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Invitro Propagation of Pomegranate (*Punica Granatum L*.) Cv. Wonderful Cultivar

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ABSTRACT

The conventional method of propagation of pomegranate is time-consuming and tiresome. It does not ensure disease-free and healthy plants. The Present investigation aimed to optimize the conditions of culture medium, shoot proliferation, and rooting for mass production of pomegranate trees (*Punica granatum L*.) cv. 'Wonderful Cultivar'. For sterilization of axillary bud explants and shoot apexes of wonderful pomegranate treatment involving $HgCl_2$ (0.1 %) for 3 min gave better sterilization. An efficient in vitro propagation for wonderful pomegranate using shoot tip and axillary bud explants is described. The best media for the establishment was observed on DKW medium for the first three weeks. After that, explants were subcultured on a WPM medium for proliferation and elongation. ½MS and full MS were used for rooting. The maximum number of shoots (3.9) per explant and shoot length (1.5 to 2.95 cm) were obtained on WPM medium supplemented with 0.8 mg/l B A P + 0.01 mg/L IBA in the medium. The highest rooting number was recorded on ½MS medium containing IBA 1mg/l—(1.4) roots per explant. A significantly higher number of shoots (2.5) and maximum length (2.95cm) of the shoot were recorded in 3 percent sucrose, (3000 lux) light intensity, and pH 5.8. Rooted plantlets were adopted and transferred to the soil successfully.

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Introduction

Pomegranate (*Punica granatum* L.) belongs to the family "Punicaceae." It is native to Iran and spread throughout the Mediterranean region of Asia, Africa, and Europe (Sepulveda *et al.*, 2000). It has 2n=2x=16, 18 chromosomes (Smith, 1976). The pomegranate was domesticated in 2000 BC and was one of the first five fruit crops (date palm, fig, olive, grape, and pomegranate) to be domesticated by humanity. Pomegranate has different ecotypes *viz.*, cultivated (*Punica granatum* L.), wild types (*Punica protopunica*), and ornamental forms

(Japanese Dwarf pomegranate - *Punica granatum* var. Nana). The wild pomegranate is confined to Socotra islands, whereas ornamental forms are popular in Southeast Asian countries. Iran is the world's largest producer of pomegranates globally, followed by India, Turkey, Spain, Tunisia, Morocco, Afghanistan, China, *etc.* Over the last decade, the country has registered sizeable increase in area and production. The export of pomegranates from Iran estimated that some 915,000 tons of pomegranates will be produced in Iran in the current Iranian year, which ends on March 20, 2019, registering a 22% increase compared with the previous year. "Last year (March 2017-18), nearly 1,500 tons of pomegranates worth around $1.5 million were exported from the country. Our main export destinations were Armenia, the UAE, South Korea, Iraq, Turkmenistan, Qatar, Germany, Switzerland, Russia, Austria, India, France, and Pakistan," Director General of the Ministry of Agriculture's Tropical and Subtropical Fruits Affairs Bureau, Masoud Latifian, was quoted as saying.

Tissue culture is a technique that can be used to grow plants in all seasons without restrictions, and it has been widely used for propagating many fruit trees. (Skiada et al., 2010; Perez-Tornero et al., 2010). There are reports of in vitro cultivation of *P. granatum*. *P. granatum* Var. "Mels Saveh" and "Yousef Khani" were cultivated in WPM (Woody Plant Medium) and MS (Murashige and Skoog) culture media. It has been reported that **½** WPM culture medium, with appropriate growth regulatory treatments, has been able to create favorable cell divisions and root induction, resulting in stronger and longer branches and successful branching and rooting (Naik et al., 2000; Murkute and Mayakumari, 2003; Chauhan and Kanwar, 2012; Singh et al., 2012).

Due to the development and importance of the plant tissue culture method and the need to develop and propagate the *P. granatum* cv. wonderful as a new cultivar, the micro-growth of this plant in different culture media has been investigated in this study.

Due to its nutritional, nutraceutical, and medicinal properties, there has been a marked shift towards consuming pomegranate worldwide. Pomegranate has wider adaptability ranging from normal soil type, saline soil, and drought conditions. Although pomegranate can be grown under varied climatic conditions of the country, it performs better in semi-arid and arid regions. Pomegranate is commercially cultivated in Iran in Fars, Central, Khorasan Rezavi, and Yazd. These provinces cover about 64 percent of Iran's total pomegranate production (Iran Agri-Statistics, 2016). 'Wonderful'–originated as a cutting in Florida and propagated in California in 1896. The fruit is oblate, very large, dark purple-red, with medium-thick rind; deep-red, juicy, winey pulp; medium-hard seeds. The plant is vigorous and productive.

Materials and Methods

Experimental Details and Treatments

Plant Collection. This work was done at Yazd University, School of Natural Resource and Desert Studies & Department of Agricultural Biotechnology, from February 2018 to February

2019. *Punica granatum* (Wonderful) was collected from the high-yielding 1-year-old tree growing in the Yazd province Pomegranate Research Center, Iran.

Washing of the Explant and Sterilization. Isolated nodal segments were cleaned under running tap water for about 15 to 20 min. Each is under a laminar air flow hood, followed by rinsing three times in sterile distilled water. Then, it was again washed with sterile distilled water. Finally, 0.1 mg/L mercuric chloride solution for 3 min. It was used to treat these explants, followed by rinsing them three times with sterile distilled water to sterilize nodal explants completely. Completely sterilized explants were inoculated on establishment media. After establishing transferred explants on proliferation media for growth, completely proliferated explants were transferred to rooting media.

Culture media and Treatments. MS medium was tested for micropropagation of the pomegranate wonderful cultivar. Media was prepared as a basal medium supplemented with organic acids and vitamins. Sucrose was added at 30.0 g/L. The pH of the prepared media was adjusted between 5.6 and 5.8, and agar-agar (Hi-Media) was added at 8.0 g/L for media solidification. The experiment was treated in plant growth regulators incorporated in combinations and singly for establishment and proliferation stages By BAP (0, 0.1, 0.5, 0.8, and 1.5). And BAP/IBA (0 + 0.01, 0.1 + 0.01, 0.5 + 0.01, 0.8 + 0.01 and 1.5 + 0.01 mg/l) were tested. Also, for the rooting stage, two different mediums, ½ MS and Full MS, were tested at IBA (0.1, 0.8. 1, 1.5 mg/l) on MS medium at full strength and ½ MS.

Statistical analysis. Data were subjected to analysis of variance (ANOVA) based on a completely randomized experimental design. Each treatment consisted of 10 replicates (culture flasks), and each experimental unit had four explants per flask. Each arrangement is in a completely randomized design. If ANOVA indicated statistically significant differences, mean comparisons were conducted using Duncan's multiple range test (DMRT) at a probability level of $P \leq 0.05$.

Results and Discussion

Different explants, especially shoot apex and nodal segments, were obtained from Yazd Province Pomegranate Research Center, Iran. These explants were taken from healthy plants of pomegranate cv. Wonderful, which were irrigated well. Each explant was prepared for incubation as per the procedure described in the material and methods of this manuscript. When different explants like shoot apex and nodal segment of pomegranate incubated on Murashige and Skoog medium without any plant growth regulators, they did not respond well.

Sterilization and Establishment

This investigation showed that Hg_2Cl is the best way to shoot apex and nodal explant sterilization to remove fungal and bacterial contamination. A concentration of 0.1 percent mercuric chloride within 3 minutes is the best treatment for sterilization of shoot apex and nodal explants. 0.1 percent concentration of mercuric chloride showed (66.5%) survival (Fig

1), and the period (3 min) gave the best survival with (59%) observed. The highest contamination was observed in the (0.5%) concentration of Hg2Cl (59.2%) and period (1 min), with (56.6%) contamination of explants.

Table 1: (ANOVA) Effect of different concentrations of Hg2Cl and Time period on Sterilization of the pomegranate explant (Wonderful cultivar).

Sig	F	MS	SS	df	SV
0.000	21.16** 16.71	50.15	3	time	
0.000	85.15**	67.22	134.45	2	Hg_2Cl Concentration
0.000	32.0** 24		106.01	6	Hg_2Cl*time
		0.790	85.3	108	Error
			3765	120	Total

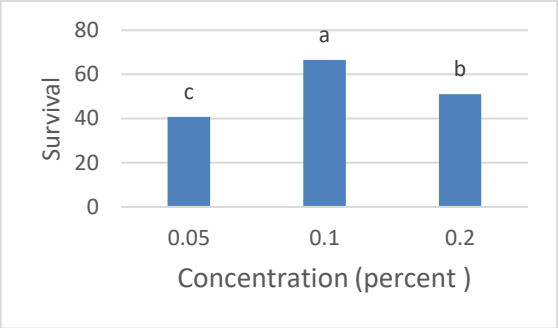

Fig 1: Effect of different concentration of Hg_2Cl on Survival

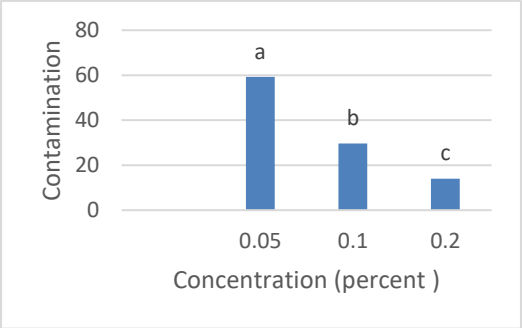

Fig 2: Effect of different concentration of Hg_2Cl on Contamination

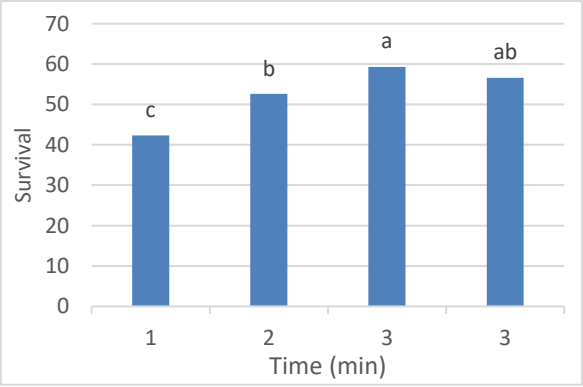

Fig 3: Effect of different time period on Survival

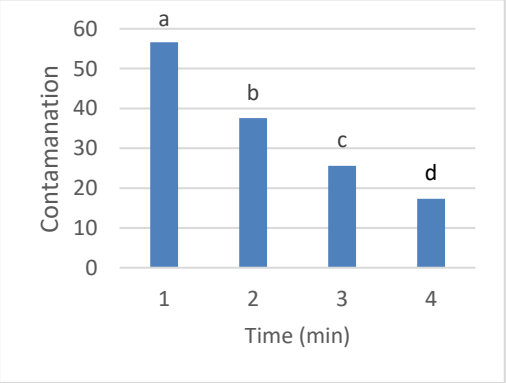

Fig 4: Effect of different time period on Contamination

Note: Columns with different letters are significantly different from each other at P< 0.05 (Duncan's multiple range test). Bars represent SE value.

Proliferation

Effect of cytokinin (BAP) + auxin (IBA) Shoot Length. BAP (0, 0.1, 0.5, 0.8 and 1.5) singly and in combination with BAP/ IBA 0 + 0.01, 0.1 + 0.01, 0.5 + 0.01, 0.8 + 0.01 and 1.5 + 0.01 mg/l) induced only shoot buds in nodal segment explants within 15 – 17 days of vaccination with 100 percent frequency at all the levels of combinations.

Highest shoot length (2.95 cm) was observed at 0.8 mg/l BAP + 0.01 mg/l IBA (Fig. 5).

Table 2. *(ANOVA) Effect of different concentrations of BAP and IBA on Length of Stem of the pomegranate cultivar, Wonderful.*

SOV	df	SS	MS	F	Sig
BAP	9	20.643	2.294	5.213	0.00
Error	90	39.599	.440		
Total	99	60.242			

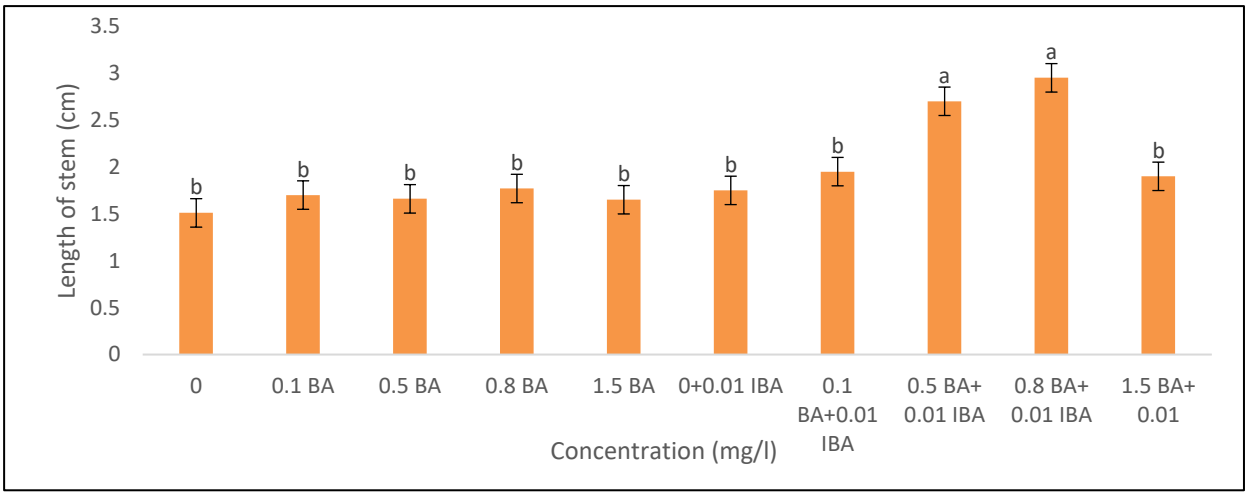

Figure 5. Effect of different concentrations of BAP and IBA on Length of Stem of the pomegranate cultivar, Wonderful. Columns with different letters significantly differ at P< 0.05 (Duncan's multiple range test). Bars represent SE v

Effect of BAP and IBA on shoot proliferation. WPM media were used to grow nodal explants for different concentrations of BAP/IBA (0 + 0.01, 0.1 + 0.01, 0.5 + 0.01, 0.8 + 0.01 and 1.5 + 0.01 mg/l). The highest average growth response (3.9 shoots) in WPM medium containing BAP 0.8 + IBA 0.01 mg/L (Fig 6), whereas 2 to 3.9 shoots per explant having the highest shoot length (2.95 cm) was recorded (Table 3, Fig 6).

Table 3. *(ANOVA) Effect of different concentrations of BAP and IBA on Number of Shoots of the pomegranate cultivar, Wonderful.*

SOV	df	SS	MS	F	Sig
BAP	9	56.560	6.284	13.897	0.000
Error	90	40.700	.452		
Total	99	97.260			

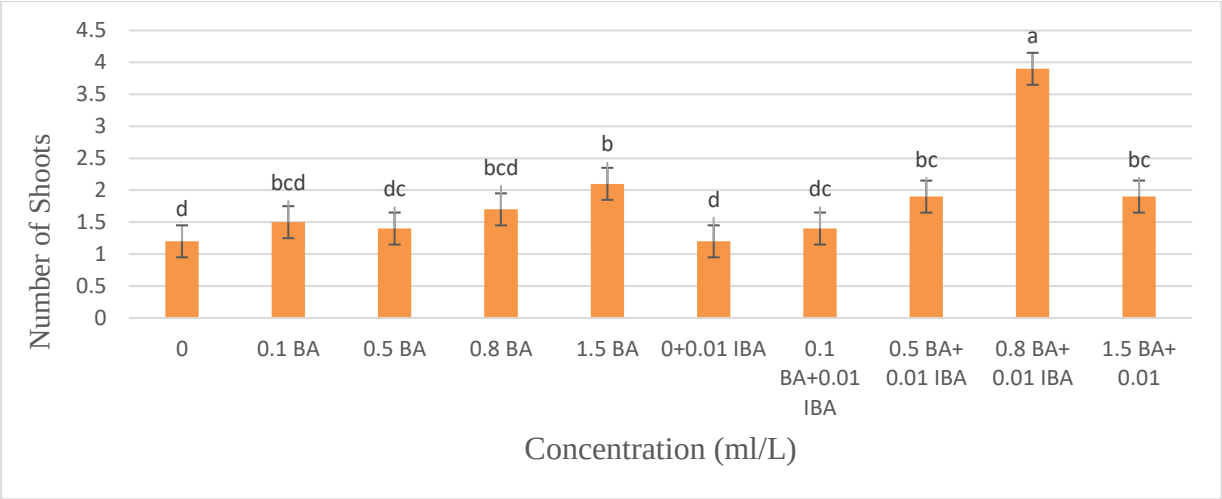

Figure 6. The effect of different concentrations of BAP and IBA on in vitro Numbers of shoots of the pomegranate cultivar is wonderful. Columns with different letters significantly differ at P< 0.05 (Duncan's multiple range test). Bars represent SE

Effect of BAP and IBA in MS medium on number of leaves. This survey showed that the best treatment for several leaves is 0.8 BAP + 0.01 IBA and showed the highest number of leaves (11 leaves) and lowest number of leaves observed in 0.1 BAP with (3.8 leaves) per explants.

Table 4. (ANOVA) Effect of different concentrations of BAP and IBA on Number of Leaves of the pomegranate (Wonderful cultivar).

SOV	df	SS	MS	F	Sig
BAP	9	560.160	62.24	13.709	0.000
Error	90	408.600	4.540		
Total	99	968.760			

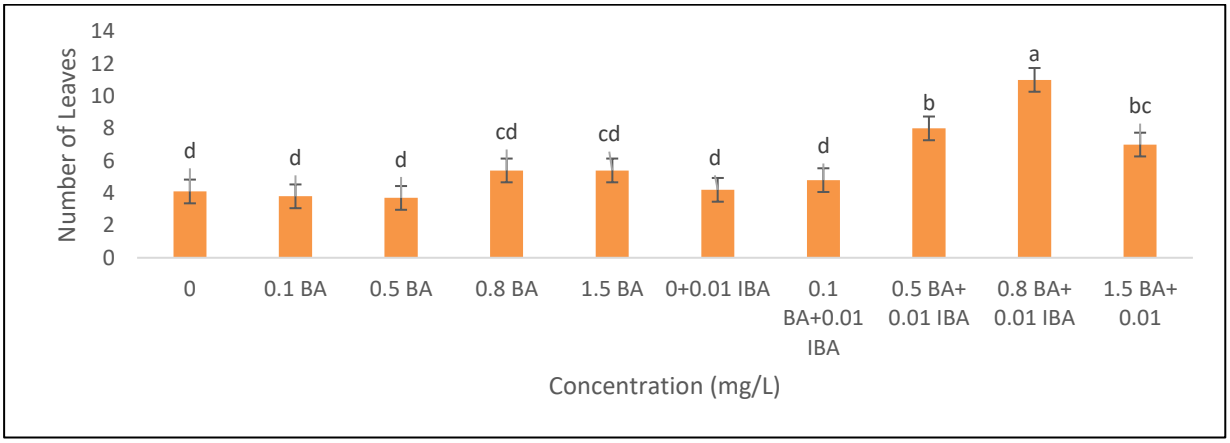

Figure 7. Effect of different concentrations of BAP and IBA on Number of Leaves of the pomegranate (Wonderful cultivar). Columns with different letters significantly differ at P< 0.05 (Duncan's multiple range test). Bars represent SE

Rooting

Three to five centimeters long shoots were excised individually from the proliferated shoot clumps and cultured on a rooting medium. The present investigation assessed root induction in the MS media supplemented with different concentrations (0.1, 0.8, 1, and 1.5 mg/l) of auxins (IBA). Most *in vitro* shoots developed roots within 20 – 22 days of incubation. IBA made different morphologies of roots and different root frequencies. Very thin and long roots were observed at 0.1 – 0.8 -1 mg/l IBA, whereas thin and medium-long roots were observed at 1 – 0.8 mg/l IBA. Thick and small roots were observed at 0.1 mg/l and 1 mg/l IBA. Root frequency ranged from 20-100 percent. Highest roots were observed at 1.0 mg/l IBA with 100 percent frequency. Thin and medium-long roots were observed at 0.1 – 0.8 mg/l IBA, whereas thick and medium-long roots were observed at 1 and 0.8 mg/l IBA.

A: Effect of IBA in MS, ½ MS medium on rooting

The containing of IBA (0.1- 0.8- 1 and 1.5 mg/l) in the MS medium showed the highest rooting response. The data shows that the highest rooting was recorded on ½MS medium containing IBA 1mg/l—(1.4) roots per explant. Therefore, IBA showed a rooting response.

Table 5. *(ANOVA) Effect of different concentrations of IBA on Root Number of the pomegranate (Wonderful cultivar).*

SOV	df	SS	MS	F	Sig
IBA	3	9.750	3.250	4.06	0.025
Error	16	12.800	0.800		
Total	19	22.550			

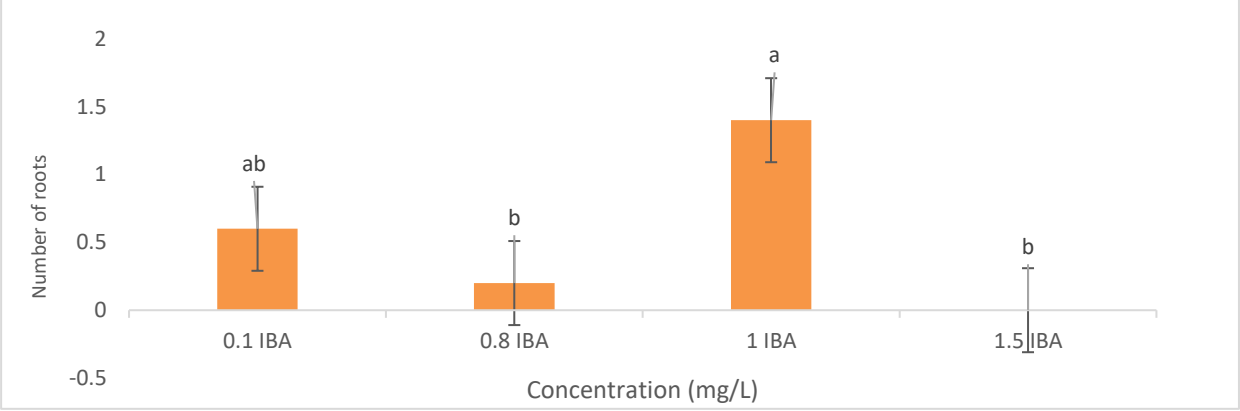

Figure 8. Effect of different concentrations of IBA on Root Number of pomegranate cultivar, Wonderful. Columns with different letters significantly differ at P< 0.05 (Duncan's multiple range test).

B: Effect of IBA in MS and ½ MS medium on root length

Root formation was observed in a medium containing 1 IBA mg/l after 16 days. Root length in 1mg/l concentration IBA was up to 1.84 cm, recorded on ½ MS medium containing IBA.

Table 6. (ANOVA) Effect of different concentrations of IBA on root length of the pomegranate (Wonderful cultivar).

SOV	df	SS	MS	F	Sig
IBA	3	21.514	7.171	6.293	0.005
Error	16	18.232	1.140		
Total	19	39.746			

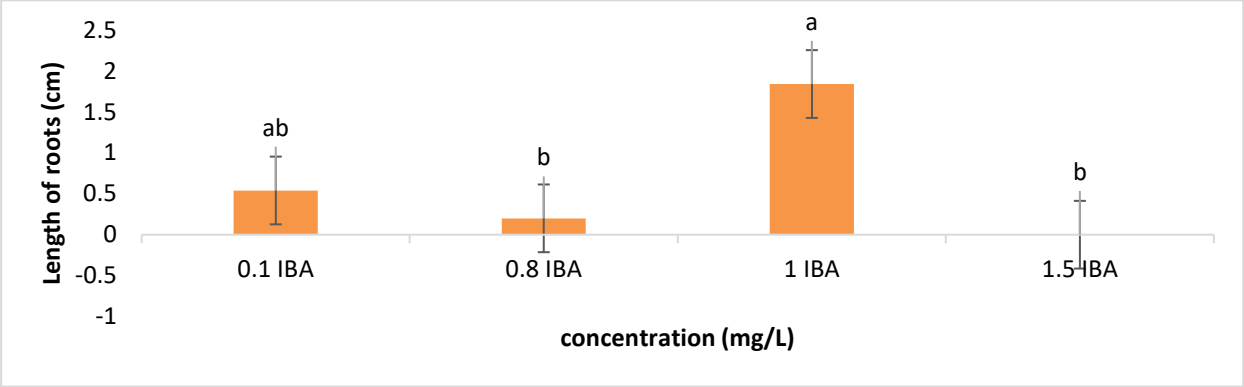

Figure 9. Effect of different concentrations of IBA on Root Length of pomegranate cultivar, Wonderful. Columns with different letters significantly differ at P< 0.05 (Duncan's multiple range test).

Rooted plantlets were successfully acclimatized and established in soil vermicompost with 80% survival frequency. All the established plants were uniform and showed no detectable variation.

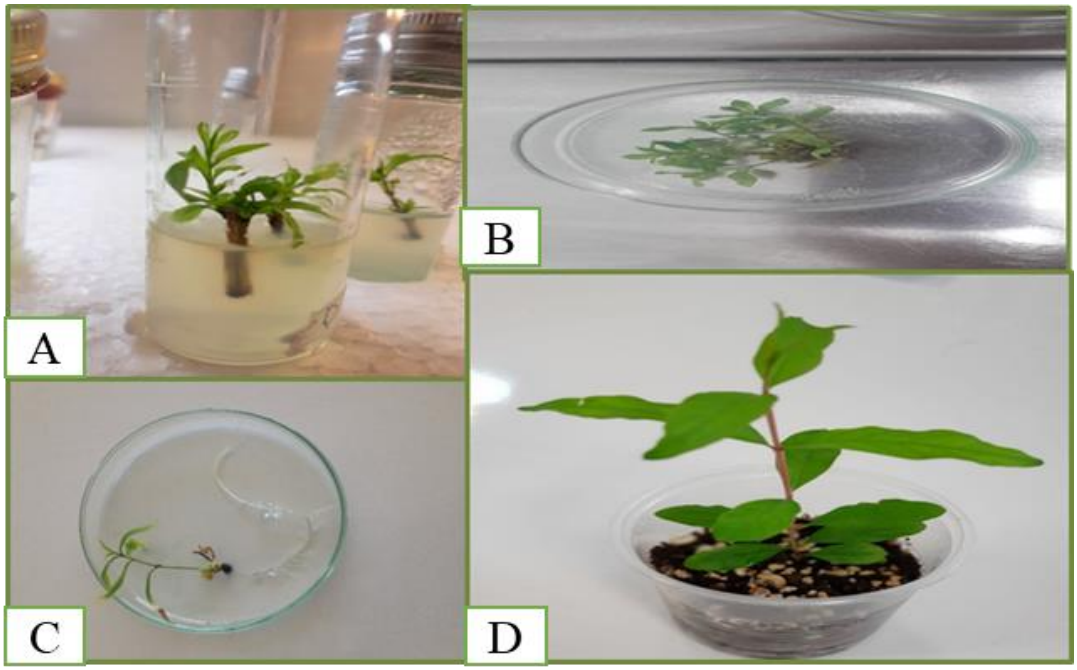

***Fig 10**. **A**: Establishment of explants. **B**: Proliferation of explant **C**: Rooting Stage **D**: Hardening of plantlet*

Conclusion

The study investigated the effects of different concentrations of $HgCl_2$, BA, and IBA on the in vitro regeneration of nodal explants. The results showed that a 0.1% concentration of $HgCl_2$ for 3 minutes was the most effective treatment for sterilizing shoot apex and nodal explants. Additionally, BA and IBA induced shoot bud formation, with the highest frequency observed at a combination of 0.8 $mg.L^{-1}$ BA and 0.01 $mg.L^{-1}$ IBA. The highest number of shoots and leaves were obtained at 0.8 $mg.L^{-1}$ BA and 0.01 $mg.L^{-1}$ IBA. Moreover, the highest rooting response was observed at 1 $mg.L^{-1}$ IBA in MS and ½ MS media, with 1.4 roots per explant. The study provides an optimized protocol for the in vitro regeneration of nodal explants, which could be useful in the large-scale production of uniform and disease-free plantlets (Fig10). Plant tissue culture is a technique used to propagate plants in a controlled environment by growing them from small fragments of plant tissue. In plant tissue culture, there is no need to import expensive seeds from other countries. Several advantages contribute to its self-sufficiency and Sustainable Agriculture: plant tissue culture offers various advantages that promote self-sufficiency in plant propagation, including rapid multiplication, disease-free plants, genetic stability, space efficiency, and year-round production. Tissue culture techniques allow for year-round production of plants, irrespective of seasonal limitations. By providing a controlled environment, tissue culture enables continuous production, increasing self-sufficiency in plant availability throughout the year.

Conflicts of Interest

The author(s) declared no conflict of interest.

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References

Kaji, B. V., Ershadi, A., & Tohidfar, M. (2013). In vitro propagation of two Iranian commercial pomegranates (Punica granatum L.) cvs. 'Malas Saveh' and 'Yusef Khani.' Physiology and Molecular Biology of Plants, 19(4), 597–603.

Kalalbandi, B. M., Waskar, D. P., Khandare, V. S., & Gorad, D. S. (2014). Micropropagation studies on pomegranate var. Bhagwa. Indian Journal Horticulture, 71(4), 564-566.

Naik, S. K., Pattnaik, S., & Chand, P. K. (2000). High-frequency axillary shoot proliferation and plant regeneration from cotyledonary nodes of pomegranate (Punica granatum L.). Scientia Horticulturae, 85(4), 261-270.

Nazir, U., Gul, Z., Shah, G. M., & Khan, N. I. (2022). Interaction effect of auxin and cytokinin on in vitro shoot regeneration and rooting of endangered medicinal plant Valeriana jatamansi jones through tissue culture. American Journal of Plant Sciences, 13(2), 223-240.

Pagliarulo, C., De Vito, V., Picariello, G., Colicchio, R., Pastore, G., Salvatore, P., & Volpe, M. G. (2016). Inhibitory effect of pomegranate (Punica granatum L.) polyphenol extracts on the bacterial growth and survival of clinical isolates of pathogenic Staphylococcus aureus and Escherichia coli. Food Chemistry, 190, 824-831.

Parmar, N., Kanwar, K., & Thakur, A. K. (2012). In vitro organogenesis from cotyledon derived callus cultures of Punica granatum L. cv. Kandhari Kabuli. National Academy Science Letters, 35(3), 215-220.

Parmar, N., Kanwar, K., & Thakur, A. K. (2013). Direct organogenesis in Punica granatum L. cv. Kandhari Kabuli from hypocotyl explants. Biological Sciences, 83(4), 569–574.

Parmar, N., Kanwar, K., & Thakur, A. K. (2015). High efficiency plant regeneration from cotyledon explants of pomegranate (Punica granatum L.) cv. Kandhari Kabuli. Vegetos, 28(2), 160-165.

Pérez-Tornero, O., Tallon, C. I., & Porras, I. (2010). An efficient protocol for micropropagation of lemon (Citrus limon) from mature nodal segments. Plant Cell, Tissue and Organ Culture, 100(3), 263-271.

Prabhuling, G., & Huchesh, H. (2018). In vitro regeneration in pomegranate (Punica granatum L.) cv. Bhagwa using double nodal segments. Research Journal of Biotechnology, 13, 8.

Sherawat, A. R., Uppal, S., & Chowdhury, J. B. (2002). Establishment of plantlets and evaluation of differentiated roots for alkaloids in Rauwolfia serpentine. Journal of Plant Biotechnology, 11, 105-108.

Singh, N. V., Singh, S. K., & Patel, V. B. (2007). In-vitro axillary shoot proliferation and clonal propagation of 'G 137' pomegranate (Punica granatum). Indian Journal of Agricultural Sciences, 77(8), 505-508.

Singh, P., & Patel, R. M. (2014). Factors influencing in vitro growth and shoot multiplication of pomegranate. The Bioscan, 9(3), 1031-1035.

Singh, P., & Patel, R. M. (2016). Factors affecting in vitro degree of browning and culture establishment of pomegranate. African Journal of Plant Science, 10(2), 43-49.

Singh, P., Patel, R. M., & Kadam, S. (2013). In vitro mass multiplication of pomegranate from cotyledonary nodal explants cv. Ganesh. African Journal of Biotechnology, 12(20), 2863-2868.

Soni, M., & Kanwar, K. (2016). Rejuvenation influences indirect organogenesis from leaf explants of pomegranate (Punica granatum L.) 'Kandhari Kabuli.' The Journal of Horticultural Science and Biotechnology, 91(1), 93-99.