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Development of embryo culture protocol for cherry laurel (*Prunus laurocerasus* L.)

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Abstract

Cherry laurel (*Prunus laurocerasus* L.) has attracted attention because of many different usage such as fresh fruit production and processing material for medicinal and pharmacy industry. This study was carried out to obtain protocols for embryo culture of cherry laurel (*Prunus laurocerasus* L.). Cherry and black types of cherry laurel embryos were cultured on MS media. This study was organized in two stages. In the first experiment the effects of the hormone combinations BAP (1.0, 2.0, 4.0 or 6.0 mg/l) and IBA (0.5 or 1.0 mg/l) were investigated. Shoot formation was not observed in cherry type embryos. Shoot and root formation occurred from black type embryos but did not have enough length or quality for transfer to the acclimatization stage. Seedlings had rudimentary roots which did not show any growth even after their transfer to fresh media. The best hormone combination was 2.0+0.5 mg/l BAP+IBA for production of plantlets of both types. In the second experiment, two cold stratification times (30 or 60 days) were applied to embryos. The interaction between cold stratification time and hormone combinations was found important. Embryo germination rates and seedlings growth both increased in embryos stratified for 60 days. Embryos stratified for 30 days required high hormone concentration to continue to the next growth. Cold stratification and hormone combinations affected success in the growing and acclimatization stages. Embryo culture of cherry laurel prevented loss of material during the germination and shortened the time to obtain seedlings in the same season that the seeds were collected.

Key words: *Prunus laurocerasus*, cherry laurel, embryo culture, BAP, IBA, cold treatment, acclimatization.

Introduction

Prunus laurocerasus L. (cherry laurel) is grown throughout the Black Sea region countries and is widely spread in the northern part of Turkey, so there are many cultivars which show different characteristics [25]. Fruit breeding is an expensive and lengthy process mainly due to the long juvenile period, the long generation time and the large size of fruit trees [9]. The traditional way to obtain seedlings in breeding programs of fruit tree species is based on stratification of seeds at low temperatures for several months during winter, and germination the following spring [12]. Low seed germination, percentage and long propagation cycle are the main constraints in the development of highly yielding cultivars through hybridization. *In vitro* methods could be used to overcome these problems. *In vitro* culture of embryos has been widely used in fruit breeding with different objectives, such as overcoming reproductive barriers in the production of inter-specific hybrids and virus-free plants or germination tests [23]. Successful embryo culture depends on the culture medium and conditions [4]. In addition, plant growth regulators enhance embryo growth [14-20]. Genotypes determine the effectiveness of the *in vitro* technique[21]. Germination of mature embryos has been successfully accomplished in walnut, cherries, myrobalan and apricot [2-27].

All the hindrances prevent the germination of mature seeds. Isolation of embryos eliminates some of these barriers located in the seed coat [1-5] whereas certain other blocks that are responsible for dormancy of the embryo, are removed as a result of a changed hormonal equilibrium [18]. Embryos isolated from non-stratified seeds remain in a state of embryonic dormancy and are characterized by lower germination percentage, developmental morphological anomalies such as hypertrophy of the main root, shortening of hypocotyls and asymmetrically growth and greening of cotyledons [6-28]. One of the most frequent abnormalities was the weak development of primary roots, which means that it was not possible to transfer the grown embryo into pots. In several *Prunus* species, different stratification temperatures and durations have been used; temperatures varying between 0 and 5°C and durations of 30-120 days [7-17].

P. laurocerasus L. is mainly propagated by the vegetative method using suckers and cuttings [24]. To date only two tissue culture studies have been reported for this species and no *in vitro* protocols have yet been developed for cherry laurel embryos[15-22]. Enhancement of *P. laurocerasus* seed germination is important in the propagation and breeding program as well as for testing and using germplasmas. Therefore, the aim of the study was to determine the combination of some growth regulators and cold storage duration times for *in vitro* embryo growth, germination and plant development in two cherry laurel types.

Material and Methods

The experiment was conducted in 2009-2011. Cherry laurel embryos (cherry and black type) were used as material in the study.

Explant preparation and embryo culture: Morphologically well developed embryos were isolated from completely ripened fruits. The seeds were removed from the fruit flesh and endocarps were washed with tap water. After the endocarp was cracked, embryos with testa were removed and surface sterilized in 20% sodium hypochlorite (with a few drops of Tween 20) for 25

min, followed by rinsing 3 times in sterile distilled water. The seeds were dissected with transversal cut to separate the seed coat. Half of the cotyledons were cut and the testa was removed carefully from the embryo under sterile conditions. Embryos with half of their cotyledons were taken and replaced to the culture media. This technique was adopted for easier execution.

In the first experiment, embryos were cultured in Petri dishes (50 mm x 10 mm) for 40 days and hormone effects were examined for embryo germination and growth. The Petri dishes were kept in a growth chamber at 25 ±1°C.

In the second part of the experiment, hormone and cold stratification effects on *in vitro* growth of *P. laurocerasus* embryos were investigated. Embryos were placed in Petri dishes (50 mm x 10 mm) and were kept in a refrigerator and stratified at 4°C in the dark for 30 and 60 days to overcome the rest period. Thereafter they were kept in a cool room for 24 h and then the chilled embryos were carefully transferred to the same (but fresh) medium in test tubes of 2 cm x 15 cm containing 10 ml of medium. The test tubes were kept for further development in a growth chamber at 25 ±1°C. Germinated embryos giving plants with rudimentary roots and shoots were carefully transferred into test tubes, again containing the same medium, and cultured for the next elongation of shoot and root for a month.

At the end of the experiments the following parameters were measured. Survival rate of embryos (%) were determined in all experiments. An embryo was considered as germinated if its radicules or shoots had extended into the medium 5 mm or more. Percentage germination (%) (percentage of root or leaf creating embryos), percentage of root and no leaf creating embryos (%), and percentage of leaf and no root creating embryos (%) were determined after three weeks of embryo culture in light. Shoot and root length (cm), and percentage of albino plantlets production (%) (germinated or non-germinated) were determined. In the acclimatization stage, transferred plantlets rate (%) and survival rates (%) of transferred plantlets were recorded.

Culture media for embryos: In all experiments, the nutrient medium of MS [19] containing 6-benzylaminopurine (BAP) (1.0, 2.0, 4.0 and 6.0 mg/l) and indole-3-butyric acid (IBA) (0.5, 1.0 mg/l) combinations was used. Gibberellic acid (GA_3) (0.2 mg/l) was added to all hormone combinations. Hormone free medium was used as a control. The media was supplemented with 30 g/l sucrose and 7 g/l agar and the pH was adjusted to 5.6 with 0.1 N NaOH and autoclaved at 2 atm. at 121°C for 20 min.

Acclimatization: *In vitro* developed plantlets were transferred into a small plastic glass containing a 2:1 mixture of peat moss and perlite and acclimatized in a culture room maintained at 25 ±1°C under 16/8 h (light/dark) photoperiod for 3 weeks by covering with small plastic bags; periodically the size of the holes in these bags was increased in order to gradually reduce the humidity. They were fertilized weekly with half-strength MS inorganic solution for 2 weeks, and after waiting a further 2 weeks were transferred to a greenhouse.

Experimental design and statistical analysis: All experiments were conducted in a completely randomized design in which three replicates were performed, each containing 15 embryos. Each experiment was repeated at least once. Data were subjected to ANOVA using Minitab Software (MINITAB Inc.). Data was transformed by the arcsine square for percentage means. The means were separated by Duncan's Multiple Range Test ($P<0.05$).

Results and Discussion

Effect of hormone combinations: The effects of hormones were different on survival rates, germination rates and growth characters in embryos of cherry laurel types. The survival rate (78.52%) and germination rate (11.11%) of cherry type embryos were lower than for black type embryos (respectively 100%, 30.37%) according to average values. The best germination rate was observed in 2.0+0.5 mg/l BAP+IBA combination (66.67%) with cherry type embryos and it was statistically significant as well. The best combination was 2.0+0.5 mg/l BAP+IBA for black cherry type embryos, for which these combinations increased the production of shoot and root as well. Shoot formation did not occur in cherry type embryos. Shoot and root formation increased in hormone-added media while no shoot or root formation was observed with embryos of either cherry laurel type grown in control media (Tables 1 and 2). Plant growth regulators enhanced embryo growth. Successful germination results were obtained with media including BAP and IBA for walnut embryos [20]. Beneficial effect of BA and GA_3 on germination of embryo and development of seedling was reported researchers too [16].

A different situation in growth and development of plantlets from embryos was observed during the hormone treatment

Table 1. Effect of hormones on cherry type embryo culture.

BAP+IBA (mg/l)	Survival (%)	Germination (%)*	Shoot formation (%)**	Root formation (%)***	Albino plants (%)
0+0	40.00 d	0.00 c	0.00	0.00 c	100
1.0+0.5	53.33 cd	0.00 c	0.00	0.00 c	80.00
1.0+1.0	86.67 ab	0.00 c	0.00	0.00 c	86.67
2.0+0.5	100 a	66.67 a	0.00	66.67 a	46.67
2.0+1.0	93.33 a	0.00 c	0.00	0.00 c	80.00
4.0+0.5	80.00 abc	0.00 c	0.00	0.00 c	73.33
4.0+1.0	100 a	33.33 b	0.00	33.33 b	66.67
6.0+0.5	93.33 a	0.00 c	0.00	0.00 c	60.00
6.0+1.0	60.00 bcd	0.00 c	0.00	0.00 c	100
Average	78.52	11.11	0.00	11.11	77.03

*Values in each column followed by different letters are significantly different ($P\leq0.05$).
* root or leaf creating embryos; ** root and no leaf creating embryos; *** leaf and no root creating embryos.

Table 2. Effect of hormones on black type embryo culture.

BAP+IBA (mg/l)	Survival (%)	Germination (%)*	Shoot formation (%)**	Root formation (%)***	Albino plants (%)
0+0	100	0.00 c	0.00 c	0.00c	100 a
1.0+0.5	100	60.00 a	0.00 c	60.00 a	86.67 b
1.0+1.0	100	46.67 a	20.00 b	46.67 a	60.00 cd
2.0+0.5	100	53.33 a	46.67 a	53.33 a	40.00 cd
2.0+1.0	100	53.33 a	46.67 a	46.67 a	46.67 cd
4.0+0.5	100	33.33 ab	20.00 b	33.33 ab	20.00 e
4.0+1.0	100	0.00 c	0.00 c	0.00 c	100 a
6.0+0.5	100	6.67 c	0.00 c	6.67 c	40.00 d
6.0+1.0	100	20.00 b	0.00 c	20.00 b	73.33 bc
Average	100	30.37	14.82	29.63	62.96

*Values in each column followed by different letters are significantly different ($P\leq0.05$).
* root or leaf creating embryos; ** root and no leaf creating embryos; *** leaf and no root creating embryos.

experiments. The embryos started to produce roots but the apical part did not develop (Fig. 1A). Shoot and root formation were observed on black type embryos but length and quality of plantlets were not sufficient for transfer to the acclimatization stage. These seedlings had rudimentary roots which did not show any growth even after transfer to fresh media (Fig. 1B). Some of the plantlets showed aberrant growth and morphological changes were observed in the roots. The hypocotyls and cotyledons enlarged drastically, and the normal root and shoot development was blocked (Fig. 1C). Albino plant production was very high and the hormone effect on albino production rate was statistically significant for black type embryos. All black type embryos were regenerated as albino in 4.0+1.0 mg/l BAP+IBA hormone combination and this is statistically significant. None of the cherry or black type embryos obtained chlorophyll in hormone-free media either.

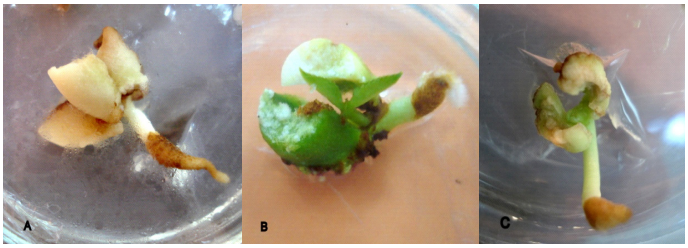

Figure 1. (A) Embryos produced roots but the apical part did not develop in cherry type embryos during the hormone treatment experiments. (B) Seedlings had rudimentary roots of black type. (C)The hypocotyls and cotyledons enlarged drastically and the normal root and shoot development was blocked after sub culturing.

Effect of cold treatments: Cold stratification played a significant role in the growing of embryos and all of the cold stratified embryos survived. Interaction between cold stratification and growth regulators was found statistically significant in all parameters observed.

Embryos of the cultured cherry type germinated better than black type embryos, and more shoot and root formation occurred in the stratification times of both (Figs 2 and 3). The highest germination rates (100%) were observed from embryos cultured in 4.0+0.5 and 2.0+0.5 mg/l BAP+IBA hormone combinations with 30 days stratification and 4.0+0.5 and 4.0+1.0 mg/l BAP+IBA combinations in 60 days stratification with cherry type embryos. The maximum shoot and root lengths (3.21 and 1.21 cm, respectively) were obtained in 2.0+1.0 mg/l BAP+IBA hormone combinations with 60 days stratified embryos and all plantlets obtained chlorophyll (Table 3).

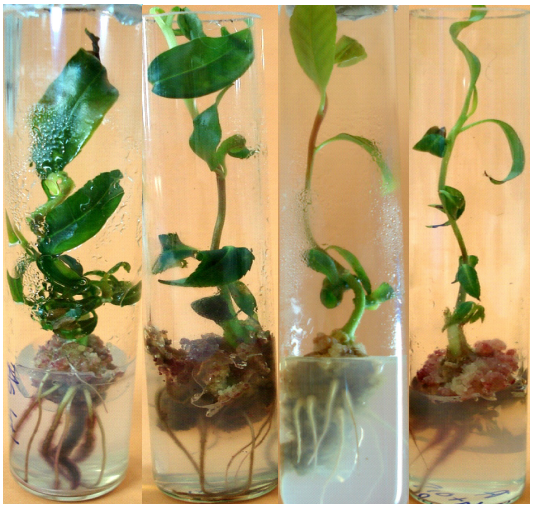

Figure 3. Seedling growing from 60 days stratified embryos on cherry type cherry laurel (2.0+0.5, 2.0+1.0, 4.0+0.5, 4.0+1.0 BAP+IBA mg/l).

The best germination was observed in 6.0+0.5 mg/l BAP+IBA combinations with 30 days stratified embryos (100%) and 2.0+1.0 and 4.0+1.0 mg/l BAP+IBA hormone combinations with 60 days stratified embryos (100%) of black type (Table 4). Albino plant production was not obtained in media including these combinations. The longest roots (3.47 cm) occurred in 4.0+1.0 mg/l BAP+IBA hormone combination with enough quality shoot length, but the whole plant formation (with shoot and root) rate was just 46.67%, so the best growing hormone combination was 2.0+1.0 mg/l BAP+IBA (3.82 cm shoot length and 1.90 cm root length) for 60 days stratified embryos of this type (Table 4).

Germination was highly influenced by the stratification time; 60 days stratification increased the germination rate of embryos. Initially, the embryos developed a rudimentary root, but after transfer to sub-culture exhibited an adventitious root system. The hypocotyls and cotyledons of the embryos turned green and the first part of the leaves became visible after a few weeks. Similar studies suggested that to attain successful germination rates of seeds a cold pre-treatment was necessary to overcome dormancy and induce shoot elongation [3-11]. The data presented here allows us to attribute to cold stratification a particular role in the control of events leading to embryo growth. Germination was not only stimulated as a result of cold treatment-hormone

Figure 2. Seedling growing from 30 days stratified embryos on cherry type cherry laurel (0+0, 1.0+0.5, 1.0+1.0, 2.0+0.5, 2.0+1.0, 4.0+0.5, 4.0+1.0, 6.0+0.5, 6.0+1.0 BAP+IBA mg/l).

Table 3. Cold stratification and hormones interaction effects on cherry type embryo culture.

BAP+IBA	Germination rate (%)*		Shoot formation (%)**		Root formation (%)***		Shoot length (cm)		Root length (cm)		Albino plants (%)	
(mg/l)	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days
0+0	40.00 bA	66.67 abA	6.67 cA	33.33 cA	40.00 bcA	66.67 abA	0.50 aA	0.55 cA	0.50 bA	0.50 cA	86.67 aA	53.33 aB
1.0+0.5	86.67 aA	80.00 abA	86.67 aA	40.00 cB	86.67 aA	80.00 abA	0.68 aA	0.75 bcA	0.61 abA	0.56 cA	0.00 cB	40.00 abA
1.0+1.0	93.33 aA	80.00 abA	6.67 cB	46.67 cA	93.33 aA	60.00 abB	0.50 aA	0.58 cA	0.63 abA	0.58 cA	0.00 cB	26.67 abA
2.0+0.5	100 aA	93.33 abA	46.67 bB	93.33 aA	100 aA	93.33 aA	0.83 aB	2.93 aA	0.99 aB	1.45 aA	0.00 cA	0.00 cA
2.0+1.0	26.67 bB	93.33 abA	20.00 bcB	100 aA	26.67 bcB	93.33 aA	0.50 aB	3.21 aA	0.75 abB	1.21 abA	73.33 aA	0.00 cB
4.0+0.5	100 aA	100 aA	86.67 aA	86.67 abA	93.33 aA	80.00 abA	1.13 aA	0.99 bcA	1.08 aA	0.81 bcA	0.00 cA	13.33 bcA
4.0+1.0	20.00 bB	100 aA	6.67 cB	93.33 aA	13.33 cB	66.67 abA	0.50 aB	1.35 bA	0.50 bB	1.35 aA	13.33 bcA	0.00 cA
6.0+0.5	53.33 bA	66.67 bA	40.00 bB	66.67 bcA	53.33 bA	53.33 bA	0.58 aA	0.82 bcA	0.95 abA	0.70 cA	6.67 bcA	0.00 cA
6.0+1.0	20.00 bB	60.00 bA	20.00 bcB	60.00 bcA	13.33 cA	40.00 bA	0.60 aA	0.87 bcA	0.50 bA	0.82 bcA	33.33 bA	20.00 bcA
Average	60.00	82.22	35.56	68.89	57.78	70.37	0.65	1.34	0.72	0.89	23.70	17.04

*Values in the same column (hormone combinations) with different lower-case letters and values in the same row (cold stratification) with different capital letters are significantly different (P≤0.05) for each observation. * root or leaf creating embryos ; ** root and no leaf creating embryos; *** leaf and no root creating embryos.

Table 4. Cold stratification and hormone interaction effects on black type embryo culture.

BAP+IBA	Germination rate (%)*		Shoot formation (%)**		Root formation (%)***		Shoot length (cm)		Root length (cm)		Albino formation (%)	
(mg/l)	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days	30 days	60 days
0+0	40.00 bA	46.67 cA	13.33 bA	46.67 bcA	40.00 bA	33.33 cA	0.50 aA	0.83 dA	0.50 a A	0.75 c	100 aA	53.33 aB
1.0+0.5	60.00 bA	60.00 bcA	40.00 bA	53.33 bcA	53.33 bA	46.67 bcA	0.50 aA	0.74 dA	0.50 aA	0.86 cA	100 aA	20.00 bB
1.0+1.0	40.00 bA	60.00 bcA	20.00 bA	33.33 cA	26.67 bA	46.67 bcA	0.63 aA	0.83 dA	1.00 aA	0.73 cA	86.67 aA	40.00 abB
2.0+0.5	40.00 bB	93.33 abA	13.33 bB	93.33 abA	33.33 bB	86.67 aA	0.50 aB	3.26 abA	0.50 aA	1.08 cA	20.00 cA	0.00 cB
2.0+1.0	53.33 bB	100 aA	13.33 bB	100 aA	53.33 bB	93.33 aA	0.50 aB	3.82 aA	0.50 aB	1.90 bA	0.00 dA	0.00 cA
4.0+0.5	60.00 bA	93.33 abA	33.33 bB	80.00 abcA	46.67 bA	80.00 abA	0.60 aB	1.74 cA	0.50 aB	1.31 cA	0.00 dA	6.67 cA
4.0+1.0	73.33 bB	100 aA	53.33abB	100 aA	60.00 bA	46.67 bcA	0.50 aB	2.63 bA	0.61 aB	3.47 aA	0.00 dA	0.00 cA
6.0+0.5	100 aA	86.67abcA	86.67 aA	66.67 abcA	93.33 aA	80.00 abA	1.19 aA	0.77 dA	0.78 aA	1.07 cA	0.00 dB	26.67 abA
6.0+1.0	33.33 bB	73.33 abcA	20.00 bA	53.33 bcA	40.00 bB	73.33 abA	0.50 aA	0.81 dA	0.50 aA	0.81 cA	60.00 bA	33.33 abA
Average	55.55	79.26	35.59	69.63	49.63	65.19	0.62	1.71	0.60	1.33	40.74	20.00

*Values in the same column (hormone combinations) with different lower-case letters and values in the same row (cold stratification) with different capital letters are significantly different (P≤0.05) for each observation. * root or leaf creating embryos ; ** root and no leaf creating embryos; *** leaf and no root creating embryos.

combinations but also several morphological characteristics of the seedling were improved. These results were supported by the other research, which also indicated that number and growth of seedlings were considerably increased by stratifying embryos [13-17]. One of the most frequent growth anomalies was the weak development of primary roots, which meant that it was not possible to transfer the embryo-grown plants into pots. Cold stratification decreased this anomaly, too. The cold treatment also resulted in decreased percentages of seedlings with asymmetrically growing cotyledons, as well as in decreased numbers of cotyledons showing asymmetrical greening.

Acclimatization stage: The growing hormone combination affected the transplanted seedlings amount and survival rate in the acclimatization stage, but statistical analysis could not be made because of insufficient and unequal material number. In the acclimatization stage, the shoot quality directly affected the survival rate of transplanted embryos. Seedlings developed from 60 days cold stratified embryos were stronger than seedlings developed from 30 days cold stratified embryos for both of the types (Tables 5 and 6). The transplanted and survival plantlets rate was highest in seedlings produced by 60 days stratified embryos (Fig. 4) in 2.0 or 4.0 mg/BAP including culture media for both types in the acclimatization stage, and these concentrations also produced the best quality of shoot and root in the embryo culture stage. The maximum transferable plantlets rate was obtained in 2.0+0.5 mg 7 l BAP+IBA hormone combinations (80.00%) for the cherry laurel type, 75.00% of the plantlets survived after transfer to *in vivo* conditions (Table 5). All of the transplanted plantlets of the black cherry type produced in 2.0+1.0 mg/l BAP+IBA hormone combination survived in the acclimatization stage (Table 6).

The growth of leafy shoots under *in vitro* conditions was first characterized by reduced shoot elongation and by increased size

Table 5. Survival rates in acclimatization stages of cherry type plantlets.

(BAP+IBA)	30 days stratification		60 days stratification	
(mg/l)	Transplanted seedling (%)	Survival seedling (%)	Transplanted seedling (%)	Survival seedling (%)
0+0	0.00	-	0.00	-
1.0+0.5	0.00	-	0.00	-
1.0+1.0	0.00	-	6.67	0.00
2.0+0.5	15.38	50.00	80.00	75.00
2.0+1.0	0.00	-	66.67	60.00
4.0+0.5	33.33	40.00	26.67	25.00
4.0+1.0	0.00	-	33.33	60.00
6.0+0.5	6.67	0.00	13.33	50.00
6.0+1.0	0.00	-	6.67	0.00
Average	18.46	30.00	25.93	38.57

Table 6. Survival rates in acclimatization stages of black type plantlets.

(BAP+IBA)	30 days stratification		60 days stratification	
(mg/l)	Transplanted seedling (%)	Survival seedling (%)	Transplanted seedling (%)	Survival seedling (%)
0+0	0.00	-	13.33	50.00
1.0+0.5	0.00	-	0.00	-
1.0+1.0	0.00	-	13.33	50.00
2.0+0.5	0.00	-	46.67	71.43
2.0+1.0	0.00	-	73.33	100
4.0+0.5	6.67	-	46.67	57.14
4.0+1.0	0.00	-	26.67	63.44
6.0+0.5	40.00	50.00	6.67	0.00
6.0+1.0	0.00	-	6.67	0.00
Average	5.19	50.00	25.93	49.03

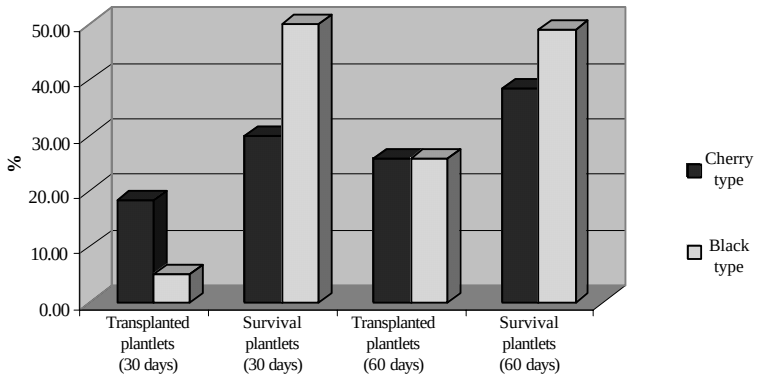

Figure 4. Transplanted and survival plantlets of cherry types in acclimatization stages (average values).

of leaves, which thus covered the shoot tips of seedlings. However, growth of the axis started later, when the rooted plantlets were transferred for acclimatization (Fig. 5). Cultivation in sterile compost in pots increased the growth of plantlets similarly [13]. Regenerated plants of cherry laurel established in soil were stable and there was no apparent phenotypic difference between them and *in vivo* grown seedlings.

The best plant production was supplied with cold stratified cherry laurel embryos. Embryos of cherry and black types gave different results and growth characteristics showed difference. The effect of genotype on embryo germination and the growing characteristics of roots and shoots was also reported in different plant species by other researchers [7, 20-26].

Figure 5. Acclimatization stage of seedlings.

Conclusions

Mature embryos of cherry laurel were successfully stimulated to develop *in vitro* to seedlings using different BAP+IBA combinations with cold stratification. Cold treatment caused an increase in the main length of hypocotyls, shoots and in the length of main roots of all types. Hormone requirement decreased in culture media with the time of stratification. The type of cherry laurel is the other important factor for successful embryo culture as well. All of these factors have to be taken into consideration for future studies. Embryo culture of cherry laurel prevented loss of material during the germination and shortened the time to obtain seedlings in the same season that the seeds were collected. The reported information would also be useful in future breeding experiments with variety.

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