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# In vitro propagation of pomegranate (Punica granatum l.) Cv. 'Males Yazdi'

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**Abstract**

Conventional method of propagation of pomegranate is time consuming and tiresome, and it does not ensure disease free and healthy plants. In vitro technique is the only prospect of plant tissue culture that has the potential to circumvent these problems. An efficient in vitro propagation is described for pomegranate using shoot tips and nodal explants. The influence of two basal medium, WPM and MS, and different plant growth regulators was investigated on micropropagation of the Iranian cultivar of pomegranate 'Malas Yazdi'. For proliferation stage, media supplemented with different concentrations (2.3, 4.7, 9.2 and 18.4 µM) of Kinetin along with 0.54 µM 1-naphthaleneacetic acid (NAA) was used. WPM proved to be more efficient medium compared to MS. The best concentration of Kinetin was 9.2 µM, resulting in the highest number of nodes, shoot length and leaf number. Half-strength WPM medium supplemented with 5.4 µM NAA was most effective for rooting of shoots. Rooted plantlets were successfully acclimatized and transferred into the soil.

**Keywords**: micropropagation, plant growth regulator, Iranian cultivar.

## 1. Introduction

Pomegranate is an economically important fruit crop of the tropical and subtropical regions of the world that is cultivated for its delicious fruits. In addition, the tree is also valued for its pharmaceutical properties and ornamental usage [11].

Pomegranate is native to Iran [27] and Iran with an annual pomegranate production of about 700,000 tones, ranks first in pomegranate production in the world [28]. Pomegranate is conventionally propagated by hard wood and soft wood cuttings. But, this traditional propagation method does not ensure disease-free and healthy plants. In addition, this method is a very time-consuming and labor-intensive process [12]. Hence, there is need to develop an efficient in vitro technique for the propagation of this fruit trees. During the past decade, tissue culture techniques have been widely used for the propagation of some important tropical and subtropical fruit trees [1, 29, and 20]. Micropropagation of pomegranate is also an essential step in the success of regeneration of transgenic lines and determines the effectiveness of a transformation protocol [2].

Protocols for regeneration of pomegranate have been reported by several researchers [18, 19, 25, and 5]; however, these protocols display several problems related to establishment of plant material and in vitro responsiveness [5]. It is therefore very consequential to improve the existing tissue culture protocols to refer these problems. The most commonly used techniques for organogenesis have employed seedling derived plant material [5], but this method is not suitable for elite cultivars. Therefore, the present investigation was undertaken to develop an efficient micropropagation protocol of 'Malas Yazdi', an Iranian leading pomegranate cultivar.

## 2. Materials and Methods

### *2.1. Plant material and explants preparation*

Shoot tip and nodal segments (0.5 - 1.0 cm long) were excised from two-year-old plants grown in a greenhouse. Following removing of the leaves, explants were washed with running tap water for 30 minutes, then were disinfected for 10 min in a 0.1% (w/v) calcium hypochlorite solution with 2 - 3 drops of Tween 20 and rinsed three times in autoclaved distilled water. The sterilized explants (three explants/jar) were vertically cultured in induction medium.

### *2.2. Culture media and culture conditions*

Two different media; MS (16) and WPM (15) were used. Sucrose was added at 30 g/L and myo-instol at 0.1 g/L. The pH of the prepared media was then adjusted to 5.6 to 5.8 with 0.1 N NaOH, before adding 0.6% agar.

For proliferation stage, Kinetin was employed at 0, 2.3, 4.7, 9.2 and 18.4 µM along with 0.54 µM NAA

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For proliferation stage, Kinetin was employed at 0, 2.3, 4.7, 9.2 and 18.4 µM along with 0.54 µM NAA (0.54 µM NAA was chosen based on the preliminary experiments). Media were then poured in 200 ml jars; media volume for each jar was 25 ml. The jars containing media were autoclaved at 121°C and 1.5 kg/cm$^2$ pressure for 15 min and left to be air cooled for media solidification. Explants cultured upright with the basal end of the node inserted a few millimeters into the culture medium. Explants were incubated in the dark for the first 7 days and then cultures were grown at 25 ± 1°C with white light (40 µmol/m$^2$/s$^1$) and a 16 hour photoperiod. Each cytokinin treatment consisted of seven replicate jars with three explants in each and repeated three times. After 28 days, shoot tips originating from explants were transferred to fresh media of the same kind and grown under above photoperiod condition. Subcultures were initiated by cutting shoots into nodal sections. Sprouted buds were further grown on the same media.

### *2.3. In vitro rooting*

Shoot tips with 2 - 4 cm long developed *in vitro* were excised and cultured in half-strength WPM medium containing 50 mg/L myo-inositol, 15 g/L sucrose and 3 g/L agar. The medium was further supplemented with 0, 2.5, 4.9 and 9.8 µM IBA or 0, 2.7, 5.4 and 10.8 µM NAA. After 8 - 10 days, the rooted shoots were transferred to an auxin-free half-strength medium for further elongation of the roots. Each auxin treatment consisted of seven replicate jars with one shoot in each. After 4 weeks in culture, number and length of roots per rooted shoot were evaluated.

### *2.4. Acclimatization of regenerated plantlets*

Well rooted explants were removed from the culture medium. The roots were washed gently with tap water to remove agar and then transferred to small plastic pots containing autoclaved cocopeat-perlite mixture. The pots were covered with polyethylene bags to maintain high humidity and kept at 25 ± 1°C in artificial light (50 µmol/m$^2$/s) provided by white fluorescent tubes for 3 to 4 weeks. To harden the plants, polyethylene bags opened gradually, from a few minutes a day until normal conditions. Plants were then transferred to larger pots (18 cm diameter) containing garden soil (soil: compost, 1: 1); kept under shade for another 2 weeks and then transferred to direct sunlight condition. The survival rate was examined 40 days after transfer.

### *2.5. Data analysis and measurements*

For proliferation stage, the experiment was conducted under completely randomized design (CRD) with two types of culture media (MS and WPM) and five concentrations of Kinetin. A completely randomized design (CRD) with eight concentrations of auxin (IBA and NAA) was used for rooting experiments. The data were analyzed using SAS Version 9.1 [24]. Significant differences were assessed using Duncan's multiple range test at $P < 0.05$.

Shoot number, length of shoots, node number, leaf number, root number and length of roots were recorded. Data included only new shoots longer than 0.5 cm.

## 3. Results

### *3.1. Culture media*

Culture media significantly affected length of shoots, number of nodes and the number of leaves, but there was no significant difference in the number of shoots (Figure 1). The plantlets produced in WPM medium were apparently more vigorous, because the length of shoots were longer, which is an important feature in micropropagation. MS was inferior compared to WPM, shown by the length of shoots, number of nodes and number of leaves (Figure 1). Therefore, WPM medium was subsequently used in rooting studies.

### *3.2. Effect of growth regulators*

Shoot buds were induced on medium with or without Kinetin within 1 to 2 weeks; however, shoot buds failed to elongate on medium without Kinetin and shoot buds elongation was observed only on media containing Kinetin (Figure 2). Multiple shoots were induced on explants cultured on media supplemented with Kinetin in different concentrations (2.3, 4.7, 9.2 and 18.4 µM) along with 0.54 µM NAA.

The supplementation of media with 9.2 µM Kinetin and 0.54 µM NAA seemed to favor proliferation, producing vigorous plantlets with the highest leaf number and length of shoot. (Figure 2).

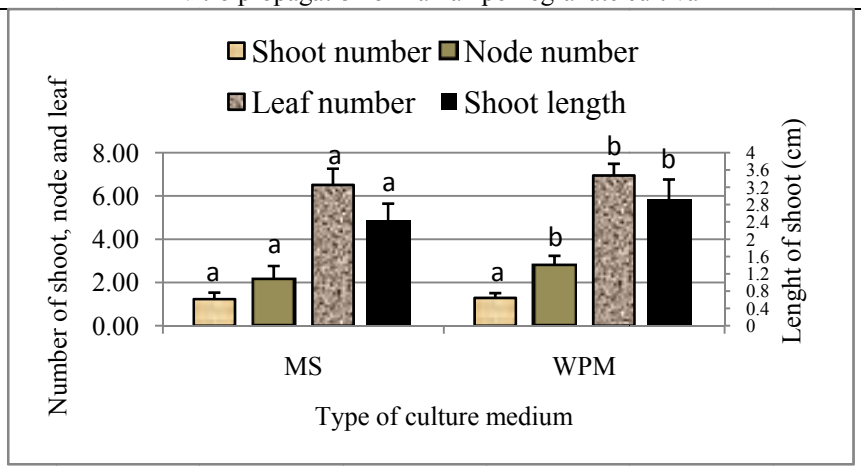


**Figure 1.** Effect of culture medium type on *in vitro* shoot proliferation of the pomegranate cultivar, 'Malas Yazdi'. Columns with different letters are significantly different from each other at $P \leq 0.05$ (Duncan's multiple range test). Bars represent SE values.

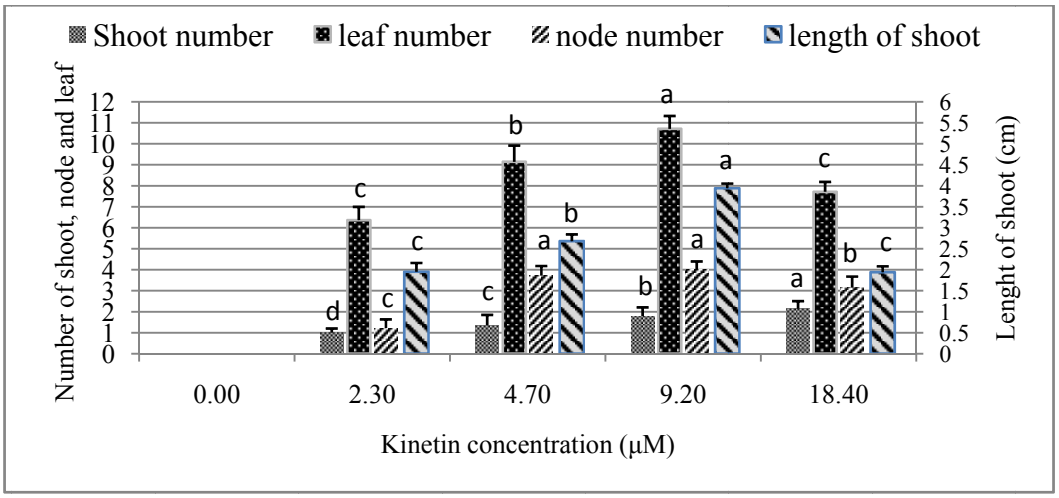


**Figure 2.** Effect of different concentrations of Kinetin on *in vitro* shoot proliferation of the pomegranate cultivar, 'Malas Yazdi'. Columns with different letters are significantly different from each other at $P \leq 0.05$ (Duncan's multiple range test). Bars represent SE values.

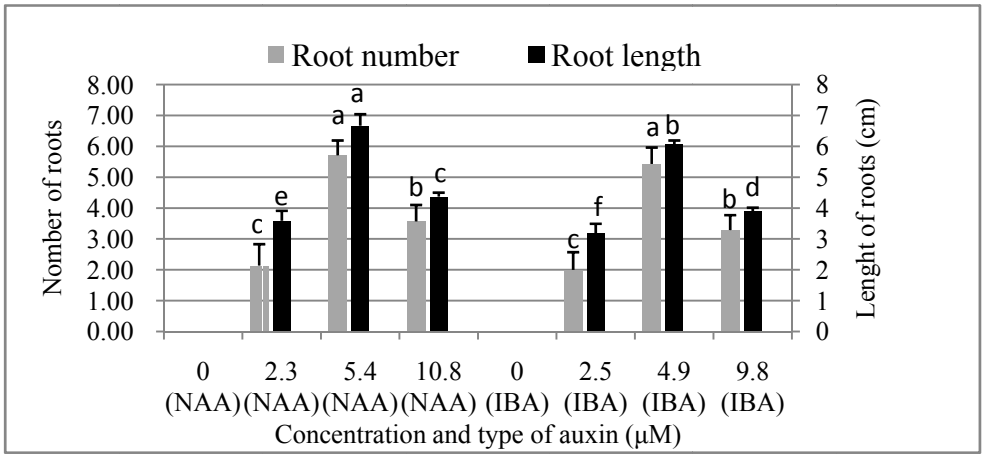


**Figure 3.** Effect of different concentrations of IBA and NAA on rooting of shoots of 'Malas Yazdi'. Columns with different letters are significantly different from each other at $P \leq 0.05$ (Duncan's multiple range test). Bars represent SE values.

*3.3. In vitro rooting and acclimatization of plantlets*

WPM medium devoid of NAA or IBA failed to induce rooting and root induction was encouraged by addition of either NAA or IBA to medium (Figure 3). NAA had a more pronounced effect than IBA on rooting of shoots. Among all the treatments tested, half-strength WPM medium supplemented with 5.4 μM NAA was most effective for rooting of shoots (Figure 3). In general, rooting of shoots was slightly increased when NAA was used (Figure 3).

Rooted plantlets were successfully acclimatized and established in soil with 80% survival frequency. All the established plants were apparently uniform and did not show any detectable variation.

## 4. Discussion

The main problems usually faced in culturing tissues from fruit trees are contamination, recalcitrance of adult trees, hyper hydration, and browning or blackening of the medium and/or the explants due to leaching of phenolics which may be harmful and cause necrosis of the explants [3, 13]. In this study, shoot tip and nodal segments obtained from two-year-old plants were used as explants. Better plant regeneration from node of young trees was also reported in *Oroxylum* [6], *Boswellia ovalifoliolata* [4], *Pterocarpus santalinus* [23], *Stereospermum personatum* [26]. Browning of explants due to exudation of phenolics has been reported as a critical problem in establishing cultures of tree species [13]. However, this problem was not observed in this study where cultures were initiated from young explants. This is probably because young plants do not synthesize phenolic substances [22].

The use of plant growth regulators in pomegranate tissue culture is of fundamental importance. Many studies have been conducted in order to identify the optimal composition of plant growth regulators, for shoot and root differentiation as well as plant regeneration in pomegranate [19, 25].The inclusion of cytokinins in the media is generally considered necessary for proliferation of apical and axillary shoots [17]; However, when only Kinetin was added, without auxin, shoot proliferation and elongation were decreased (about 30%; unpublished data).

Supplementing auxins in the culture medium is necessary for some cultivars, while others can be proliferated without this growth regulator [17]. The first seemed to be the case for 'Malas Yazdi' as shoot proliferation was favored in the presence of NAA. According to Ibáñez et al., [10] when auxin is not necessary the explants function as active centers of auxin biosynthesis, which did not occur for our cultivar.

Media compositions have a key role in morphogenesis; MS in full salt strength was the most commonly used basal medium for pomegranate micropropagation while half strength MS (1/2 MS), WPM and B5 [8] were useful in some cultivars [17]. The fact that low concentrations of Kinetin and NAA were demanded in WPM, further supports the hypothesis that this medium was the most suitable to 'Malas Yazdi'.

NAA or IBA has been the most commonly used auxin for promoting rooting of *in vitro* regenerated shoots of pomegranate. The type of auxin seems to strongly affect the rooting of explants [9]. It has been reported that NAA, especially in high concentrations, caused severe toxicity problems in explants [7]. Similar results were obtained in this study with both auxins, NAA and IBA. In pomegranate, MS and WPM, mostly in half-strength, have been attempted with varying success [17]. In the present study, half-strength WPM medium was efficient enough for root induction. Relatively low salt concentrations in medium are known to enhance rooting of shoots in several plant species [21].

After 40 days of hardening no survival problems were exhibited. The survival rate paralleled the physiological appearance of plants after auxin treatment, which enforces the suggestion that the concentration and the type of rooting growth regulator highly affect the plants after acclimatization [14].

Results obtained in this study are considered satisfactory for providing a potential commercial micropropagation protocol of 'Malas Yazdi', ensuring proper conditions for the *in vitro* maintenance of this important Iranian pomegranate cultivar, with unique characteristics. *In vitro* propagation of pomegranate cultivars has been reported earlier [17]. However, the limitations of previous protocols are browning of the culture medium followed by necrosis of the explants which influenced the establishment of shoot cultures, while this problem was negligible in the present study.

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