***IN VITRO* PROPAGATION OF *PRUNUS AVIUM* L.**

**FROM CLONAL SEED ORCHARD**

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**Abstract**

A total of 24 genotypes (selected adult plus trees) were introduced from clonal seed orchards of wild cherry (*Prunus avium* L.) in the process of *in vitro* production. The possibility of optimizing routine micropropagation methods of the clones at all stages was explored. Several techniques were established that allow the introduction of the initial culture throughout the year. The specific structure of culture media and the unique combination of growth regulators were determined in all *in vitro* production stages, leading to the growth of high-quality plants with very good survival during the acclimatization process. BAP–1,0 mg/L, Kinetin-0,5 mg/L and IAA-0,5 mg/L were used for micropropagation, which resulted in the multiplication rate of 3-9 with plant height from 1,5 to 3,0 cm. Microplant rooting was achieved by a combination of IAA-2,0 mg/L, IBA-2,0 mg/L with the addition of GA3-0,2 mg/L. The rooted plantlets of wild cherry developed normal internodes and leaf blades, and the roots were well formed with 3 to 7 roots per plant, whose length increased the longer they remained in the culture medium. The micropropagated selected plus trees manifested strong apical dominance and the majority of the young plants reached a height of over one metre in the period of seven months.

***Key words****: Prunus avium L., in vitro* propagation*,* selected plus trees, clonal seed orchard.

**INTRODUCTION**

Improvement of wild cherry (*Prunus avium* L.) with conventional cultivation methods could be a very slow process impeded by the difficulty of finding regular and sufficient quantities of natural seed. Although the establishment of clonal seed orchards ensures a more regular yield of good quality seed, it does not eliminate the dependence on weather conditions which affect blossoming and seed yield, nor does it solve difficulties related to seed germination. Certain biotechnological *in vitro* methods accelerate the process and ensure genetic stability. Microclonal propagation of rejuvenated individuals, adult elite genotypes with well developed root systems, is the fastest and the best method of wild cherry improvement. Process optimization facilitates the problems and reduces production costs. Both additive and non-additive components of genetic variability are thus preserved. Good quality seedlings with known characteristics that are obtained over a short period could be used not only for the establishment and replenishment of clonal seed orchards, but also for direct afforestation of certain areas or for restocking forest stands with elite genotypes, as well as for further improvement.

A number of investigations into *in vitro* propagation of *Prunus avium* L. have been carried out by Druart et al. (1981), Pevalek-Kozlina and Jelaska (1987), Cornu (1990), Hamerschlag and Scorza (1991), Gruselle et al. (1995), Ružić et al. (2003), Szczygiel and Wojda (2008), Mahmout at al. (2009), primarily because of the high timber value of this species. In the nature, the existence of wild cherry types of up to 50 individuals is exceptionally rare and occurs only in several cases. The *in situ* conservation of such genotypes may be difficult if they are privately owned or if they are overmature, since the tree trunk progressively begins to decay after 80 years of age. The *ex situ* conservation in clonal seed orchards makes it possible to conserve either the unmodified genetic constitution or genetic constitution with minimal possibility of change through mutations, selections, drift or gene contamination of plant material. The introduction of new conservation methods of wild cherry genetic resources, such as *in vitro* vegetative propagation or cryopreservation, would increase the possibility of controlling genetic stability, especially if molecular characterization of each clone is carried out.

Meristem reproduction of selected elite trees of wild cherry was set up by Ivanička and Pretova (1986), Douglas (1999). In Croatia, primary research into microclonal propagation of wild cherry for the purpose of obtaining rapid *in vitro* micropropagation was conducted by Pevalek-Kozlina and Jelaska (1987). The experiment included several differently-aged genotypes.

Variability of elite wild cherry trees in combination with *in vitro* mass production of seedlings was studied by Vornam and Gebhardt (2000), Ďurkovič (2006), Hammatt and Grant (1997). An annual height increment of 1.8 – 2.0 m of these wild cherry seedlings was recorded. The *in vitro* culture of wild cherry with meristem or shoot explants is performed by organogenesis through three stages of the process. These include differentiation of the explants into the leaf rosettes, micropropagation and shoot elongation, as well as their rooting, similarly to other woody species, and finally their outplanting.

In Croatia, a clonal seed orchard of wild cherry with 27 selected clones was established in Kutina (Kajba et al. 2006, Kajba et al. 2012). The ramets of this orchard provided the starting, planting stock (ortets) for the research done in this work. To introduce the selected material into the *in vitro* procedure and mass vegetative propagation, twigs with formed buds in the dormant stage were used, as well as those in the growth stage.

Drawing on previous research into microprogatation of cultivated and wild cherry, the possibility of optimizing the routine of tissue culture methods for clone propagation (plus trees) of wild cherry at all the stages was attempted, starting from the initial culture, micropropagation, elongation, rooting and acclimatisation and *in vivo* hardening of the plants to the stage of commercial seedlings.

**MATERIALS AND METHODS**

***Disinfection and introduction into the initial culture***

The grown seedling shoots collected during different seasons of the year provided the initial material. Plant material consisted of axillary and terminal buds of a total of 24 clones of wild cherry from the clonal seed orchard in Kutina. Dormant buds or buds in the vegetation stage were taken as the initial material. The material was disinfected accordingly. The establishment of the initial *in vitro* culture for the needs of this research began at the end of July 2007 and lasted until the beginning of June 2008. Twigs that were used to isolate axillary buds were excised in different developmental stages of plant material during all the four seasons of the year. Disinfection included different periods of washing the material with Tween 20, Domestos and Izosan G chlorine solutions, 70 % alcohol supplemented with ascorbic acid and antibiotics (Table 1).

***Standard conditions for the preparation of culture mediums***

The culture medium for the establishment of the initial culture of axillary and terminal meristems of wild cherry consisted of half-strength medium according to Murashige and Skoog (1962), as presented in Tables 2 and 3. The universal culture medium for axillary bud multiplication of all the 24 clones of wild cherry consisted of our own combination of Murashige and Skoog (1962), Lloyd and McCown (1981) – WPM and OM (Rugini 1984), supplemented with Staba modified vitamins. Axillary shoot elongation before rooting was performed on the multiplication base supplemented with kinetin and GA3. Microplant rooting was done on a modified Murashige and Skoog (1962) culture medium, supplemented with auxin combinations.

**RESULTS AND DISCUSSION**

***Introduction into the initial culture and disinfection of plant material***

In several of the published papers on micropropagation of wild cherry (Hammat and Grant 1997, Harrington et al. 1994, Pevalek et al, 1994, Hammat 1999, Osterc et al. 2004, Ďurković 2006, Mansseri-Lamrioui et al. 2009), the initial explants were established by means of axillary or terminal buds, as well as layers. Initial explants are generally established in the initial culture during active growth of the mother plant in early spring. Fidanci et al. (2008) reports on the introduction of explants from the end of April to the beginning of June, since later collection of explants leads to a high degree of contamination, browning of clumps and reduced growth accompanied by a low multiplication rate. The introduction of woody plant material of wild cherry into the initial micropropagation culture may also present a problem due to overmature ortets or the infected initial material growing in natural forest habitats.

The need to produce wild cherry *in vitro* may also occur during other seasons, particularly if a certain genotype should be preserved. For this reason, it is of utmost importance to disinfect the initial material of wild cherry throughout the year.

The disinfection procedure was established for all the 24 clones of wild cherry in all the four seasons of the year. Research was initiated in July 2007. To isolate the explants, 30-cm long twigs were collected from the ramets with vegetative axillary buds in the dormant stage. The largest number of clones was established from the material taken in January 2008, when clones ĐU1, PŽ, R2, KC1, KC2, L4, N3 and KP3 were established.

According to the results of different research, the best time of collection and establishment is at the beginning of vegetation and intensive growth in the spring (Pevalek-Kozlina and Jelaska 1987), Sedlak et al. (2008), Scaltsoyiannes et al. (2009). Such material is healthy and is hardly ever contaminated with pathogens or external agents. The research resulted in a high survival rate of the initial explants (70 – 80 %), and all the clones were introduced into the initial culture.

*In vitro* research with woody plant material frequently reports on the use of mercury chloride for disinfection. To disinfect axillary buds from 10-year-old material, Sedlak et al. (2008) used 0.15 % solution of mercury chloride for one minute, while Mansseri-Lamrioui et al. (2009) used 2.5 gL for 20 minutes. In the works of Szygel and Wojda (2008), disinfection with calcium hypochlorite was unsuccessful, but disinfection with 0.1 mercury chloride was successful. Pevalek-Kozlina and Jelaska (1987) and Scaltsoyiannes et al. (2009) successfully disinfected wild cherry with HgCl (mercury chloride) applied over a shorter time interval and in lower concentrations. Apart from chloride solutions and mercury chloride, some newer disinfectants have been used more recently, such as DICA (dichloroisocyanuric acid sodium salt) in combination with 70 % alcohol (Osterch et al. 2004).

In this research, chloride-based solutions Domestos and Izosan G were used for reasons of their lesser toxicity during handling. Clone L3 was not successfully disinfected and was not introduced into the initial culture with this type of disinfectant.

***Multiplication***

Different concentrations of growth regulators were tested and the formation of new shoots, multiplication rates and duration were monitored, and so was the abundance of young plants. Five plantlets from every clone were set up in each concentration and were replicated twice in order to reliably determine the effect of growth regulator.

The use of cytokinin Kinetin to stimulate axillary branching in the multiplication phase had a positive effect on the formation of morphologically regular, firm, exceptionally luscious plants with nicely formed leaf blades, without additional callus formations and without the occurrence of vitrified plants. The plants were 1.5 to 3 cm tall and the internodes were adequately arranged. The multiplication rate was 1 at a concentration of 1 and 2 mgL, whereas it amounted to 2.5 at a concentration of 4 mgL. The morphologically formed plants achieved 100 % *in vitro* rooting later on. These kinetin concentrations did not interrupt apical dominance and did not stimulate the formation of axillary shoots regardless of the duration of multiplication. The plants in the culture medium experienced the most intensive growth in the first 14 days of subcultivation, but subsequently started losing their juvenile appearance.

The application of cytokinin BAP in the *in vitro* multiplication phase of wild cherry resulted in the formation of plant clumps with several formed axillary shoots, depending on the used concentration and on clonal origin. Concentrations of 0.5 mgL BAP formed morphologically regular clumps with 2-3 axillary shoots, 1.5 – 2.0 cm tall, with no callus in the base, without any vitrification and with regularly formed internodes and leaf blades. Concentration of 1.0 mgL BAP proved to be the most efficient. Properly shaped clumps with visible interruption of apical dominance and with new axillary shoots were formed. The internodes were of normal appearance, the leaf blades were of exemplary size, and the colour was natural green with very little formation of unidentified callus in the clump base. The clumps retained their juvenile appearance up to the 27th day of subcultivation, when they were transferred to fresh culture medium. The highest multiplication rate was recorded at a concentration of 1.5 mgL BAP but with a lower microplant quality. The clumps turned yellowish in colour, the leaves and stems became brittle and the internodes were short. The newly formed axillary shoots ranged between 0.7 and 1.2 cm in height after 28 days of subcultivation, and occurred in all the axillaes. A small callus growth was noticed at the base of the clump. The plants were fragile and broke during the excision process. A concentration of 2 mgL BAP led to decreased multiplication and increased callus formation at the clump base. The leaves were irregularly developed. Older leaves had partially increased and disproportionate leaf blades in relation to the clump. The axillary shoots in the leaf axillae were shorter and, although rare, the tips of the newly formed shoots were vitrified as a result of high BAP concentrations.

The vitrification process is irreversible and such shoots cannot be further multiplied. Since they cannot root, they represent a loss in the *in vitro* production process of any plant species. This was confirmed by Deberg and Maene (1981), who found a solution to the problem by decreasing the quantity of ammonic nitrate, decreasing the BAP concentration or increasing the agar concentration.

The use of BAP in the multiplication process of both wild and cultivated cherry is common. Concentrations range from 0.2 mgL to a maximum of 2.0 mgL (Garin et al. 1997, Hammat and Grant 1997, Grant and Hammat 1999, 2000, Sedlak et al. 2008, Fidanci et al. 2008, Scaltsoyiannes et al. 2009).

The following cytokinins have so far been used in wild cherry multiplication: BAP, kinetin, Tidiazuron. However, there are no data on the use of 2iP; for this reason, multiplication with two concentrations was attempted in this work in order to study its effects.

The use of cytokinin 2iP in concentrations of 5 and 10 mgL did not stimulate multiplication, and concentration of 5 mgL did not initiate the growth of the planted explants. The plants remained undeveloped and had small yellowish tips. At a concentration of 10 mgL 2iP the plants grew well and retained their green colour, but there was no multiplication or callus formation. Experiments with concentrations above 10 mgL are required since the use of 2iP in the multiplication of black chokeberry (*Aronia melanocarpa* L.) resulted in the development of tall plants (unpublished data), taller than 4 cm, which is very important in the rooting stage, and later on for survival in the acclimatisation stage.

Drawing on the results of favourable effects of kinetin on plant height growth and the role of BAP in interrupting apical dominance and forming numerous axillary shoots, BAP and kinetin were combined in the culture medium with the purpose of establishing a production model for *in vitro* mass propagation of wild cherry. BAP concentrations of 1.0 mgL were used, which yielded the best results when supplemented with 0.5 mgL of kinetin. A combination of different concentrations of growth regulators stressed their importance in the stage of wild cherry multiplication. In spite of identical composition of culture media in all the experiments and of clonal origin of the explants, accurately determined and very large differences occurred in the morphological appearance of the clumps, in the colour, firmness/juvenility of the material, and in the number of newly formed shoots. This selection eventually resulted in the clumps of natural appearance, with regularly formed internodes, a multiplication rate of 3-9 in dependence of clonal origin, and the duration of subcultivation of 22 - 27 days. Auxins IAA in concentrations of 0.5 mgL were present in the culture media for wild cherry multiplication in all the tests.

Although past literature mentions only one cytokinin for multiplication and only one auxin for rooting, in this research the quality of multiplied shoots was improved with a combination of cytokinin with auxin IAA through all the subcultures of multiplications, which had a favourable effect on plant rooting later on.

The success of microcuttings through *in vitro* cultures depends on a number of factors. The mineral and hormonal structure of the culture medium, as well as the age of initial explants is of high importance (Chikh 2000). Different mineral systems are used for micropropagation of the selected species, and formulations vary greatly depending on the cultivar, genotype or source of plant material (Rifaud and Cornu 1981, Tricoli et al. 1985, Druart 1992, Dolcet-Sanjuan et al. 2004).

There have been various studies on the effect of different compositions of culture mediums for *in vitro* production of root stock for grafting wild cherry and cultivated cherry. Culture mediums MS, ½ MS, QL (Quorin and Lepoivre 1977), ½ QL, Knop, Heller, commonly fortified with agar 7gL i pH 5,8 are cited in the works of Ivanička and Pretova (1986), Hammat (1999), Sedlak et al. (2008), Pevalek-Kozlina and Jelaska (1987), Fotopoulos and Sotiropoulos (2005), Scaltsoyiannes et al., (2009). Drawing on different experience, including our own, and on the results from literature, new culture mediums were formed for this research for all the stages of *in vitro* cultivation of wild cherry (Tančeva Crmarić 2011). They are presented in Tables 2 and 3.

The composition of all culture mediums satisfied the requirements of young plants at all stages. Regardless of their clonal origin, the plants were morphologically well developed and luscious, and vitrification occurred only as a result of high BAP concentrations. The selected culture mediums and the selected combination of growth regulators in the multiplication stage proved efficient for all the 24 clones of wild cherry. For mass production of only one clone or a selected clone group, slight modifications of cytokinin concentrations produce maximal multiplication. Namely, clumps of microplants manifested different behaviour patterns in dependence of certain geographic/regional clone origin. After 27 days of subcultivation on a shoot multiplication medium, there were differences in height, size of internodes and leaf blades, as well as in multiplication rates, as shown in Figure 1.

No correlation was established between the heights of formed clone microplants during multiplication with the heights of the same clones preserved *in situ*.

Protocols developed for micropropagation of wild cherry and other species of the genus *Prunus* (Yang and Schmidt 1994, Al-Sabbagh et al. 1999, Pruski et al. 2000, Ruzič et al. 2003, Ruzič and Vujovič 2008) are not broadly used; rather, the protocols are determined by the genotype. In this research, the selected culture medium and a combination of growth regulators resulted in acceptable multiplication of morphologically well formed plants, with no vitrification, for all the 24 genotypes of wild cherry under study.

Numerous investigations of *in vitro* mass production involved the use of antibiotics in the process for the purpose of eliminating bacterial contamination and preserving healthy planting stock. Sedlak et al. (2008) reports on the application of antibiotic Cefotaxime 200 mgL to overcome bacterial contamination during multiplication. In this research, 3 mgL concentration of a broad-spectrum antibiotic Rifampicin was successfully used in the multiplication phase and no bacterial contamination was recorded.

***Elongation of wild cherry in* in vitro *conditions***

Clumps obtained from wild cherry multiplication, especially clones which did not reach 1.5 cm in height, were subcultivated on the elongation medium, where an increase of shoots in the clump was observed. After 20 days, the plantlets were excised and transferred to the rooting medium. The elongation phase is exceptionally important because tiny rooted plants cannot acclimatise successfully, thus incurring severe losses. Zilkah et al. (1992) supplemented the medium with GA3 for elongation. In order to achieve elongation, Scaltsoyiannes et al. (2009) put a red cover over the pots with microplantlets during the multiplication stage.

In this research, subjecting the plantlets to dark periods proved less efficient because a very low percent of microplantlets started growing. At the same time, plant quality decreased as the plants lost a healthy green colour. Plants that were elongated in the dark abruptly increased their internodes and became thinner, while the leaf blades remained small and pale. It is very likely that dark periods in the elongation stage could result in the deterioration of microplantlet tips. This method of plant elongation yields potted microplants of exceptionally uneven appearance.

In order to overcome these problems, the clumps were transferred to a culture medium supplemented with Kinetin 0.5 mgL and GA3 0.2 MgL for 20 days, despite the fact that one more elongation subcultivation raised the cost of the process. This resulted in uniformly elongated clumps with elongated internodes. The leaves retained their green colour, the plants did not thin out and no tips were degenerated. These plantlets rooted more rapidly and achieved a very high survival percentage in the acclimatization stage.

***Rooting***

Culture medium MS ½ supplemented with 20 gL saccharose and a combination of growth regulators IAA – 2.0 mgL and IBA – 2.0 mgL supplemented with gibereline GA3 – 0.2 mgL were used to root wild cherry microplantlets.

*In vitro* rooting is estimated to account for up to 75 % of production costs in laboratories (Debergh and Maene 1981). For this reason, a number of authors used *in vivo* rooting or concentrated on obtaining tall plants of normal appearance, whose survival was high during the acclimatisation stage.

Fotopoulos and Sotiropoulos (2005) used MS or lowered concentrations of 1/2MS supplied with 1 or 2.5M IBA. The length of the *in vitro* formed adventitious roots corresponded to the applied concentration.

An increase in sugar concentrations in the culture medium led to more resistant plants capable of better adaptation to the transfer since an increase in plant size and total dry matter content was confirmed (Wainwright and Scarce 1989). However, there are opposite views which argue that sugar quantities should be lowered if CO2 sources and photon flux density are increased (Kozai and Iwanami 1988). In order to obtain plants with closed stomata, tests aimed at reducing humidity in apple microplantlets were carried out. The procedure involved the use of desiccants in the elongation and rooting stage, mechanical cooling of glass walls or opening the tops of glass containers (Brainerd and Fuchigami 1991).

There were also experiments involving the addition of growth retardants to reduce the leaf area. The most commonly used retardant for apple was paclobutrazol (Swietlik and Miller 1983). In their experiments, Wardle et al. (1983) added ABA to force plants to lose their leaves and form new leaves in the acclimatisation stage.

It is important to obtain good quality plants with sturdy leaves and with root systems capable of performing their role in the acclimatisation stage. In this research, the selected medium for *in vitro* rooting of wild cherry and a combination of growth regulators IAA and IBA in the *in vitro* condition proved successful.

After 14 days, wild cherry plantlets derived from the 24 studied clones were successfully rooted and continued growing and developing their roots. In the *in vitro* rooting stage they grew up to 5 cm in height.

The rooted plantlets of wild cherry developed normal internodes and leaf blades, and the roots were well formed. There were 3 to 7 roots per plant, whose length increased the longer they remained in the culture medium.

***Acclimatisation***

Plant acclimatisation is the key step to successful production of *in vitro* plants. Although extensive research has been dedicated to *in vitro* production, efforts in overcoming production difficulties in the laboratory will be futile if the problem of transferring the plants from *in vitro* to *in vivo* conditions is not solved. Plant acclimatisation is always the bottleneck of this technology (Hazarika 2003). In order to sustain successful plant survival in the acclimatisation substrate, adequate conditions should be ensured, including air and substrate moisture by means of fog and mist irrigation systems and substrate temperature by means of winter heating and summer cooling systems. Additional light should also be provided during short winter days, as well as shade during summer months when light intensity is very high. Moreover, plant nutrition and protection during the entire acclimatisation period is of outstanding importance. After 30 days, the plants are transferred from acclimatisation to container pots in the glasshouse, where they remain on the benches until they reach a height of 10 cm. The conditions in the greenhouse are partially controlled and include watering and nutrition, heating and cooling, as well as meticulous plant material protection.

The plant material is later stored in open sites where, protected with a net from weather conditions, it remains until delivery. The material can be delivered in the growth or dormant stage. The material is characterized by excellent health (regularly controlled by the Plant Protection Institute) and by well developed roots.

Research into *in vitro* cultures of noble forest tree species has since the very beginning been aimed at exploring the possibility of clonal propagation of mature, elite genotypes and the production of plants with their own roots. Rooting and acclimatisation stages in the species *Juglans nigra, Quercus rubra* and *Castanea dentata* are reported to be the limiting factors of micropropagation production (Pijut et al. 2007).

A certain number of wild cherry plants, one plant from each clone produced *in vitro* and acclimatized to *in vivo* conditions during the winter of 2009 – 2010 were pot planted in the spring and monitored. Intensive growth was recorded during spring, and most of the young plants reached a height of over 1 meter (Figure 2). Identically to the studied micropropagated seedlings of wild cherry (Vornam and Gebhardt 1999), they manifested strong apical dominance. After three years, the plants were 3.75 m tall, reaching 6.5 metres after ten years, with dbh of 9-10 cm at age 10.

The micropropagated plus individuals obtained in this research have characteristics that are comparable to both German micropropagated plants and French clones presented by Santi et al. (1998). Compared to the plants obtained from seed, the *in vitro* plants showed an improved root system with more than three roots per plant. Their quality can be corrected with *in vitro* treatments (Gebhards 1985, Meier – Dinkel 1986).

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**Table 1. Duration of plant material desinfection and concentrations**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Summer | Spring | Autumn | Winter |
| Running water supplemented  with Tween 20 | 10' | 10' | 15' | 30' |
| Sterile water supplemented  with vitamin C | 30 – 120' | 10 – 30' | 60' | 60 – 120' |
| Ethyl alcohol 70 % | 20'' | Quick deep | 30'' | 30'' |
| Izosan solution | 0.8 – 1.5% - | 0.7 – 0.9% - 8' | 1.2% – 15' | 1.0-2.0% - 15-20' |
| Washing with sterile water | 3 × 5´ | 2 × 3' | 3 × 5' | 3 × 10’ |
| Introduced clones of wild cherry | KP5, G1 | L5, N1, K3, L6, | K5, R1, L1, ĐU1 | G2, K3, K1, KP2, L4, NB1, ĐU1,  PŽ, R2, KC1, KC2, L4, N3, KP3 |

**Fig. 1.** Height of newly formed axillary shoots per clump and shoot multiplication according to clonal origin during *in vitro* multiplication stage of wild cherry

**Table 2. Structure of culture mediums for *in vitro* production of wild cherry per stage**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| ***Macroelements*** mg/L-1 | Initial culture | Axillary shoot multiplication | Shoot elongation | Rooting of microplantlets |
| KNO3 | 950 | 190 | 190 | 950 |
| Ca(NO3).4H2O | / | 600 | 600 | / |
| NH4NO3 | 825 | 400 | 400 | 825 |
| NH4H2PO4 | / | 100 | 100 | / |
| KH2PO4 | 85 | 170 | 170 | 85 |
| KCl | 100 | 100 | / | / |
| K2SO4 | 445 | 330 | / | / |
| CaCl22H2O | 220 | 96 | 96 | 220 |
| MgSO4 7H2O | 185 | 370 | 370 | 370 |
| ***Microelements*** |  |  |  |  |
| MnSO4 4H2O | 22.3 | 22.3 | 22.3 | 22.3 |
| ZnSO4 7H2O | 8.6 | 8.6 | 8.6 | 8.6 |
| H3BO3 | 6.2 | 6.2 | 6.2 | 6.2 |
| KI | 0.83 | 0.83 | 0.83 | 0.83 |
| CuSO4 5H2O | 0.025 | 0.025 | 0.025 | 0.025 |
| Na2MoO42H2O | 0.25 | 0.25 | 0.25 | 0.25 |
| CoCl . 6H2O | 0.025 | 0.025 | 0.025 | 0.025 |
| FeSO4.7H2O | 27.8 | 27.8 | 27.8 | 27.8 |
| Na2EDTA | 37.3 | 37.3 | 37.3 | 37.3 |
| ***Organic supplements*** |  |  |  |  |
| Adenin sulphate | / | 50 | / | / |
| L – tyrosine | / | 50 | / | / |
| Myo – inositol | / | 100 | / | 100 |
| Thiamine HCl | 0.5 | 1.0 | 2.0 | 2.0 |
| Pyridoxine HCl | 0.5 | 2.0 | / | / |
| Nicotinic acid | 0.5 | 2.0 | 1.0 | 1.0 |
| Biotine | 0.1 | 1 | / | 0.1 |
| Riboflavin | 0.1 | 0.5 | / | 0.1 |
| Folic acid | 0.2 | 0.2 | / | 0.2 |
| Ca Pantothenate | 2.0 | / | / | / |
| Glycine | 2 | 4 | 4 | 4 |

**Table 3. Growth regulators used in different stages of *in vitro* production of wild cherry**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Growth regulator | Initial | Multiplication | Elongation | Rooting |
| IAA | 1.0 | 1.0 | / | 2.0 |
| IBA | / | / | / | 2.0 |
| BAP | 0.4 | 0.5-2.0 | / | / |
| Kinetin | 0.2 | 1.0 – 4.0 | 0.5 | / |
| GA3 | / | / | 0.2 | 0.2 |
| Agar | 6 500 | 6 500 | 6 500 | 6 500 |
| Saccharose | 25 000 | 30 000 | 30 000 | 20 000 |

**Fig. 2**. Seven months old micropropagated plants of *Prunus avium* L.