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# Preserving and propropagation of the most important cherry and plum rootstocks introduced from Italy using plant tissue culture technique in Syria

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## ABSTRACT

Plant tissue culture has become an important technique in plant production. This research was carried out in the Laboratory of Biotechnology for Medicinal Plants of the National Commission for Biotechnology/Damascus, during the period between 2022-2023, to study the effect of some growth regulators on multiplication and rooting of the most important cherry rootstocks (Weiroot 10, Maxma 14) and clonal rootstock (Marianna GF8-1) Introduced From Italy and determine which combination of growth regulators lead to the highest rate of multiplication (in terms of number of shoots and length), and the best rooting (in terms of rooting percentage, number of roots and length). The highest survival and uncontaminated explants percentages were 82.26%, 70% and 66.5% obtained with 0.1% HgCl2 for 1 min in rootstocks Weiroot 10, Maxma 14 and Marianna GF8-1, respectively. The results indicated that Murashige and Skoog (MS) medium supplemented with 1.0 mg/l BA , 0.1 mg/l IBA and 0.2 mg/l GA3, achieved the highest shoot multiplication with an average of 4.85 and 4.62 shoots per explant and an average of 3.94 cm and 3.85 cm shoot length in rootstock Weiroot 10 and Maxma 14 respectively, and MS medium supplemented with 1.0 mg/l BA and 0.2 mg/l GA3, achieved the highest shoot multiplication with an average of 5.55 shoots per explant and an average of 3.62 cm shoot length in rootstock Marianna GF8-1. The greatest rooting percentages were 97%, 95% and 82.5% and the largest average number of roots were 5.12, 3.15 and 4.55 obtained when using IBA auxin at a concentration of 0.5 mg/l for studied rootstocks Maxma 14, Weiroot 10 and Marianna GF8-1, respectively with an average of 4.65 cm root length. The acclimatization percentages ranged between 74% and 91.5% in both rootstocks Marianna GF8-1 and Weiroot 10, respectively.

**Keywords: Cherry, Plum, Rootstocks, Micropropagation, Cytokinin, Auxin, Rooting.**

## Introduction

Prunus is a large, diverse genus in the Rosaceae family, commonly referred to as stone fruits. Principal commercial crops in this genus include peaches, nectarines, plums, prunes, pluots, apriums, apricots, cherries and almonds (Giovannini et al., 2014). Rootstocks are playing an increasingly crucial role in determining orchard efficiency and sustainability in fruit crops. Also, Selecting the right combination of rootstock and cultivar is important for optimizing fruit quality parameters (Kumar et al., 2024). Prunus rootstocks are commercially produced through stem seeds or cuttings. The production of rootstocks through seeds result in segregation and therefore uniform plants cannot be obtained and the mother plant characteristics cannot be maintained. Alternatively, rootstocks can be produced by cuttings and this clonal propagation method is the

favored in many parts of the world, because it allows the production of uniform propagules. However, propagation by cuttings is difficult in some Prunus genotypes due to the low rooting potential (Fachinello, 2000) and propagation of Prunus spp. by cutting does not guarantee healthy and disease-free plants (Holtz et al., 1995). In these circumstances, in vitro propagation of rootstocks emerge as a viable alternative way of propagation because it is not dependent on season, provides clean, disease and virus-free planting material. Micropropagation is a convenient and rapid procedure to obtain a large number of genetically identical plants (Antonopoulou et al., 2005). Basic stages of micropropagation technique are selection of plant to be explanted, taking plant samples, preparation stage including the planting of samples and preparation of nutrient media, culture stage where the explants are planted in an artificial nutrient media, propagation and development of shoots in a culture media, rooting and adaptation to the external environment [George et al., 2008].

Kodad et al. (2020) showed that the regulators'best concentration and type depends on the genotype to get a successful multiplication rate.

Several in vitro studies have been carried out using shoots and different tissues of prunus rootstocks and cultivars. For example; Sauer (1985) cultivated the meristems of the top buds on young shoots of cherry rootstock, Mazzard, in MS media containing 0.1 mg L-1 NAA and 2 mg L-1 BAP and provided the development of side shoots. Similarly, PevalekKozlina and Jelaska (1987) used shoot tips and side buds of wild cherry rootstock (Prunus avium L.) in vitro production. The best shoot propagation was achieved by adding 2.2 µM BA, 2.5 µM IBA and 0.3 µM GA3 to the modified WPM base nutrient media. In another in vitro micrografting study, Özzambak and Schmidt (1991) successfully propagated the Early Burlat and Viola cherry cultivars and F 12/1 and 209/1 rootstock shoot tips in the MS media containing 1.0 mg L-1 BAP. Akbas et al.(2009) mentioned that the explants of Amygdalus communis L. which were cultured on MS medium containing various concentration of BA, Kn, for shoot multiplication were best achieved from explants on MS medium containing 30 g/l sucrose, 7g/l agar and 2.0 mg/l BA. This amount of BA (2.0 mg/l) gave the best multiple shoot formation response with average of 16.10 shoots per explant. Abou Rayya et al. (2010) found that the most effective cytokinine for enhancing in vitro growth was BA followed by kinitine and zeatin respectively. Lower concentration of BA and kinitine at (0.5 and 1.0 mg/l) gave healthier plants than 2.0 or 4.0 mg/l. Unek et al. (2011) found that the highest number of shoots was produced by medium containing 1.0 mg / l BAP. Higher BAP (2.0 & 4.0 mg / l) concentration decreased number of shoots with Ganrem rootstock. Indole butyric acid (IBA) is the most widely used auxin to stimulate the rooting process in cuttings because of: 1) its high ability to promote root initiation (Weisman et. al., 1988) and 2) its weak toxicity and great stability in comparison to naphthalene acetic acid and indole- 3-acetic acid (Hartmann et. al., 1997). many workers who used different concentrations of IBA for rooting of in vitro micro shoots during micropropagation of various cherry and plum rootstocks (Sarropoulou et al., 2014; Zamanipour et al., 2015; Vujovi et al., 2018). Similarly, Mir et al. (2010) and Aghaye et al. (2013) achieved in vitro rooting in half strength MS medium supplemented with IBA in Mazzard, Mahaleb and Gisela 6 rootstocks. Very less rooting was observed when different concentrations of NAA (40% rooting) and IAA (30% rooting) were used. Sisko (2011) reported low percentage rooting with 1mg/l NAA as compared to IBA in Gisela 5. Kumar et al. (2020) achieved maximum rooting (100%) of clonal cherry rootstock Gisela 5 on full strength MS medium fortified with 0.5 mg/l IBA with thin, long roots devoid of callus. Wolella et al. (2017) reported that the best results for rooting of Prunus domestica cv. Stanley was obtained from half strength MS medium supplemented with 1.0 mg/l IBA, with an average number of 4.25 roots per shoot and 3.6 cm average root length. Therefore, the aim of this research was to develop an effective sterilization protocol for in vitro propagation of the most important cherry rootstocks (Weiroot 10, Maxma 14) and plum rootstock (Marianna GF8-1) Introduced from Italy, and determine the best combination of growth regulators to use in order to achieve the highest rate of multiplication and find a reproducible method for the successful rooting of studied rootstocks.

## Materials and Methods

**Plant Materials:** This research was carried out in the Laboratory of Biotechnology for Medicinal Plants of the National Commission for Biotechnology/ Damascus, during the period between 2022–2023. The source of plant materials was the nodal segments of two entrance cherry rootstocks (Maxma 14 and Weiroot), and plum rootstock (Marianna GF8-1) that are grown at the Sarghaya Research Station of the General Commission for Scientific Agricultural Research.

**Maxma 14:** is the result of a cross between Mazzard and Mahaleb. It is about three-fourths the size of Mahaleb, which lends itself to higher density plantings. It is compatible with most varieties and is precocious and productive. It is one of the leading rootstocks in use in Europe. Maxma 14 rootstock is tolerant to wet soils and is resistant to iron chlorosis (Lynn and Clive, 2010).

**Weiroot 10:** (Wei=Weihenstephan, root-rootstock) selection were developed out of wild cherry types (Prunus cerasus L.) from mountainous regions of Bavaria (Weihenstephan, TU München). Weiroot is known to have a graft incompatibility with some cherry varieties, be early fruiting and be easily propagated by with green buds (Büyükyılmaz and Öz, 1994).

**Marianna GF.8-1 (*P. cerasifera* P. munsoniana*):** is a suitable rootstock for plums and many nectarines and apricots. Good resistance to root nematodes, crown gall, and *Armillarea mellea*. Some suckering. Matures earlier then Myrobalan. Dwarfing stock producing a tree 50% of seedling (Lanauskas, 2006).

**Surface disinfection (Surface sterilization of explants**): New shoots of 15-20 cm in length were collected in mid-May from studied rootstocks and brought to the laboratory. After removing the leaves, shoots were cut into nodal cuttings, which contained 1-2 lateral buds and with lengths that ranged from 0.5 to 1 cm. For the purpose of disinfection, the explants were washed with running water for one hour. Next, they were surface sterilized by dipping in 70% (v/v) ethanol for 30 seconds, then in different sterilant for varying time duration.

- In the first experiment, the explants were surface sterilized with 30% sodium hypochlorite (NaOCl) solution containing 2 drops of Tween for (10, 20, 30) minutes.
- In the second case, the explants were surface sterilized with 0.1% mercuric chloride (HgCl2) solution for (1, 2) minutes. Finally, In all the above cases, the explants after surface sterilization process were rinsed three times (for five minutes each time) in distilled water. Surface sterilization was performed in sterilized conditions.

**Shoot initiation:** After sterilization, each explant was cultured in an MS medium (Murashige and Skoog, 1962) (Table 1). All the cultured tubes were placed and observed for four weeks in a growth chamber at 22±1 °C, with 16 h of photoperiod, and at a light intensity of 30 µmol m-2 s-1 provided by cool daylight fluorescent lamps (Figure 1).

**Shoot multiplication:** For shoot multiplication, the newly-formed microshoots were sub-cultured in MS medium including various types of growth regulators in order to achieve shoot multiplication:

- MS1: MS + 0.5 mg/l BA + 0.2 mg/l GA3.
- MS2: MS + 1.0 mg/l BA + 0.2 mg/l GA3.
- MS3: MS + 2.0 mg/l BA + 0.2 mg/l GA3.
- MS4: MS + 1.0 mg/l BA + 0.2 mg/l GA3 + 0.1 mg/l IBA.

All of the cultivated tubes were placed in a growth chamber at 22±1 °C, with 16 h of photoperiod, and a light intensity of 30 µmol m-2 s-1 provided by cool daylight fluorescent lamps. Number of shoots per explant and Shoot length per explant were recorded after 30 days of sub-culturing.

In vitro regenerated shoots were micropropagated and sub-cultured every three weeks.

**Rooting of shoots:** Uniform proliferated shoots (2- 3 cm) were transferred to half MS medium supplemented with various concentrations of IBA:

- R1: ½MS without plant growth regulators served as a control.
- R2: ½MS+ 0.5 mg/l IBA
- R3: ½MS+ 1.0 mg/l IBA
- R4: ½MS+ 2.0 mg/l IBA

All the treatments were maintained in the dark for one week and then were transferred to photoperiod of 16/8 h light/dark for three weeks. The rooting parameters: Rooting percentage (%), average number of roots and average length of roots (cm) were recorded after 4 weeks of in vitro culture.

**Acclimatization:** In vitro rooted shoots were removed from cultured tubes and the roots were gently washed in distilled water to remove any residual medium. Subsequently, they were transplanted into individual commercial plastic pots filled with an autoclaved mix of perlite and peat [1:2 (v/v)]. Plantlets were covered with clear borosilicate beaker to maintain a 90±5% relative humidity, for 4 weeks before transferring into the growth room. Relative humidity was slowly decreased by gradually removing beakers. Plantlets were acclimatized after 3 weeks in a green house at 25±2 ºC under natural daylight conditions.

**Figure 1. Preparation of rootstocks explants**

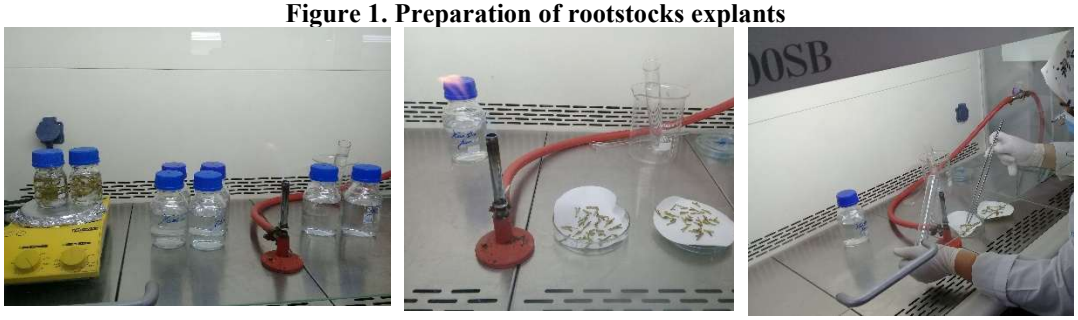

**Figure 2. Surface sterilization of explants and Shoot initiation**

**Figure 3. Acclimatization of *In vitro* rooted shoots**

**Experimental Design and Statistical Analysis:** All experiments were carried out according to completely randomized design. The multiplication experiment contained three rootstocks, Five Surface sterilization treatment, four proliferation treatments (medium), three rooting treatments, three replicates per each treatment, and 20 explants per replicate. The results were analyzed using the analysis of variance (ANOVA) method to determine the significant differences between the means of all treatments. Duncan's multiple range test was used at 1% level of significance to assess the significance of difference among means using the Genstat 12 statistical program. Means followed by the same letter are not significantly different.

## Results and Discussion

**Surface sterilization of explants:** Several factors can affect success of sterilization such as season of year, position of culture, location of explant on mother plant, method of sterilization, both type and concentrations of sterilization chemical materials and finally exposure period to sterilization materials. As shown in Table (1) cleared the effect of sodium hypochlorite and mercuric chloride on surface sterilization of studied rootstocks explants for micro propagation. The responses of explants to various types and concentrations of sterilization agents were different. The highest percentages of uncontaminated and survival explants (66.5%, 70%, and 82.26%) were recorded when explants of

studied rootstocks (GF 8-1, Weiroot 10 and Maxma 14) were disinfected with 0.1% $HgCl_2$ for 1 min, Meanwhile, the lowest concentration of NaOCl (1.5%) at all exposure periods possessed the lowest percentage of uncontaminated survival explants. These results clearly revealed that the variable impact of sterilizing agents and success of sterilization is dependent on genotype used. The results obtained in this study revealed that the sodium hypochlorite alone was the least effective in the sterilization process, while the use of mercury chloride $HgCl_2$ with sodium hypochlorite were the most effective, although $HgCl_2$ is extremely toxic (Alizadeh et al., 2020).

**Table 1. Effects of sterilizing agents used in a different concentration with varying time of sterilizing nodal cuttings of the studied rootstocks.**

| Treatments | Uncontaminated and survival explants percentage | | | Mean |
|---|---|---|---|---|
| | GF 8-1 | Weiroot 10 | Maxma 14 | |
| 1.5% NaOCl for 10 min | 0 k | 1.22 k | 6.67 j | 2.63 E |
| 1.5% NaOCl for 20 min | 5.64 j | 10.5 i | 16.66 h | 10.93 D |
| 1.5% NaOCl for 30 min | 18.23 h | 35.5 f | 28.33 g | 27.35 C |
| 0.1% $HgCl_2$ for 1 min | 49.12 e | 55.12 d | 53.33 d | 52.52 D |
| 0.1% $HgCl_2$ for 2 min | 66.5 c | 82.26 a | 70 b | 72.87 A |
| Mean | 27.90 C | 36.89 A | 35 B | …… |
| L.S.D 0.01 | Rootstocks | 0.97 | | |
| | Treatments | 1.25 | | |
| | Interaction | 2.17 | | |

**Figure 4. New shoot of Maxma 14 rootstock at the second week of initiation stage.**

**Figure 5. New shoot of Weiroot rootstock at the end of initiation stage.**

**The Effect of Different Plant Growth Regulator Combinations on the Shoot Multiplication of studied rootstocks:** The data in Table 2 show the effect of different concentrations of BA in combination with GA3 at 0.1 mg/l alone or with GA3 at 0.1 mg/l and IBA at 0.1 mg/l on the average number of shoots produced per explant as well as the mean length of the shoots. According to the data, the MS medium culture supplemented with 1.0 mg/l BA plus 0.2 GA3 plus 0.1 IBA, resulting in a mean number of 4.20 shoots with an average shoot length of 3.28 cm, is the most suitable treatment. As for the interactions between studied rootstocks growth regulator combinations, the results clarified that the highest significant average number of shoots/explant (4.85 and 4.62) and the longest shoot (3.94 and 3.85 cm) for both Weiroot 10 and Maxma 14 rootstocks, respectively, were obtained in a medium supplemented with BA at 1 mg/l plus GA3 at 0.2 mg/l plus IBA at 0.1 mg/l (Figure 6), while the highest significant average number of shoots/explant (5.55) and the longest shoot (3.62 cm) for GF 8-1 rootstock were obtained in a medium supplemented with BA at 1.0 mg/l plus GA3 at 0.2

mg/l. Prior research indicates that each plant species propagated in vitro needs different requirements and concentrations of plant growth regulators. Most of them are based on BAP and auxins IBA, IAA and NAA (Channuntapipat, 2002). Brison et al. (1995) found that the simultaneous presence of cytokinin, giberellin and auxin in the medium was more effective for Prunus rootstocks in vitro. It was indicated that the cytokinin, such as BA, encourages cell division by activating DNA synthesis, inducing growth of lateral buds, and promoting shoot formation (Dobranszki and Silva, 2010). The selection of BA as a cytokinin was due to its effect in vitro with several woody plants (Bennett and Davies, 1986). In this regard, it is important to observe that the multiplication medium should be supplied with more cytokinin in relation to auxin (Murashige, 1974). The effect of cytokinins on tissue or organ cultures differs based on the culture type, the rootstock used, and explant age (George et al., 2007). The auxins control cytokinin levels through repressing its synthesis ratio and its gathering size (Nordstrom et al., 2004).

**Table 2. Micropropagation of studied rootstocks explants in MS medium supplemented with different plant growth regulators combinations**

| Media | Avg No of shoots/ explants | | | | Avg length of shoots (cm) | | | |
|---|---|---|---|---|---|---|---|---|
| | Maxma 14 | Weiroot 10 | GF 8-1 | Mean | Maxma 14 | Weiroot 10 | GF 8-1 | Mean |
| MS1 | 1.43 c | 1.65 c | 2.10 c | 1.72 B | 1.12 b | 2.33 ab | 2.15 ab | 1.87 BC |
| MS2 | 1.65 c | 2.15 c | 5.55 a | 3.42 A | 2.25 b | 2.55 ab | 3.62 a | 2.47 AB |
| MS3 | 3.22 bc | 3.40 bc | 3.05 bc | 3.22 A | 0.63 b | 1.4 b | 1.54 b | 1.19 C |
| MS4 | 4.62 ab | 4.85 ab | 3.12 bc | 4.20 A | 3.85 a | 3.94 a | 2.05 ab | 3.28 A |
| Mean | 2.95 A | 3.01 A | 3.45 A | ..... | 1.7 A | 2.55 A | 2.34 A | ..... |
| l.S.D (0.01) | Rootstocks | 0.93 | | | 0.93 | | | |
| | Media | 1.07 | | | 1.07 | | | |
| | Interaction | 1.86 | | | 1.86 | | | |


**Figure 6. Shoot culture at the end of multiplication stage for Maxma 14 (a) and Weiroot rootstock (b) in MS medium supplemented with BA at 1 mg/l plus $GA_3$ at 0.2 mg/l plus IBA at 0.1 mg/l (b), and for GF8-1 rootstock (c) in MS medium supplemented with BA at 1 mg/l plus $GA_3$ at 0.2 mg/l**

**The Effect of Different concentrations of auxin on rooting of studied rootstocks:** The data of percentage of rooting, roots/explants, and average length of roots (cm) as affected by the concentration of auxin were presented in table 3 and figure 7. Based on the results obtained, the effect of IBA concentrations was significant on the rooting percentage (%), root number and root length (Table 3). The highest rooting percentage (91.50%), maximum number of roots/shoot (4.27), and highest root length (4.65 cm) were obtained in half strength MS medium supplemented with 0.5 mg/l IBA. However, no rooting was noticed with IBA at the control treatment for all used rootstocks. The interactions between treatments and rootstock, showed the highest rooting percentages (97 %, 95%, 82.5%) and the maximum number of roots (5.12, 3.14, 4.55) and the highest root length (4.50 cm, 4.33 cm, 5.12 cm) were obtained in half strength MS medium supplemented with 0.5 mg/l IBA for all

studied rootstocks (Maxma 14, Weiroot 10 and GF 8-1, respectively). Indole-3-butyric acid (IBA) is commonly used to promote root initiation both in vitro and with cuttings (Pan and Zhao, 1994). IBA can enhance rooting via increased internal free IBA or may synergistically modify the action of endogenous synthesis of IAA (Krieken et al., 1993). Thus, keeping cultures in the dark for a short period prior to transfer them into light condition can enhance in vitro rooting ability because photoreceptor activation in dark is one of the factors which are involved in plant growth processes (Tian et al., 2007; Lamrioui et al., 2011; Housman, 2003). Furthermore, IBA is more stable and less sensitive to auxin degrading enzymes (Riov, 1993). Sabatini et al. (1999) reported that differentiation of phloem ray parenchyma cells into root primordia depends upon the type and concentration of auxin. In the literature, the concentration of 1.0 mg/1 of IBA is usually the one mostly used (Drew et al., 1993; Kalinina and Brown, 2007). Baker and Wetzstein (2004) have reported that higher concentrations of auxin induce the higher level of degradative metabolites in tissues, thus blocking the regeneration process. Moreover, Sugiyama (1999) has reported that the effect of an auxin on rooting is promontory at low concentrations and inhibitory at supra-optimal concentrations.

**Table 3. Effect of various levels of IBA on rooting of studied rootstocks shoots on half strength MS medium.**

| IBA treatment (mg/l) | Rooting percentage (%) | | | Mean | Avg No of roots/shoot | | | Mean | Avg length of root (cm) | | | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Maxma 14 | Weiroot 10 | GF 8-1 | | Maxma 14 | Weiroot 10 | GF 8-1 | | Maxma 14 | Weiroot 10 | GF 8-1 | |
| **0.00** | 0 i | 0 i | 0 i | 0 D | 0 e | 0 e | 0 e | 0 D | 0 e | 0 e | 0 e | 0 D |
| **0.50** | 97 a | 95 b | 82.5 c | 91.50 A | 5.12 a | 3.15 bc | 4.55 ab | 4.27 A | 4.50 ab | 4.33 ab | 5.12 a | 4.65 A |
| **1.00** | 76 e | 80 d | 75 e | 77 B | 4.33 ab | 2.44 c | 3 bc | 3.26 B | 3.24 bc | 2.55 cd | 3.67 abc | 3.15 B |
| **2.00** | 20.24 g | 32.5 f | 15 h | 22.58 C | 1.75 cd | 0.5 de | 1.66 cd | 1.30 C | 1.37 de | 1.22 de | 1.10 de | 1.23 C |
| **Mean** | 48.31 B | 51.88 A | 43.12 C | ..... | 2.80 A | 1.52 B | 2.30 A | ..... | 2.27 A | 2.03 A | 2.47 A | ..... |

| | | | | |
|---|---|---|---|---|
| **I.S.D (0.01)** | **Rootstocks** | **0.93** | 0.74 | 0.80 |
| | **Media** | **1.08** | 0.85 | 0.93 |
| | **Interaction** | **1.86** | 1.47 | 1.61 |


**Figure 7. Rooting of studied rootstocks on half strength MS medium containing 0.5 mg/l IBA ( a. Maxma 14., b. Weiroot., c. GF8-1);**

**Acclimatization:** The data in Table 4 show the survival percentage ranged between 74% and 91.5%. The Rootstock Weiroot 10 showed the highest survival rate (91.5%). It has now been observed that the process of acclimatization depends on a number of crucial factors, including genotype, which not only influences the response of the explant to different culture media, but also the organogenesis and ability of the regenerated plants to withstand the ex-vitro growing conditions (Hazarika et al., 2006). Figure 8 shows the survival percentage at 30 days after transfer to an autoclaved mix of perlite and peat.

**Table 4. Percentage Survival Rate of Rootstocks Plantlets at 30 Days after Transfer to an autoclaved mix of perlite and peat.**

| Rootstock | Percentage Survival Rate (%) |
|---|---|
| **Maxma 14** | 85 b |
| **Weiroot 10** | 91.5 a |
| **GF 8-1** | 74 c |
| L.S.D 0.01 | 3.03 |

**Figure 8. Acclimatized of studied rootstocks plantlets after 1 months**

## Conclusions

1. In this study, a protocol for surface sterilization of three stone fruit rootstocks was developed using nodal segments.
2. MS medium containing 1.0 mg/l BA, 0.1 mg/l IBA, and 0.2 mg/l GA3 was chosen as the optimum medium for multiplication and development of Weiroot 10 and Maxma 14 shoots.
3. MS medium containing 1.0 mg/l BA and 0.2 mg/l GA3 was chosen as the optimum medium for multiplication and development of GF 8-1 shoots.
4. half strength MS medium containing 0.5 mg/l IBA was chosen as the optimum medium for rooting of Weiroot 10, Maxma 14 and GF 8-1 shoots.

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