TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2014) 38: 293-301
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doi:10.3906/bot-1302-55

Melatonin and other factors that promote rooting and sprouting of shoot cuttings in *Punica granatum* cv. Wonderful

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Received: 25.02.2013 • **Accepted:** 23.09.2013 • **Published Online:** 17.01.2014 • **Printed:** 14.02.2014

Abstract: The agricultural and medicinal plant pomegranate (*Punica granatum* L. cv. Wonderful) was studied to examine the effects of wounding of cuttings and to test the effects of different concentrations of indole-3-butyric acid (IBA), gibberellic acid (GA_3), hydrogen peroxide (H_2O_2), melatonin (MEL), and ascorbic acid (ASC) on rooting of the shoot cuttings under mist. The data indicated that IBA had a positive influence on the rooting percentage in wounded and nonwounded cuttings. The best rooting was achieved with very thin cuttings (3–4 mm in diameter). The percentage of rooting (100.0 ± 0.0) and the number of roots per plant (15.2 ± 2.4) were positively affected if the point of severing was at an internode of the cutting. It was found that 17 mg L^{-1} H_2O_2 in combination with 1000 mg L^{-1} IBA produced the longest roots, whereas 500 mg L^{-1} GA_3 in combination with the same IBA concentration (1000 mg L^{-1}) reduced root length. In addition, the human hormone MEL at an application of 1.16 mg L^{-1} can be substituted for IBA to produce positive effects on rooting, while 352.24 mg L^{-1} ASC alone or in combination with IBA also promoted rooting.

Key words: Cutting thickness, rooting, melatonin, mist propagation, pomegranate

1. Introduction

Pomegranate (*Punica granatum* L.) has been studied for the nutritional value of its fruit and the medicinal applications of various parts of the tree. Hence, it has been shown that the fruit is a rich source of minerals, vitamins, antioxidants, and tannins, while the juice of the fruit is an excellent source of vitamins (C, B), sugars, minerals (K, Fe), and antioxidant polyphenols (ellagic acid and punicalagin) that may lower the risk of heart disease (Aviram et al., 2004; Karimi and Mirdehghan, 2013). Pomegranate juice is one of the foods that has been found to have high antioxidant content (Miguel et al., 2010).

In the last few years many pomegranate orchards have been established in Greece with the self-rooted plants of the cultivar Wonderful. Generally, pomegranate is propagated commercially from cuttings (Melgarejo et al., 2008); however, there is great variability in the rooting ability of the various cultivars (Owais, 2010). Propagation from cuttings is an easy, quick, and economical method. Largely due to the medicinal and nutritional attributes of pomegranate, there is an increasing demand for superior plant material. Mist propagation has been a valuable technique for high throughput propagation of uniform and healthy clonal plants for some time now. Cuttings for mist propagation are usually taken from late spring to early summer, and auxins can be used to promote adventitious root initiation (Saroj et al., 2008).

It has been noted that apart from promoting rooting, auxins also play a key role in a number of developmental processes, where they act as an indicator for the division, elongation, and differentiation of cells (Pasternak et al., 2002; Erdağ et al., 2010).

Studies have shown that wounding increases the rooting of the stem cuttings of *Arbutus andrachne* (Al-Salem and Karam, 2001). Su et al. (2006) have also shown that both nitric oxide (NO) and hydrogen peroxide (H_2O_2) play a crucial role in the modulation of several physiological processes. Hydrogen peroxide is a messenger in the formation and development of plant roots, and it is a mediator for various physiological and biochemical responses, including lateral root (Su et al., 2006) and cell wall development (Potikha et al., 1999). Studies to date have not been able to clearly show whether H_2O_2 is directly involved in indole-3-acetic acid (IAA)-induced adventitious root formation or if it mediates in the processes responsible for adventitious rooting (Berleth and Sachs, 2001).

Melatonin (MEL) is a human hormone that is produced in the pineal gland. The precursor for melatonin in plants is tryptophan, which is also transformed into IAA (Murch

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et al., 2000). Melatonin in plant tissues has been shown to affect root growth and promote rooting in cherry rootstocks at a concentration of 1 μM (Sarropoulou et al., 2012a, 2012b).

Ascorbic acid (ASC) is fundamental for plant growth. For some time now it has been known that ASC plays a role in plant growth, and there is strong evidence that wall ascorbate is linked to cell expansion (Smirnoff, 2000). Little is known, however, about the influence of ASC on rooting and, in particular, the effects of its exogenous application. ASC has been shown to enhance superoxide dismutase (SOD) and catalase (CAT) activities, which were related to the rooting of cuttings (Li et al., 2007). It has been reported that ASC is a cofactor of several enzymes involved in ethylene and gibberellin biosynthesis (Prescott and John, 1996). Kato and Esaka (1999) found that ASC, as a necessary factor in cell cycle progression during cell division, affects meristem expansion.

Although some studies on propagating other cultivars of pomegranate from stem cuttings have been conducted (Owais, 2010), there are very limited data on new practices and chemical treatments to enhance rooting in the cultivar Wonderful.

The objectives of this study were to determine the influences of wounding, IBA, GA_3, H_2O_2, ASC, and MEL on rooting and sprouting of shoot cuttings of pomegranate (*Punica granatum* L. cv. Wonderful). Due to the cost of the chemical substances used, improvement in the rooting parameters of this cultivar will reduce the cost per plant and, subsequently, the cost per acre of new plantings for fruit production as well as plants grown for medicinal purposes.

2. Materials and methods

2.1. Plant material and growth conditions

The experiment was conducted at the Experimental Farm of the Aristotle University of Thessaloniki (Northern Greece) from the end of April to the end of June, 2010. Semihardwood shoots collected early in the morning from 4-year-old pomegranate plants (Wonderful) from the experimental orchard were used for the preparation of cuttings. Each cutting was 15 cm in length and had 4 leaves. The cuttings were disinfected by dipping them in a fungicide solution (0.6 mg L^{-1} Benlate). After being treated with IBA, GA_3, H_2O_2, ASC, and MEL the cuttings were planted in plastic containers (30 × 45 cm) containing moistened perlite (Perloflor) as the rooting substrate. The containers with the shoot cuttings were then placed under intermittent mist on a propagation bench in a greenhouse with an average temperature of 20–22 °C. The light intensity was reduced to 50% full sun by white-washing the outside of the greenhouse to avoid temperature increase. Each day misting started at 0700 and terminated at 2100; it lasted for 5 s every 15 min and was controlled by an electric timer. Every week the cuttings were sprayed with the Benlate fungicide (0.6 mg L^{-1}) in order to reduce mortality. Four experiments were conducted and their means reported.

2.2. Experiment 1: the effects on rooting of IBA concentration and wounding at the base of cuttings

The experiment included 8 treatments with 4 IBA concentrations (0, 500, 1000, and 2000 mg L^{-1}) in combination with 2 wounding treatments. Wounding was done by making 2 opposite longitudinal incisions at the base of each cutting. Nonwounded cuttings were used as the control. Subsequently, the basal part (1.5 cm) of the cuttings was treated for 5 s with IBA, at the concentrations referred to above, in 50% ethanol. These IBA concentrations were chosen after preliminary experiments indicated that the greatest rooting percentage was achieved within this range. The base of each control cutting was treated with 50% ethanol for 5 s. All the experiments included 5 replicates of 20 cuttings each, i.e. a total of 100 cuttings per treatment. After a period of 30 days had elapsed on the mist propagation bench and maximum rooting had been achieved, the experiment was terminated. The rooting percentage, number of roots per rooted cutting, root length (mm), percentage of shoot sprouting, and number and length (mm) of shoots per cutting were measured.

2.3. Experiment 2: the effects of cutting thickness on rooting

Cuttings with similar morphological characteristics, as in Experiment 1, were divided into 4 categories depending on the diameter (mm) of their base (6–7, 4–5, 3–4, and less than 3 mm). The base of each cutting was treated with 1000 mg L^{-1} IBA, which is the average efficient IBA concentration, for 5 s. After a period of 30 days on the mist propagation bench the experiment was terminated, and the same parameters as in experiment 1 were measured.

2.4. Experiment 3: the effects of GA_3 and H_2O_2 concentration combined with 1000 mg L^{-1} IBA on rooting

The experiment included 8 treatments (control, 1000 mg L^{-1} IBA, 1000 mg L^{-1} IBA + 250 mg L^{-1} GA_3, 1000 mg L^{-1} IBA + 500 mg L^{-1} GA_3, 17 g L^{-1} H_2O_2, 1000 mg L^{-1} IBA + 17 g L^{-1} H_2O_2, 17 mg L^{-1} H_2O_2, and 1000 mg L^{-1} IBA + 17 mg L^{-1} H_2O_2). Each treatment had 5 replicates of 20 cuttings, and the experiment was terminated after 30 days from planting. At the termination of the experiment the rooting percentage, number and length of roots, as well as number of shoots per cutting were measured. Gibberellic acid was applied by leaf spraying with GA_3 solution containing liquid soap as a surfactant. Hydrogen peroxide was applied by dipping the base of the cuttings (1.5 cm) for 5 s. After drying the leaves and the bases of cuttings, the bases (1.5 cm) were immersed in a solution of 1000 mg L^{-1} IBA for 5 s.

2.5. Experiment 4: the effects of MEL and ASC on rooting

The experiment included 9 treatments (control, 1000 mg L^{-1} IBA, 17 mg L^{-1} H_2O_2, 1.16 mg L^{-1} MEL, and 352 mg L^{-1} ASC with and without 1000 mg L^{-1} IBA). The treatment 352 mg L^{-1} ASC + 17 mg L^{-1} H_2O_2 was also included. The solutions of MEL, ASC, and H_2O_2 were applied alone by dipping the base (1.5 cm) of each cutting for 5 s and after drying, dipping for another 5 s in 1000 mg L^{-1} IBA in 50% ethanol solution.

2.6. Statistical analysis

The experimental layout was completely randomized, and the data were analyzed with analysis of variance (ANOVA) using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The experiments were conducted twice, and the reported data are the means of the 2 experiments. For mean comparison, Duncan's multiple range test and standard error (SE) were used at $P \leq 0.05$ to establish significant differences among the treatments.

3. Results and discussion

3.1. The effects of exogenously applied IBA and wounding on rooting and sprouting of pomegranate cuttings

The data show that IBA exerted a positive influence on percentage of rooting and root number per cutting both in wounded and nonwounded cuttings, compared to the control (Table 1). These results are in accordance with Blakesley and Chaldecott (1993), who reported that auxins are very important for initiating rooting. Melgarejo et al. (1998) also observed an increase in the rooting percentage of pomegranate with 12,000 mg L^{-1} IBA. Similar positive effects on pomegranate rooting due to auxins were reported by Hamooh (2005). In the present study, both wounded and nonwounded cuttings treated with IBA had an equal root number per rooted cutting, with the exception of the control (0 mg L^{-1} IBA). The nonwounded cuttings treated with 2000 mg L^{-1} IBA produced the greatest number of roots per cutting, which was, however, not statistically significant compared to wounded cuttings; furthermore, the roots had a burned appearance. This could be ascribed to the toxic effects of the relatively high IBA concentration (2000 mg L^{-1}) on root primordial cells (Cardarelli et al., 1987).

Rooting success is determined by the number and length of roots. In the present study, when the IBA concentration was increased, the length of the roots produced was found to decrease in both wounded and nonwounded cuttings (Table 1). Generally, wounding is effective in increasing the rooting of cuttings by blocking the downward movement of carbohydrates and other root-promoting factors (Hartmann and Kester, 1968). Stoltz and Hess (1966) reported that girdling increased the total sugar and starch content of hibiscus cuttings. Since H_2O_2 acts as a second messenger in response to wounding (Orozco-Cárdenas et al., 2001), removing a slice of bark may promote the generation of a wound-induced signal and further increase H_2O_2 in pomegranate cuttings. Contrary to our results, other studies (Hamooh, 2005) found that when IBA concentrations were increased up to a certain level there was also an increase in root length. In contrast to rooting, the nonwounded control cuttings showed the greatest percentage in sprouting, while shoot length increased when 500 and 1000 mg L^{-1} IBA was applied (Table 1).

Table 1. Effect of 4 IBA concentrations and wounding of the base of *Punica granatum* L. cv. Wonderful cuttings on rooting percentage, number of roots per rooted cutting, average root length (mm), percentage sprouting, number of shoots per cutting, and average shoot length (mm); W: wounding.

Treatment IBA (mg L^{-1})	Rooting %	Number of roots per rooted cutting	Average length of roots (mm)	Sprouting %	Number of shoots per cutting	Average length of shoots (mm)
0.0	45.0 ± 5.9	4.3 ± 1.1	13.2 ± 3.3	70.0 ± 6.3	2.6 ± 0.2	28.1 ± 5.1
0.0 W	50.0 ± 8.1	8.1 ± 1.6	16.2 ± 2.2	53.3 ± 7.6	3.0 ± 0.7	30.9 ± 6.4
500	85.0 ± 4.3	16.0 ± 2.6	12.6 ± 1.2	55.0 ± 8.1	2.1 ± 0.2	46.0 ± 6.3
500 W	61.7 ± 8.6	17.2 ± 3.8	13.1 ± 3.1	61.7 ± 4.0	1.9 ± 0.2	30.4 ± 8.5
1000	75.0 ± 9.3	19.0 ± 0.9	11.5 ± 1.0	50.0 ± 5.2	1.6 ± 0.3	43.0 ± 4.8
1000 W	60.0 ± 7.8	21.3 ± 2.6	12.5 ± 0.6	55.0 ± 6.2	2.0 ± 0.2	43.0 ± 6.9
2000	60.0 ± 8.1	22.8 ± 1.8	10.4 ± 0.5	36.7 ± 9.6	1.0 ± 0.4	20.5 ± 7.7
2000 W	51.7 ± 8.9	17.3 ± 3.8	8.6 ± 1.9	45.0 ± 12.8	1.3 ± 0.3	21.7 ± 5.1

Values are means (n = 5) ± SE according to Duncan's multiple range test ($P \leq 0.05$).

3.2. The effects of cutting thickness on rooting and sprouting

Ontogenetic age plays an important role in the rooting efficiency of cuttings (Melgarejo et al., 2008). It is well-known that the interaction of cutting diameter with IBA concentration is very important (Poupard et al., 1994; Husen, 2004). The present data showed that the percentage of rooting increased with a decrease in the diameter (Table 2; Figure 1). This finding does not agree with the results of other authors, who reported the exact opposite, i.e. an increase in the rooting percentage of cuttings with the greatest diameter (Melgarejo et al., 2008; Saroj et al., 2008). However, there were no significant differences observed in the present study in regards to the number and length of roots as a function of cutting thickness. In previous reports it is well documented that factors such as the age of the donor plants, within-shoot position, and growth regulators affect the rooting ability of *Acacia mangium* shoot cuttings (Darus, 1989; Poupard et al., 1994). Hartmann et al. (1990) also reported that endogenous auxin is assumed to be synthesized close to the terminal bud. In our study this negative correlation between the base-diameter of the cuttings and the rooting percentage may be due to variation in the physiological status of the shoot tissues, a different level of lignifications and deficiency in endogenous auxin promoters, or an excess in inhibitors of adventitious root induction in the cuttings with the greatest base-diameter. This within-shoot, basipetal, decreasing rooting ability may also be associated with anatomical and histological differences. The cuttings with the greatest base-diameter, obtained from the lower parts of the shoots, are more differentiated and ontogenetically older, and according to Darus (1989) the cylinder of sclerenchymatous cells of older shoots may constitute an obstacle to root formation.

The greatest percentage in sprouting as well as number and length of shoots was recorded in cuttings with the greatest diameters. According to Sun and Bassuk (1993), IBA-induced root formation in rose cuttings provoked an increase in endogenous ethylene concentration that was significantly correlated with the number of roots formed in cuttings and inversely correlated with budbreak. They also suggested that exogenously applied IBA is transported from the basal to the upper part of the cuttings, where it causes increased ethylene production, and, as a result,

Table 2. Effect of cutting thickness of *Punica granatum* L. cv. Wonderful on rooting percentage, number of roots per rooted cutting, average root length (mm), percentage sprouting, number of shoots per cutting, and average length (mm) of shoots. Cuttings were treated with 1000 mg L^{-1} IBA.

Base diameter (mm)	Rooting %	Number of roots per rooted cutting	Average length of roots (mm)	Sprouting %	Number of shoots per cutting	Average length of shoots (mm)
6–7	57.5 ± 6.3	13.3 ± 2.7	24.4 ± 1.9	75.0 ± 10.4	2.1 ± 0.2	67.2 ± 7.3
4–5	78.3 ± 7.0	12.9 ± 1.4	21.3 ± 1.5	38.3 ± 6.0	1.8 ± 0.3	39.7 ± 4.7
3–4	86.7 ± 7.2	13.1 ± 1.6	21.8 ± 2.5	21.6 ± 5.4	1.3 ± 0.3	32.4 ± 4.0
<3	98.3 ± 1.6	12.1 ± 1.3	26.0 ± 2.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Values are means (n = 5) ± SE according to Duncan's multiple range test ($P \leq 0.05$).

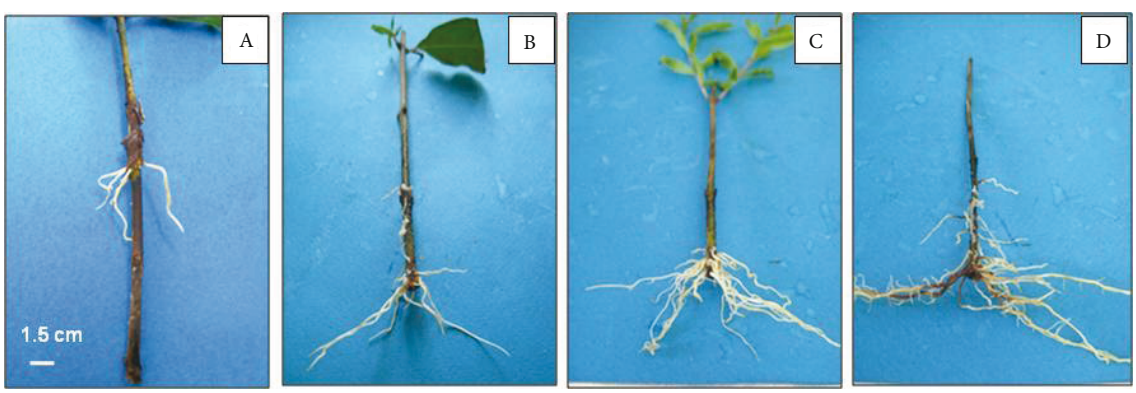

Figure 1. Representative photographs showing the effect of base diameter on the rooting of cuttings: A- 6–7 mm, B- 4–5 mm, C- 3–4 mm, and D- <3 mm. Scale bar = 1.5 cm. Photographs were taken 30 days after planting.

budbreak of cuttings is inhibited. These results may explain the positive correlation between sprouting parameters and base-diameter of the cuttings in our study.

3.3. The effects of node and internode severing on rooting and sprouting of pomegranate cuttings

When the base of each cutting was treated with 1000 mg L^{-1} IBA, the point of the cut of internode appears to have significantly influenced the percentage of rooting (100.0 ± 0.0) and number of roots per plant (15.2 ± 2.4), while the nodal point of cut provoked an increase in the percentage of sprouting (90.2 ± 7.1) (Table 3). Thus, internodal cuttings are recommended for rooting and nodal ones for sprouting. This finding is of great importance as it allows for nodal and internodal cuts to be made to the plant, thus improving efficiency through the production of many more cuttings from the initial shoot material. Hansen (1986), on the other hand, observed that the internode showed poor rooting in *Schefflera arboricola* (Hayata), where there was a lower percentage in both rooting and number of roots per cutting than in nodal cuttings. In the present study, more shoots were produced in nodal, compared to internodal cuttings (Table 3). This partially agrees with the study by Yadav et al. (2009) on the nodal and internodal segments of *Populus deltoides* W.Bartram ex Marshall.

3.4. The effects of GA_3 and H_2O_2 on rooting and number of shoots produced in pomegranate cuttings

Studies have shown that the rooting of cuttings is controlled by a fine balance of endogenous stimulatory and inhibitory factors (Eliasson, 2006). It is generally accepted that, while auxins promote rooting, cytokinins and gibberellins inhibit it. There is no report in the literature about the effect of GA_3 and H_2O_2 alone or in combination with 1000 mg L^{-1} IBA on the number of shoots per cutting. The effects of various concentrations of GA_3 and H_2O_2 in combination with 1000 mg L^{-1} IBA and 1000 mg L^{-1} IBA alone on the rooting of pomegranate cuttings compared to control are shown in Figure 2. As seen in Table 4, there were significant differences between control and treatments with GA_3 and H_2O_2 applied alone or in combination with IBA, as regards root number. More specifically, the cuttings treated with 1000 mg L^{-1} IBA alone and in combination with 17 g L^{-1} H_2O_2 had the greatest number of roots per cutting (12.3 ± 1.2 and 10.7 ± 1.2, respectively) compared to the control. Nevertheless, the cuttings that were treated with 500 mg L^{-1} GA_3 in combination with 1000 mg L^{-1} IBA produced the shortest roots (Figure 2D), whereas those treated with 17 mg L^{-1} H_2O_2 and 1000 mg L^{-1} IBA produced the longest ones (Figure 2G). Apart from preventing cell division, gibberellins are also involved in the transformation of differentiated meristematic cells, which may explain the findings above (Brian et al., 1962). In addition, our findings showed that the application of H_2O_2 at 17 g L^{-1} together with 1000 mg L^{-1} IBA induced root growth (Table 4). Thus, H_2O_2 might enhance adventitious rooting by stimulating the activity of certain enzymes, increasing the content of carbohydrates, and repressing the production of polyphenols (Liao et al., 2010). According to Rugini et al. (1997), olive cuttings treated with H_2O_2 had a high rooting percentage. A positive effect of H_2O_2 in rooting concerning root number after putrescine application was also observed in the study by Hartmann et al. (1990). In addition, the results of Sebastiani and Tognetti (2004), who reported significantly greater root numbers in olive cuttings treated with 4000 mg L^{-1} IBA + 3.5% (w/v) H_2O_2, agree with our findings, which also indicated that IBA alone or in combination with H_2O_2 produced greater root numbers than the control. As regards number of shoots per cutting, control and cuttings treated with 1000 mg L^{-1} IBA had the greatest number of shoots (3.3 ± 0.7 and 2.7 ± 0.4, respectively). However, treatments with GA_3 and H_2O_2 alone or on combination with 1000 mg L^{-1} IBA had no positive influence on number of shoots per cutting (Table 4).

Table 3. Effect of point of severing (node or internode) and 1000 mg L^{-1} IBA in *Punica granatum* L. cv. Wonderful cuttings on rooting percentage, number of roots per rooted cutting, average root length (mm), percentage sprouting, number of shoots per cutting, and average length (mm) of shoots.

Point of cutting severing	Rooting %	Number of roots per rooted cutting	Average length of roots (mm)	Sprouting %	Number of shoots	Average length of shoots (mm)
Control	57.1 ± 9.3	6.3 ± 1.2	24.7 ± 1.8	34.9 ± 5.3	1.9 ± 0.4	44.6 ± 3.0
Node + 1000 mg L^{-1} IBA	87.5 ± 7.5	8.9 ± 1.3	26.0 ± 2.5	90.2 ± 7.1	1.6 ± 0.1	44.8 ± 6.1
Internode + 1000 mg L^{-1} IBA	100.0 ± 0.0	15.2 ± 2.4	29.2 ± 3.6	60.0 ± 9.5	1.0 ± 0.2	36.3 ± 2.5

Values are means (n = 5) ± SE according to Duncan's multiple range test ($P \leq 0.05$).

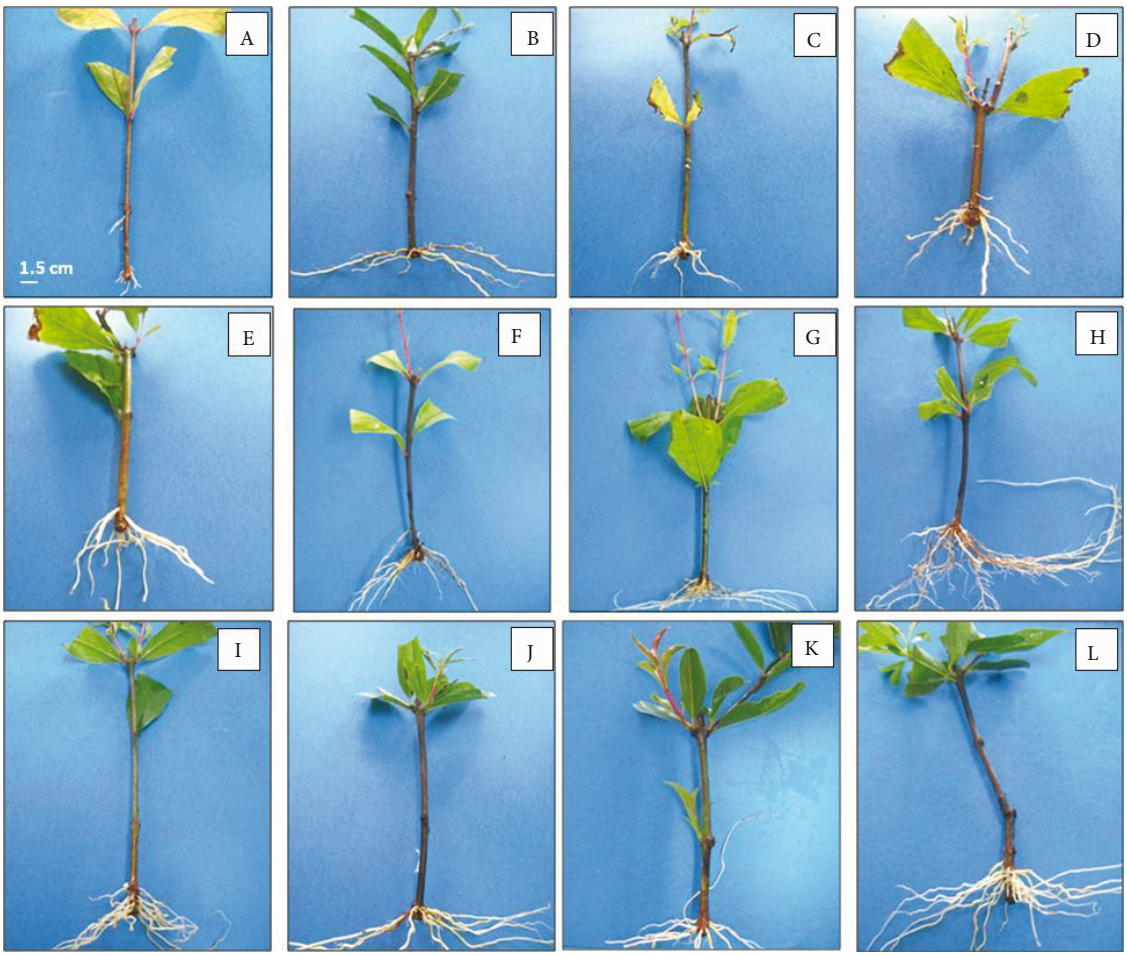

Figure 2. Representative photographs showing the effect of various treatments on rooting of *Punica granatum* stem cuttings. A- control, B- 1000 mg L^{-1} IBA, C- 1000 mg L^{-1} IBA + 250 mg L^{-1} GA_3, D- 1000 mg L^{-1} IBA + 500 mg L^{-1} GA_3, E- 17 g L^{-1} H_2O_2, F- 1000 mg L^{-1} IBA + 17 g L^{-1} H_2O_2, G- 17 mg L^{-1} H_2O_2, H- 1000 mg L^{-1} IBA + 17 mg L^{-1} H_2O_2, I- 1.16 mg L^{-1} MEL, J- 1.16 mg L^{-1} MEL + 1000 mg L^{-1} IBA, K- 352 mg L^{-1} ASC, and L- 352 mg L^{-1} ASC + 1000 mg L^{-1} IBA. Scale bar = 1.5 cm. Photographs were taken 30 days after planting.

Table 4. Effect of 1000 mg L^{-1} IBA and different concentrations of GA_3 and H_2O_2, alone or in combination with 1000 mg L^{-1} IBA, in *Punica granatum* L. cv. Wonderful cuttings on rooting percentage, number of roots per rooted cutting, average root length (mm), and number of shoots per cutting.

Treatment	Rooting %	Number of roots per rooted cutting	Average length of roots (mm)	Number of shoots per cutting
Control	74.0 ± 8.1	6.4 ± 0.52	18.6 ± 2.1	3.3 ± 0.7
1000 mg L^{-1} IBA	88.0 ± 4.9	12.3 ± 1.2	18.9 ± 3.0	2.7 ± 0.4
1000 mg L^{-1} IBA + 250 mg L^{-1} GA_3	60.0 ± 7.1	9.4 ± 0.8	19.1 ± 4.6	2.1 ± 0.1
1000 mg L^{-1} IBA + 500 mg L^{-1} GA_3	42.0 ± 6.6	9.5 ± 1.3	13.8 ± 1.9	1.9 ± 0.2
17 g L^{-1} H_2O_2	80.0 ± 6.3	6.6 ± 0.9	18.4 ± 3.3	2.2 ± 0.2
1000 mg L^{-1} IBA + 17 g L^{-1} H_2O_2	83.0 ± 3.7	10.7 ± 1.2	22.5 ± 4.9	2.1 ± 0.2
17 mg L^{-1} H_2O_2	86.0 ± 5.1	9.5 ± 0.5	22.1 ± 3.0	1.9 ± 0.1
1000 mg L^{-1} IBA + 17 mg L^{-1} H_2O_2	77.0 ± 6.8	9.4 ± 0.4	26.1 ± 3.8	2.2 ± 0.2

Values are means (n = 5) ± SE according to Duncan's multiple range test ($P \leq 0.05$).

3.5. The effects of MEL and ASC on rooting and number of shoots per cutting

A very limited number of studies have been conducted on the effects of MEL on rooting and proliferation of fruit trees. MEL appears to play a role in the first stages of plant growth (Hernández-Ruiz et al., 2004). In the present study it was found that the application of MEL alone to cuttings can have the same positive role as IBA on rooting (Figures 2I, 2J). Furthermore, in combination with IBA, MEL seems to have a synergistic effect on rooting, not only producing a large number of roots but also the longest ones, which differed significantly from control (Table 5). Similar results have been reported in other studies (Sarropoulou et al., 2012a, 2012b), which found that the balance between MEL and serotonin, in combination with an auxin, promoted morphogenesis in plants. Changes in the concentrations of MEL in plant tissues have been shown to influence the root growth rate (Sarropoulou et al., 2012a). Chen et al. (2009) found that the exogenous application of MEL at low concentrations in *Brassica juncea* promoted root growth, whereas at high concentrations rooting was inhibited. Reactive oxygen species have been reported to activate Ca^{2+} influx, which enhances cell elongation (a key process in root growth), making cell membranes more permeable (Foreman et al., 2003).

In the present study, when 352 mg L^{-1} ascorbic acid was applied, either alone or in combination with 1000 mg L^{-1} IBA, it promoted the rooting of cuttings (Table 5; Figures 2K, 2L). Similar results have been reported for *Tylophora indica*, where 100 mg L^{-1} ASC promoted root and shoot organogenesis (Sharma and Chandel, 1992). This could be ascribed to the fact that ASC controls the synthesis of hydroxyproline, which is contained in the proteins that are essential for the growth of cells in the G1 and G2 phases (Liso et al., 1984). Ascorbic acid is also one of the most important factors in H_2O_2 detoxification of cells (Noctor and Foyer, 1998), and at high concentrations it helps cells to maintain constant redox potential. Furthermore, ASC is a strong antioxidant which reduces the effects of the phenolic compounds that are released due to wounding.

In conclusion, because of its high economic value, propagation techniques that lead to the rapid multiplication of the cultivar Wonderful are greatly sought after. Mist propagation in combination with certain chemicals, such as MEL, ASC, and H_2O_2; growth regulators; wounding; and cutting thickness have all been shown to promote rooting. Our findings indicated that rooting of cuttings can be improved by using a 1000 mg L^{-1} IBA concentration on very thin shoot cuttings (3–4 mm). Furthermore, it was found that 1.16 mg L^{-1} MEL can be a substitute for IBA, as it has the same positive effects, while H_2O_2 and ASC promote rooting of cuttings. Finally, it appears that in cultivar Wonderful, internodal cuttings can be used more profitably than nodal cuttings.

Table 5. Effect of 1000 mg L^{-1} IBA, H_2O_2, MEL, and ASC, alone or in combination with IBA, in *Punica granatum* L. cv. Wonderful cuttings on rooting percentage, number of roots per rooted cutting, average root length (mm), percentage sprouting, and number of shoots per cutting.

Treatment	Rooting %	Number of roots per rooted cutting	Average engthof roots (mm)	Sprouting %	Number of shoots per cutting
Control	80.0 ± 5.6	3.4 ± 0.8	24.3 ± 1.8	88.3 ± 3.9	0.5 ± 0.1
1000 mg L^{-1} IBA	89.4 ± 3.2	7.4 ± 0.8	33.2 ± 3.2	87.6 ± 2.1	2.1 ± 0.2
17 mg L^{-1} H_2O_2	74.5 ± 5.3	4.1 ± 0.6	31.3 ± 2.4	83.6 ± 5.3	2.2 ± 0.4
1000 mg L^{-1} IBA + 17 mg L^{-1} H_2O_2	94.6 ± 3.6	8.7 ± 0.3	30.5 ± 4.3	89.1 ± 5.3	2.1 ± 0.3
1.16 mg L^{-1} MEL	92.7 ± 3.4	5.8 ± 0.7	34.6 ± 3.7	83.6 ± 5.3	2.1 ± 0.2
1000 mg L^{-1} IBA + 1.16 mg L^{-1} MEL	98.2 ± 1.8	8.9 ± 0.4	37.5 ± 4.8	81.8 ± 6.4	1.9 ± 0.4
352 mg L^{-1} ASC	96.5 ± 2.1	9.8 ± 1.3	32.1 ± 3.7	73.0 ± 5.9	1.1 ± 0.1
1000 mg L^{-1} IBA + 352 mg L^{-1} ASC	93.2 ± 4.9	11.0 ± 1.0	26.4 ± 2.2	57.6 ± 5.7	1.0 ± 0.2
17 mg L^{-1} H_2O_2 + 352 mg L^{-1} ASC	98.2 ± 1.8	10.0 ± 1.3	30.2 ± 3.1	63.6 ± 5.7	0.9 ± 0.1

Values are means (n = 5) ± SE according to Duncan's multiple range test ($P \leq 0.05$).

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