg/l	7.46
T3	MS + BAP 2.0 mg/l	7.26
T4	MS + BAP 2.5 mg/l	7.73
T5	MS + kn 0.4 mg/l	6.73
T6	MS + kn 0.6 mg/l	5.60
T7	MS + kn 0.8 mg/l	5.86
T8	MS + kn 1.0 mg/l	6.33
T9	MS + control	0.00
S.E		0.35
C.D at 1%		1.05

4.2.3 Mean shoot length (cm) of shoot tip explants

The data regarding effect growth regulators on shoot length (cm) of shoot tip explants in different treatment are presented in Table 9. The resulting effect is shown in Plate.

It was evident from the data presented in Table 9 that significant differences were observed among the treatments with respect to shooting length (cm) per shoot tip explants. Maximum shoot length (2.80cm) was observed in treatment T4 which was statistically at par with T2 and T3. In control treatment T9 there no result of shoot length. The minimum shoot length (1.68cm) was observed in treatment T6 which was at par with T1, T5, T7, and T8.

Treatment -Treatment detail

Shoot length (cm)

Table 9: Effect of growth regulators on mean shoot length (cm) of shoot tip explants

T1	MS + BAP 1.0 mg/l	1.72
T2	MS +BAP 1.5 mg/l	2.61
T3	MS + BAP 2.0 mg/l	2.71
T4	MS + BAP 2.5 mg/l	2.80
T5	MS + Kn 0.4 mg/l	1.78
T6	MS + Kn 0.6 mg/l	1.68
T7	MS + Kn 0.8 mg/l	1.68
T8	MS + Kn 1.0 mg/l	1.92
T9	MS + control	0.00
S.E		0.14
C.Dat 1%		0.41

4.3 Standardization of growth regulators for roots growth of shoot tip explants

4.3.1 Mean number of roots per shoot of shoot tip explants

The data with regard to the effect of growth regulators on a mean number of roots per shoot in shoot tip explants are presented in Table 10 and the result was shown in Plate.

The data presented in Table10 indicated that the mean number of roots per shoot tip explants was statistically significant among the treatments under study. The highest number of roots per shoot tip of explants (4.00) was recorded in Treatment T8 which was statistically at par with Treatment T7. Lowest number of root produced (2.20) respected in Treatment T1 which was at par with T2. No result was found in control treatment T9.

Treatment -Treatment detail

Number of roots per shoot

Table 10: Effect of growth regulators on a mean number of roots per shoot of shoot tip explants

T1	1/2 MS+ NAA 2 mg/l	2.20
T2	1/2 MS+ NAA 4 mg/l	2.40
T3	1/2 MS+ NAA 6 mg/l	3.06
T4	1/2 MS+ NAA 8 mg/l	3.00
T5	1/2 MS+ IBA 2 mg/l	3.13
T6	1/2 MS+ IBA 4 mg/l	3.20
T7	1/2 MS+ IBA 6 mg/l	3.86
T8	1/2 MS+ IBA 8 mg/l	4.00
T9	1/2MS + control	0.00
S.E		0.14
C.D at 1%		0.44

4.3.2 Mean root length of shoot tip explants

Data pertaining to the effect of growth regulators on the mean root length of shoot tip explants was presented in Table 11 and the result is shown in Plate. It was evident from the data mentioned in Table 11. That the mean root length (cm) in different treatment differed significantly. Maximum root length (3.20cm) was observed in treatment T8 which was statistically at par with T1, T3, and T6.

The minimum root length (2.15cm) was observed in treatment T5 which was statistically at par with T2, T4, and T6.In treatment T9 no result was found.

Treatment -Treatment detail

Root length (cm)

Table 11: Effect of growth regulators on mean root length of shoot tip explants

T1	1/2MS + NAA 2 mg/l	2.75
T2	1/2MS + NAA 4 mg/l	2.22
T3	1/2MS + NAA 6 mg/l	2.79
T4	1/2 MS + NAA 8 mg/l	2.28
T5	1/2 MS + IBA 2 mg/l	2.15
T6	1/2 MS + IBA 4 mg/l	2.52
T7	1/2 MS + IBA 6 mg/l	2.94
T8	1/2 MS + IBA 8 mg/l	3.20
T9	1/2 MS + control	0.00
S.E		0.20
C.D at 1%		0.60

4.3.3 Number of days taken for rooting of shoot tip explants

The data representing the effect of growth regulators on a number of days taken for rooting of shoots tip explants are presented in Table 12. Data differed significantly in all treatment under study. Significantly less number of days taken for rooting in treatment T8 (8.33). The next best treatment was T3 (9.68) which were statistically at par with T2 and T4. The number of days for rooting (11.06) was observed in the treatment T6 which was statistically at par with T1, T5, and T7. Treatment T9 showed no result regarding days required for rooting.

Treatment-Treatment detail

Number of days for rooting

Table 12: Effect of growth regulators on a number of days taken for rooting of shoot tip explants

T1	1/2MS + NAA 2 mg/l	10.46
T2	1/2 MS + NAA 4 mg/l	9.80
T3	1/2 MS + NAA 6 mg/l	9.66
T4	1/2 MS + NAA 8 mg/l	9.80
T5	1/2 MS + IBA 2 mg/l	10.53
T6	1/2 MS + IBA 4 mg/l	11.06
T7	1/2 MS + IBA 6 mg/l	10.13
T8	1/2 MS + IBA 8 mg/l	8.33
T9	1/2 MS + control	0.00
S.E		0.39
C.D at 1%		1.18

4.4 Standardization of optimum condition for hardening of plantlets

4.4.1 Percent plantlets survival of shoot tip explants at hardening

Data in respect of effect of the different proportion of hardening media on plantlets survival of shoot tip explants are presented in Table 13 the results are shown in Plate.

Present data differed significantly and maximum percent survival of plantlets of shoot tip explants at hardening (71.87%) was recorded in treatment T2 (Soil + Sand (1:1 v/v) followed by treatment T1 (68.72 %).

Minimum percent survival plantlets of shoot tip explants at hardening were observed in treatment T3 (50.05 %).

Treatment-Treatment detail

Percent survival

Table 13: Effect of different proportion of hardening media on plantlets survival of shoots tip explants

T1	Sand	68.72(43.41)*
T2	Soil+sand(1:1 v/v)	71.87(46.00)*
T3	Soil+sand+vermicompost (1:1v/v)	50.05(30.04)*
S.E		0.88
C.D at 1%		2.6

(*) fig denotes arc sign value

Fig. 1: Explant (shoot tip, nodal segment, leaf segment)

Fig. 2: Response of growth regulator for Shooting

Fig. 3: Response of growth regulator for Rooting

Fig. 4: Hardening

Fig. 5: Seedling

5. DISCUSSION

Source of explants for micropropagation. Nodal segment gave better result regarding a number of the shoot (2.40), a number of leaves (7.28), length of shoot (2.53 cm) but less survival percent (35.65%) with more contamination percent (20%) shoot tip. The shoot tip was next best explants in regards to, the number of shoots per explants was (1.48), number of leaves (4.68), length of shoot (2.42cm). Significantly more survival (44.25 %) and less contamination percent (14.28 %) was recorded as compared to nodal segment explants. Similar results were reported by (Gangamma Aralikatti 2005) in jackfruit, (Patil et al., 2011) in pomegranate.

5.1 Sterilization procedure for shoot tip explants

Survival percentage of shoot tip explants

Sterilization of shoot tip explants was done by using $HgCl_2$ and $NaOCl_2$ of different concentration and a different time. In present study $NaOCl_2$, 0.5% was used for 5 minutes, 10 minutes and 15 minutes, while $HgCl_2$ 0.1% was used for 5 minutes, 10 minutes and 12 minutes for sterilization of shoot tip explants. Use of $HgCl_2$ 0.1% for 5 minutes and 10 minutes gave the highest survival percentage (90.58%) over the other treatment of $NaOCl_2$ and $HgCl_2$ of different time and concentration. Similar results were reported by (Oh et al., 1991), in C. Junos and (Singh et al., 2011) in pomegranate.

Contamination percentage of shoot tip explants

The lowest contamination percent (9.42%) was recorded in the treatment of $HgCl_2$ 0.1% for 10 minutes and it was better over the rest of the treatment. In treatment $NaOCl_2$ 0.5 %, contamination percent for 5 minutes and 15 minutes was (100%); this duration of time and concentration of $NaOCl_2$ was not beneficial for sterilization of shoot tip explants. The results obtained in the present study were on similar lines as reported by (Singh et al., 2011)

Growth regulators for shoot growth of shoot tip explants

The main purpose of the multiplication stage is to maintain the micro-culture in a stabilized state and multiply the micro shoots to large extent. The basic medium of shoot multiplication stage is similar to the rooting stage but often the growth regulators and mineral supplement levels are varied. The growth regulators are used to support a basic level of growth it is also equally important to direct the development response of the propagules (Hartmann et al., 1997) in vitro culture of higher plants with growth regulators auxin and cytokinins were very significant to get the large propagules.

Discovery of cytokinins has helped in vitro propagation of plants by shoot multiplication through regulating shoot growth (Skoog and Miller 1957). Cytokinins are often used to stimulate growth and development. Kinetin, BAP commonly used.

Number of shoots produced per shoot tip explants

About 1-2 shoots develop generally in the present study. A number of multiple shoots from the nodal segment was also reported by (Patil et al., 2011) when the mean number of shoots growing on explants was observed in the treatment (MS + BAP 2.0 mg/l) performed best by allowing the maximum number of shoots (1.73) to sprout and grow. Similar to these finding development of mean shoots from a single explant have been reported by (Naik et al., 1999), as well as (Chaugule 2002) and (Gangamma Arlikatta 2005), respectively. It has also been reported by them that some of this shoot regenerated into roots on the same medium to form complete plantlets. However, in the present study, none of the shoots could regenerate roots unless they were transferred to a different medium. A mean number of shoot (2.60) per culture with 1/2 MS+ BAP 2.0 mg/l in jackfruit was obtained by (Gangamma Arlikatta 2005) as well as (1.9) shoot per culture with 1.0 BAP + 0.5 NAA gm/l in pomegranate was obtained by (Murkute et al., 2004).

Shoot length of shoot tip explants

The shoots length growing in the in vitro culture indicated the potential for growth and response to the culture. The maximum length of the shoot was attained (2.80 cm) in the treatment MS + BAP 2.5 mg/l and this similar result was obtained by (Murkurte et al., 2004) as well as (Gangamma Arlikatta 2005) in jackfruit, respectively and (Patil et al.,2011) in pomegranate.

Mean number of leaves per shoots of shoot tip explants

The mean number of leaves from growing shoots indicated the potential for growth and response to the culture. The maximum number of leaves was attained (7.73) was in the treatment of MS + BAP 2.5 mg/l and this similar results obtained by (Patil et al., 2011).

Growth regulators for root growth of shoot tip explants

Shoots of 2.0 – 2.5 cm length or minimum with 4 leaves whichever was obtained earlier were considered as criteria and shoots were transferred for rooting (Naik et al., 2000). The function of root formation stage was to roots the micro shoots and to prepare them for transplanting out of the aseptic

protected environment of test tubes to outdoor conditions of the greenhouse, media having a low concentration of salts have proven satisfactory for rooting of shoots. But in vitro rooting of herbaceous plants is much easier than woody perennials although the progress is considerable in relation to the primary establishment and multiplication rates. Rooting remained a major problem in woody perennials.

Mean number of roots per shoot of shoot tip explants

In the present investigation best treatment for a mean number of roots per shoot was found to be half strength of medium supplemented with IBA 8 mg/l which was (4.0) shoot per explants. Results were in confirmatively with the findings of this type of (Gangamma Aralikatta2005) in jackfruit as well as (Patil et al., 2011) in pomegranate.

Mean root length (cm) of shoot tip explants

In the present investigation best treatment for the root length (cm) was found to be half strength of MS medium supplemented with IBA 8 mg/l which is (3.20 cm). Similar results were confounded by (Patil et al., 2011) and (Murkute et al., 2004) in pomegranate.

Number of days taken for rooting of shoot tip explants

The earliest root induction with (8.33) days after culture was observed in ½ MS + IBA 8 mg/l. The maximum number of days taken for rooting was (11.06) in ½ MS + IBA 4 mg/l when attained height 3-4 cm were transferred to hardening. A similar result was confounded by (Chaugule 2002) as well as (Murkute et al., 2004).

Plantlets hardening of shoot tip explants

When the plantlets in rooting medium in vitro condition attained a height of plantlet is 3-4 cm, and a number of roots 6-8 was then transferred for primary hardening containing a different mixture of hardening media.

In the present investigation, it was observed that (71.87%) survival of plantlets was recorded in treatment containing (soil + sand 1: 1 v/v). The result of the present investigation was in confirmetly of (Bhagat 2002) and (Bylatha 1993).

The plantlets obtained should be then transferred to hardening media containing (soil + sand 1:1 v/v) which was the best treatment for hardening. Based on the results the protocol suggested above for direct regeneration from shoot tip explants in (Punica granatum. L) Var. Bhagwa is the ideal method for maintaining and propagating elite parental characters in the progeny. However, further refinement is required before this culture method can be applied to commercial cultivar.

6. REFERENCES

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