Surface sterilization and *in vitro* propagation of *Prunus domestica L. cv. Stanley* using axillary buds as explants

Eyob Kassaye Wolella

Department of Biotechnology, Natural and Computational Science, Wolkite University, Wolkite, Ethiopia

Received: December 30, 2016; accepted: March 17, 2017.

The aim of the study was to investigate the factors needed for establishing an effective protocol for propagation of *Prunus domestica cv. Stanley* using axillary buds. Axillary buds were sterilized by using different concentration of sodium hypochlorite and mercuric chloride, and then cultured on Murashige and Skoog media supplemented with 6-benzoylaminopurine (BAP) or kinetin (KIN) (0.5-3.0 mg/l) alone and in combination with indole-3-butyric acid (IBA) (0.1-0.5 mg/l). The shoots were transferred to half strength MS medium supplemented with varying concentrations of IBA or indole-3-acetic acid (IAA) (0.5-3 mg/l) for root growth and development. The highest significant survival value (97%) were recorded when explants disinfected with 2% sodium hypochlorite for 15 minutes and 0.1 % mercuric chloride for 7 minutes. The highest multiplication rate as well as length of axial and lateral shoots was obtained on media with BAP. Very poor multiplication was achieved on media with KIN. Whereas, in many combinations of KIN or BAP with IBA, and particularly in those with BAP and IBA, highest shoot proliferation was achieved. The highest shoot induction was observed on MS media supplemented with 0.5 mg/l BAP in combination with 0.1 mg/l IBA with an average number of 3.08 ± 0.58 shoots per explants and 3.33 ± 0.29 cm average shoot length. The highest rooting was observed on 1.0 mg/l IBA with an average number of 4.25 ± 1.2 roots per shoot and 3.6 ± 0.1 cm average root length. Therefore, these developed protocols are recommended for *in vitro* regeneration of *Prunus domestica L. cv. Stanley*.

Keywords: plum; sterilization; *In vitro* propagation; MS media.

***Corresponding author:** Eyob Kassaye Wolella, Department of Biotechnology, Natural and Computational Science, Wolkite University, P. O. Box 07, Wolkite, Ethiopia. E-mail: eukassa2006@yahoo.com

Introduction

Plum, one of the most taxonomically diverse stone fruit, belongs to *Prunus* genus and *Rosaceae* family, adapted from temperate to tropical regions of the world [1, 2]. Plums are used for their edible fruit, ornamental purposes, and rootstocks for almost all other *Prunus* species [2-7].

Plums, and their dried form (prunes), have laxative, anticancer, antihyperglycemic, anti-hyperlipidemic, antihypertensive, anti-osteoporosis, and hepatoprotective activities because of their lower fat, carbohydrates (sorbitol, glucose, fructose, and sucrose), amino acid, organic acids (Malic acid, citric, tartaric, benzoic, and boric acid), vitamins (A, B1, B2, C, and K), minerals (potassium, calcium, magnesium, zinc, copper, manganese, selenium, and boron), dietary fibers, and polyphenolic compounds [2, 8- 12].

The plum fruit crop is produced all over the world. However, the amount of plum production fluctuates considerably from year to year. The

first recorded introduction of temperate fruit germplasms to Ethiopia was made in 1971 from California, USA to investigate their adaptability potential. Temperate fruit production in Ethiopia is highly promising even though the culture is new to the farming society and is limited to a few places in the highland areas [13, 14]. At present temperate, fruit growing farmers produce the fruits in small scale level in this country. Currently, there is a growing awareness among the highland communities, and efforts are being made to expand the production in several highland places by government organizations, non-government organizations (NGOs), and private growers. Therefore, production of planting materials would be an important business opportunity in near future.

Most of the *Prunus* seeds show poor germination percentage and genetic variability [15-20]. The major fruit species of the world are now propagated asexually by the processes of budding, grafting, and cutting. The choice of rootstocks for grafting can have a profound effect on growth, tolerance to soil and climatic variables, resistance to soil pests and pathogens, yield efficiency, anchorage and ease of propagation. The propagation by cutting is seasonal dependent, laborious, and requires large area for propagation [2, 6, 21-23].

The problems exhibited for plum propagation by seed, stem cuttings and grafting can be overcome by micropropagation. This would therefore serve to hasten the plum breeding programs [2, 4, 6].

Reports of micropropagation of *Prunus* species, such as plums, are very limited in the literature. However, micropropagation methods have been developed for some species of *Prunus* using stem node, shoot tip, axillary buds, leaf, cotyledons, and seed explants [15, 24-37].

There is no universal medium for *in vitro* culture because plant species and cultivars are genetically specific with regard to different components of the medium. Murashige and Skoog medium has been proved to be the most suitable medium for successful explant development of stone fruit (almond, apricot, and peach) [38]. One of the most important aspects of successful micropropagation is determination of an effective sterilization protocol, optimal types, and proper concentrations of plant growth regulators as medium constituents [26, 28, 32, 39-41]. Therefore, the objective of this study was to develop an effective sterilization protocol for *in vitro* propagation of *Prunus domestica L. cv. Stanley*.

Materials and methods

Stock solution and media preparation

MS media [42] were prepared by dissolving the appropriate amount of macro and micro nutrients and organic supplements. Plant growth regulators (BAP, KIN, IBA, and IAA) stock solutions were prepared by using the proportion of 1 mg : 1 ml and stored in a refrigerator at 4°C for further use. The MS culture media were prepared from its respective stock solutions by using 3% sucrose, different concentration of plant growth regulators, and agar (7 g/l) for shoot initiation and multiplication. The plant growth regulator BAP or KIN (0, 0.5, 1.0, 1.5, 2.0, and 3.0 mg/l) and in combination with IBA (0, 0.5, and 1.0 mg/l) were added separately to the media to study the effect on shoot proliferation. The medium was boiled until the agar melted completely. Then, 55 ml of the medium was dispensed in each culture jar and autoclaved at 121°C for 25 min after adjusting the pH to 5.8 with 1 N NaOH or 1 N HCl.

Plant material and effect of different sterilization treatments during the establishment stage

This experiment was made to investigate effect of sterilizing agents on percentage of the explants survival (percentage of alive explants) and percentage of non-contaminated explants. The axillary buds (as starting plant materials) were taken from young branches of *Prunus domestica cv. Stanley* trees growing in a highland

fruit nursery site in Gondar, Ethiopia. The explants were stored in an icebox before transportation to the tissue culture laboratory at Department of Biotechnology, University of Gondar. The explants were prepared by taking growing nodal segments and removing extra leaf sheaths. Explants were rinsed thoroughly in soap water, then washed by running tap water for 15 to 30 min to remove soil and other superficial contamination. The nodal segments were rinsed in sterile-distilled water for 30 min. The explants were rinsed for 20 min with sterile cold anti-oxidant solution (150 mg/l citric acid and 100 mg/l ascorbic acid) to avoid a browning problem of the tissue in the culture.

The explants were soaked for 30 min in 70% ethanol under aseptic conditions in a laminar air-flow cabinet. To develop a successful protocol for sterilization, the following sterilization treatments were used. The prepared explants were immersed in different concentrations of NaOCl (1%, 2%, and 3% (v/v)) and/or mercuric chloride ($HgCl_2$, MC) at concentrations of 0.05 %, 0.1%, and 0.2 % (w/v) for different exposure times (table 1) with a few drops of Tween-20. Each treatment consisted of three jars with each jar containing four axillary bud as explant source. After disinfection treatments, the explants were thoroughly rinsed for 4-5 times to remove all traces of the disinfectants and attached sterilizing agent by using sterile double distilled water. The sterilized bud segments were kept in fresh sterile double distilled water until final trimming and culturing them on a basal MS medium [42].

The culture jars with cultured explants were securely sealed with Parafilm™ and clearly labeled. The cultures were then transferred to the growth room with 16 hours of photoperiod (8 hours dark) and 2,700 lux light intensity at 25 ± 2 OC. Observations were recorded regularly during 30 days to identify the non-growing cultures, infected cultures, and healthy cultures. The surviving explants were taken as a source for the plant material used for the following experiments.

Effects of BAP and KIN alone and in combination with IBA for shoot initiation and multiplication

This experiment was designed to study the effect of different concentrations of BAP or KIN (0.0, 0.5, 1.0, 1.5, 2.0, and 3.0 mg/l) alone and in combination with 0.1 mg/l IBA on shootlet development of the axillary bud explants of *Prunus domestica cv. Stanley*. To obtain high proliferation of shoots, the explants were subcultured three times on the best medium to obtain stock materials for the following experiments. One month after the third subculture (after three months from the first subculture, or starting the experiment), the numbers of shootlets/explants and shootlet length (cm) were recorded.

Statistical data Analysis

All the experiments in this study were prepared in completely randomized design (CRD) with three replications. A maximum care was taken to minimize any variation in the laboratory conditions among treatments for each of the experimental material. Statistical data analysis was done by using Excel spreadsheets and SPSS (version 16.0). The analysis of variance (ANOVA) was used to detect the significance of difference among treatments at $p \leq 0.05$. Means of different treatments were compared by using Duncan Multiple Range Test at a 5% confidence interval.

Results and discussion

Protocol for sterilization of *Prunus domestica cv. Stanley* axillary bud explants for culture establishment

One of the challenges faced in *in vitro* propagation of plant was the microbial contamination at the initiation and multiplication stages. The microbes competed with plant tissue culture for nutrients; hence they increased explants' mortality, reduced shoot proliferation and rooting, and contributed to tissue necrosis and growth abnormality [43]. Most likely it is impossible to prevent

Table 1. Effects of sterilizing agents used in a different concentration with varying time of sterilizing axillary buds of *Prunus domestica*.

Treatments and exposure time (minutes)	Contaminated buds (%)	Survival buds (%)	Damaged buds (%)
1% NaOCl for 30 min	100^{a}	0^{e}	0^{b}
2% NaOCl for 25 min	94^{a}	5.53^{de}	0^{b}
3% NaOCl for 15 min	74.67^{c}	2.77^{de}	19.43^{a}
0.05% $HgCl_2$ for 15 min	91^{ab}	8.3^{cd}	0^{b}
0.1% $HgCl_2$ for 10 min	80.33^{bc}	13^{c}	0^{b}
0.2% $HgCl_2$ for 7 min	77.67^{c}	0^{e}	22.2^{a}
2% NaOCl for 15 min and 0.1% $HgCl_2$ for 7 min	3^{d}	97^{a}	2.77^{b}
1% NaOCl for 20 min and 0.2% $HgCl_2$ for 5 min	2.59^{d}	91^{b}	5.53^{b}

contamination of the *in vitro*-grown plants unless preventative measures are taken. In most commercial and scientific plant tissue culture laboratories, the losses due to contamination were between 3 to 15% for every *in vitro* subculture [44]. This issue resulted in economic losses because of waste of time, effort, and materials [45]. The elimination of microorganism in woody plant material was problematic especially [46].

Sathyanarayana and Varghese have described the methods of surface sterilization depending on plant species, surface contaminant levels, growth environment, age, and part of the plant used for micro propagation [47]. Therefore, this experiment was conducted to study the effectiveness of sodium hypochlorite and/or mercuric chloride for surface sterilization of *Prunus domestica cv. Stanley* axillary bud explants for micro propagation. After three to four days of bud transfer to sterile MS medium, the growth of microorganisms (bacteria and/or fungi) was observed around the base of the explants. This problem could have been caused by insufficient aseptic techniques during working, incomplete surface sterilization of the explants, and the microorganism available in the explants [48, 49]. The responses of explants to various types and concentrations of sterilization agents were different. The analysis of variance (ANOVA) and the level of statistical significance ($p \leq 0.05$) for the contamination, survival, and damage of explants are presented in table 1.

The data as shown in table 1 revealed that, as the concentration of sodium hypochlorite increased from 1% to 3%, contamination was decreased, and the same was true when the concentration of $HgCl_2$ increased from 0.05% to 0.2% for almost all levels of exposure time. There were non-significant differences of low contamination and minimum explant death when explants were disinfected with 1% NaOCl for 20 min in combination with 0.2 % $HgCl_2$ for 5 min, or 2% NaOCl for 15 min in combination with 0.1 % $HgCl_2$ for 5 min. The highest significant survival value (97%) was recorded when explants were disinfected with 2% NaOCl for 15 min and 0.1 % $HgCl_2$ for 7 min (figure 1). It is known that, in order to reduce the rate of explants mortality during surface sterilization, the sterilizing agent concentration should be reduced as the exposure time increased, and vice versa, to minimize the phototoxic activity of the sterilizing agents [47].

Figure 1. The best surviving and healthy explants were obtained during surface sterilization of axillary buds using 2% NaOCl for 15 min and 0.1% $HgCl_2$ for 7 min.

Table 2. Mean shoot number and mean shoot length produced at different concentration of (0-3 mg/l) BAP in combination with 0.1 mg/l IBA.

BAP (mg/l) + 0.1 mg/l IBA	Mean number of shoots/explants	Mean shoot length (cm)
0	1.00 ± 0.00^{a}	1.7 ± 0.50^{a}
0.5	3.00 ± 0.58^{b}	3.33 ± 0.29^{b}
1	2.67 ± 0.44^{bc}	2.50 ± 0.50^{a}
2	2.77 ± 0.58^{bc}	3.03 ± 0.153^{ab}
3	1.00 ± 0.58^{a}	1.33 ± 0.29a

Note: Means annotated with the same superscript letters in the same column are not significantly different at the 5% probability level.

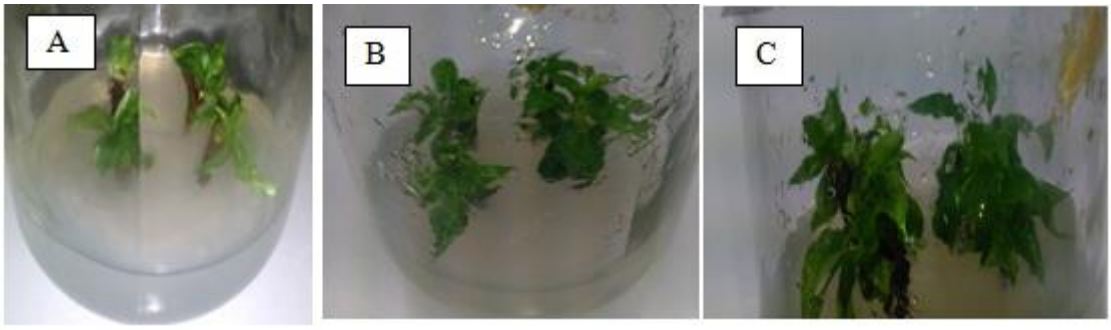

Figure 2. Shoots produced in MS medium supplemented with different concentrations of BAP (mg/l) in combination with 0.1 mg/l IBA. (A) Control. (B) 0.5 mg/l BAP in combination with 0.1 mg/l IBA. (C) 1 mg/l BAP in combination with 0.1 mg/l IBA.

None of the surface-sterilizing agents were effective for total elimination of microorganism using sodium hypochlorite alone or mercuric chloride alone [50]. In this experiment, the positive effect of the combination of methods might be due to a sufficient synergistic effect of $HgCl_2$ and NaOCl on suppression of the survival of microorganism within a short period, and hence, it did not affect the cultured explants. Similarly, other researchers used the sodium hypochlorite in combination with mercuric chloride for effective sterilization of different explants [51-53]. However, there was slight modification of concentration of mercuric chloride and sodium hypochlorite including the exposure time. This change might be due to variation of plant materials taken for sterilization.

Shoot induction and multiplication

Different concentrations of plant growth regulators such as BAP or KIN (0.0, 0.5, 1.0, 1.5, 2.0, and 3.0 mg/l) alone or in combination with 0.1 mg/l IBA were evaluated for the maximum production of multiple shoots. After 4 weeks of culture, shoot number and shoot length were observed and recorded. The cultures were subcultured at an interval of 3 weeks for three times for multiple shoot proliferation.

The results indicated that the medium containing 0.5 mg/l BAP or 1 mg/l BAP in combination with 0.1 mg/l IBA showed significant similar positive response for shoot induction and shoot multiplication. Hence, the use of the lower concentration of BAP was recommended because it was more economical.

Table 3. Effects of different concentrations of IBA for root induction on half strength MS medium in *Prunus domestica L. cv. Stanley*.

IBA (mg/L)	Percentage rooting	Mean number of roots per plantlet	Mean root length (cm)
Control	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
0.5	70.66 ± 0.10[c]	3.0 ± 1.25[c]	2.3 ± 0.3[d]
1.0	100 ± 0.00[d]	4.25 ± 1.2[d]	3.6 ± 0.1[e]
1.5	72.67 ± 0.00[c]	3.20 ± 0.9[c]	2.4 ± 0.5[d]
2.0	30.73 ± 0.00[b]	1.8 ± 0.2[b]	1.7 ± 0.45[c]
3.0	28.22 ± 0.00[b]	0.5 ± 0.4[a]	0.9 ± 0.8[b]

Note: The means followed by the same letter in a column are not statistically different according to the Duncan's multiple range test ($P \leq 0.05$).

Figure 1. Root initiation of plum shootlets on half strength MS media supplemented with 1 mg/l IBA.

The results were in line with those of prior researchers [31, 35, 36, 40, 54, 55]. In comparison to KIN, BAP has high physiological capacity to break apical dominance and promotes shoot proliferation at low concentrations [31, 32, 35, 56, 57]. MS initiation medium supplemented with different concentrations of KIN and combinations of KIN with 0.1 mg/l IBA induced a lower mean number of shoots than that of MS medium prepared with different concentrations of BAP and combinations of BAP with 0.1 mg/l IBA (table 2, figure 2).

The highest shoot induction was observed on MS media supplemented with 0.5 mg/l BAP in combination with 0.1 mg/l IBA with an average number of 3.08 ± 0.58 shoots per explant and 3.33 ± 0.29 cm average shoot length. The results of this study revealed that subculturing in the prescribed sequence of stages used here had no significant effect on the multiplication rate of the shoots. This was similar to a study by Vujovic *et al* [58].

Root initiation

The shoots produced *in vitro* during shoot proliferation were transferred to half strength MS media supplemented with different levels of IBA and IAA (0.5, 1.0, 1.5, 2.0, and 3.0 mg/l) for root induction and development of root systems. The mineral concentrations of the culture

medium contribute in the process of regulating hormonal balance for root initiation. It is well known that half strength MS media reduces callus formation. Some researchers have proposed the superiority of half strength MS medium for root induction [35, 37, 59, 60]. The data of percentage of rooting, roots/explants, and average length of roots (cm) as affected by the type of auxin concentration were presented in table 3 and figure 3. The highest rooting percentage (100%), significantly highest root number (4.25 ± 1.2), and root length (3.6 ± 0.1 cm) were obtained in half strength MS media supplemented with 1mg/l IBA. Poor root growth and development were reported in half MS media supplemented with IAA and in the control treatment. The highest concentrations of IBA proportionally encourage tissue lignification, which lead to considerable decrease in rooting ability. Similar results were previously reported in other temperate fruit species by many researchers [32, 58-61].

The lower response of IAA and higher response of IBA for root induction might be due to rapid phytochemical and enzymatic oxidization by an oxidase. IBA apparently oxidized slowly. IBA can enhance rooting via increased internal free IBA or may synergistically modify the action of endogenous synthesis of IAA. 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 [32, 40, 62].

Conclusion

In this study, a protocol for surface sterilization and micro propagation of plum (*Prunus domestica L. cv. Stanley*) was developed using axillary bud explants. During sterilization protocol establishment, the highest significant survival value (97%) was recorded when explants disinfected with 2% sodium hypochlorite for 15 min and 0.1 % mercuric chloride for 7 min. The induction and proliferation of shoots and roots of these plants were dependent on the combination and concentration of plant growth regulators that were used. The number of newly formed shoots varied with concentration of different plant growth regulators. The best shoot response and proliferation were obtained on full strength MS media supplemented with 0.5 mg/l BAP in combination with 0.1 mg/l of IBA. Whereas, the best rooting response was observed on half strength MS media supplemented with 1.0 mg/l IBA. Therefore, these concentrations are recommended for *in vitro* propagation of sufficient, true to type and disease free plants of *Prunus domestica L. cv. Stanley*.

Reference

1. Das B, Ahmed N, Singh P. 2011. Prunus diversity early and present development: Int. J. Biodivers. Conserv. 3 (14):721-734.
2. Topp B, Russell D, Neumüller M, Dalbo M, Liu W. 2012. Plum. In Badenes M, Byrne D (eds.), Fruit Breeding, handbook of plant breeding. Springer, New York, USA. 8 (3); 571–621.
3. Ramming D, Cociu V. 1991. Plums. In Moore J, Ballington J (Eds.), genetic resources of temperate fruit and nut crops. ISHS Acta Hort. 29: 235–287.
4. Okie W, Hancock J. 2008. Plums. In Hancock J (ed), temperate fruit crop breeding, germplasm to genomics. Kluwer academic publisher. Dordrecht, Holland. 337-358.
5. Ilgin M, Kafkas S, Ercisli S. 2009. Molecular characterization of plum cultivars by AFLP markers. Biotechnol. 23: 1189-1193.
6. Hartmann W, Neumuller M. 2009. Plum breeding. In Priyadarshan P, Jain S (ed), breeding Plantation Tree Crops, Temperate Species. Springer, New York. USA. 161-231.
7. Pirkhezri M, Mogadam M, Ebadi E, Hassani D.and Abdoosi V. 2014. Morph pomological study of some new Japanese plum (Prunus Salicina Lindl) cultivars grown in Iran. Int. J. Biosci., 5 (8):180-187.
8. Bofung CMF, Silou T, Mouragadja I. 2002. Chemical characterization of Safou (Dacryodes edulis) and evaluation of its potential as an ingredient in nutritious biscuits. For trees Livelihoods. 12: 105 - 118.
9. Folta KM, Gardiner S. 2009. Genetics and Genomics of Rosaceae, Plant Genetic and Genomics: Crops and Models 6.
10. Jabeen Q, Aslam N. 2011. The pharmacological activities of prunes: The dried plums. J. Medicinal Plants Res. 5(9):1508-1511
11. Prajapati P, Solanki A, Sen D. 2012. Nutrition value of plum tree for health. Int. Res. J. pharmacy. 3 (5): 54-56.
12. Nisar H, Ahmed M, Anjum M, Hussain S. 2015. Genetic diversity in fruit nutritional composition, anthocyanins, phenolics and antioxidant capacity of plum (prunus domestica) genotypes. Acta Sci. 14 (1): 45-61.
13. Gemu M, Handoro F. Temperate fruit and their disease in Ethiopia. Afr. crop sci. confer. Proce., 2006; 8: 919-924.
14. Melke A, Fetene M. 2014. Apples (Malus domestica, Borkh.), phenology in Ethiopian Highlands, plant Growth, blooming,

fruit development and fruit quality perspectives. Am. J. Exp. Agri. 4 (12): 1958-1995.
15. Druart P. 1992. In vitro culture and micropropagation of Plum (Prunus spp.). In Bajaj Y(ed), High-Tech and Micropropagation II, Biotechnology in Agriculture and Forestry. Springer, New York, USA. 18: 279-303.
16. Webster A. 1995. Temperate fruit tree rootstock propagation. New Zealand J. crop and Hort. Sci. 23 (4): 355-372.
17. Hjeltnes S, Nornes L. 2007. Germination of plum seeds. Acta Hort. 734: 83-87.
18. Esen D, Günes N, Yildiz O. 2009. Effects of citric acid presoaking and stratification on germination behavior of Prunus avium L. seeds. Pakistan Journal of Botany. 41(5): 2529-2535.
19. Ghayyad M, Kurbysa M, Napolsy G. 2010. Effect of endocarp removal, gibberelline, stratification and sulfuric acid on germination of Mahaleb (Prunus mahaleb L.) seeds. Am-Eur J. Agr. Environ Sci. 9(2):163-168.
20. Pipinis E, Milios E, Mavrokordopoulou O, Gkanatsiou C, Aslanidou M, Smiris P. 2012. Effect of pretreatments on seed germination of Prunus mahalab L. Not. Bot. Hort. Agrobo. 40 (2):183-189.
21. Botu I, Turcu E, Botu M. 2002. Advanced plum selections as rootstocks for stone fruits. Acta Hort. 658:441-448.
22. Beckman T, Lang G. 2003. Rootstock breeding for stone fruits. Acta Hort. 622:531-551.
23. Botu I, Botu M. 2007. Limits and perspectives in plum cultivar breeding using Conventional methods. Acta Hort. 734: 321–324.
24. Rosati P, Marino G, Swierczewski C. 1980. In vitro propagation of Japanese plum (Prunus salicina Lindl. cv. Calita). J. Am. Soci. Hort. Sci. 105 (1): 126-129.
25. Nedeicheva S, Ganeva D, Atanasov A. 1985. In vitro propagation of three clonal Prunus rootstocks. Rast Dui Nauki. 22 (8): 98-104
26. Mante S, Scorza R, Corts J. 1989. Plant regeneration from cotyledons of Prunus persica, Prunus domestica and Prunus cerasus. Plant Cell, Tiss. Organ Cult. 19:1–11.
27. Bassi G, Cossio F. 1991. In vitro shoot regeneration of Blufre and Susinadidro prune cultivars (Prunus domestica L.). Acta Hort. 28 (9): 82 -182.
28. Mante S, Morgens P, Scorza R, Cordts J, Callahan A. 1991. Agrobacterium-mediated transformation of plum (Prunus domestica L.) hypocotyls slices and regeneration of transgenic plants. J. Biotechnol. 9: 853–857
29. Nowak B, Miczynski K. 1997. Regeneration of plum (Prunus domestica L) cultivars Cacanska Rodna, Stanley, and Cacanska Najbolia from invitro leaf explants. Folia Hort. 9: 33–42.
30. Novak B, Miczynski K. 1996. Regeneration capacity of Prunus domestica L. cv. Wegierka Zwykla from leaf explants of in vitro shoots using TDZ. Folia Hort. 8: 41–49.
31. Silva A, Rogalski M, Guerra M. 2003. Effects of different cytokinins on in vitro multiplication of Prunus Capdeboscq rootstocks. Crop Breeding and Appl. Biotechnol. 3 (2): 149-156.
32. Tian L, Wen Y, Jayasankar S, Sibbald S. 2007. Regeneration of Prunus salicina Lindl (Japanese plum) from hypocotyls of mature seeds. In vitro cell. dev. Biol. 43: 343–347.
33. Ruzic, D, Vujovic T. 2007. A protocol for micropropagation of plum (prunus domestica L.). J. Pomol. 41: 79–85.
34. Zs J, Pamfil D, Clapa D, Fira A. 2008. In vitro regeneration and meristem culture of prunus domestica. Bulletin UASVM, Hort. 65 (1): 6
35. Zou Y. 2010. Micropropagation of Chinese Plum (Prunus salicina Lindl. cv. gulf ruby) using mature stem segments. Not. Bot. Hort. Agrobot. 38 (3): 214-218.
36. Ostadsharif O, Garoosi G, Haddad R, Nezami E. 2014. Effect of Medium, Sugar and Plant Growth Regulators on Micropropagation of Saint Julien A (Prunus domestica spp. Insititia) rootstock. Agr. Biotechnol. 13 (1): 9-18
37. Choudhary R, Chaudhury R, Malik S, Sharma K. 2015. An efficient regeneration and rapid micropropagation protocol for Almond using dormant axillary buds as explants. Indian J. Exp. Biol. 53:462-467.
38. Zaied NS. 1997. Studies on vegetative propagation of stone fruit trees. Ph.D. Thesis facult. Of Agrec., Moshtohor, Zagazig Univ.,Egypt.
39. Thorpe T, Stasolla C, Yeung E, Deklerk G, Roberts A, George E. 2008. Plant growth regulators II. Cytokinins, their Analogues and Antagonists. In: George E, Hall M, Deklerk G(eds.), Plant Propagation by Tissue Culture, Springer. 1: 115-173.
40. Mansseri-Lamrioui1 A, Louerguioui1 A, Bonaly J, Yakoub-Bougdal S, Allili N, Gana-Kebbouche S. 2011. Proliferation and rooting of wild cherry: The influence of cytokinin and auxin types and their concentration. Afr. J. Biotechnol. 10 (43):8613-8624.
41. Nazary R, Aghaye M. 2012. Micropropagation of GF 677 Rootstock. J. Agri. Sci. 4 (5): 131-138.
42. Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant. 15: 473-479.
43. Kane M. 2003. Bacterial and fungal indexing of tissue cultures.
44. Leifert C, Morrris EC, Waites MW. 1994. Ecology of microbial saprophytes and pathogens in tissue culture and field grown plants: reasons for contamination problems in vitro. Crit. Rev. Plant Sci. 13(2): 139-183.
45. Webster SK, Seymour JA, Mitchell SA, Ahmad MH. 2003. A novel surface sterilization method for reducing microbial contamination of field grown medicinal explants intended for in vitro culture. Biotechnology Centre, U.W.I, Mona, Kingston 7, Jamaica, West Indies.
46. Niedz RP, Bausher MG. 2002. Control of in vitro contamination of explants from greenhouse and field-grown trees. In Vitro Cell. Dev. Biol. 38:468-471.
47. Sathyanarayana BN, Varghese DB. 2007. Plant Tissue Culture: Practices and New Experimental Protocols. I. K. International. pp. 106
48. Constantine DR. 1986. Micropropagation in the commercial environment. In Withers L, Alderson, PG (Eds.), Plant tissue culture and its agricultural applications. Butterworth, London. pp. 175-186.
49. Buckley PM, Reed BM. 1994. Antibiotic susceptibility of plant associated bacteria. Hort. Sci. 29:434.
50. Moutia, Dookum A. 1999. Evaluation of surface sterilization and hot water treatments on bacterial contaminants in bud culture of sugarcane. Exp. Agric. 35:265 - 274.
51. Sundari UT, Sherif NA, Benjamin JHF, Rao MV. 2011. Rapid micropropagation via axillary bud proliferation of Coccinia grandis L. Voigt. from nodal segments. Plant Tiss. Cult. Biotech. 21(1): 75-82
52. Ranyaphi RA, Mao AA, Borthakur SK. 2012. In vitro plant regeneration of wintergreen (gaultheria fragrantissima wall.): Assessment of multiple nutrient formulations and cytokinin types. Indian J. Biotechnol. 11: 197-204
53. Arun KD, Swamy PS. 2015. An efficient multiple shoot induction protocol from nodal and root explants of atalantiamonophylla (l.) Dc., a medicinal plant. Int. J. Pharm Bio Sci. 6(3): 1238 – 1246
54. Emarah. 2008. Factors affecting propagation of strawberry (Fragaria spp.) through tissue cultures. J. Product. Dev. 13 (1), 191-212.
55. Ruzic D, Vujovic T. 2008. The effects of cytokinin types and their concentration on in vitro multiplication of sweet cherry cv. Lapins (Prunus avium L.). Hort. sci. 35: 12-21.

56. Pruski K, Astatkie T, Nowak J. 2005. Tissue culture propagation of Mongolian cherry (Prunus. Froticosa) and Nanking cherry (P.Tomentosa). Plant Cell, Tiss. Organ Culture. 82: 207-211.
57. Edriss M, Baghdadi G, Abdelrazek A, Abdrabboh G, Abdelaziz H. 2014. Micropropagation of Some Peach Rootstocks. Nat. Sci. 12 (3):106-114.
58. Vujovic TD, Ruzic, Cerovic R. 2012. In vitro shoot multiplication as influenced by repeated sub culturing of shoots of contemporary fruit rootstocks. Hort. Sci. 39: 101 - 107.
59. Hossain S, Munshi M, Islam M, Hakim L, Hossain M. 2003. In vitro propagation of Plum (Ziziphus jujubalam Lam.). Plant Tiss. Culture. 13 (1): 81–84.
60. Alam M, Barua R. 2015. In vitro regeneration and antibacterial activity of Prunus domestica L. J. Biosci. Biotechnol. 4 (1): 9-15.
61. Bandeira J, Thurow L, Braga E, Peters J, Bianchi V. 2012. Rooting and acclimatization of the Japanese plum tree, cv. America. Revista Brasileira de Fruticultura. 34 (2):597-603.
62. Housman J. 2003. Changes in peroxidase activity, auxin level and ethylene production during root formation by poplar shoots raised in vitro. Plant growth regulator. 13 (3):263-268.