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Effects of donor plant age and explant types on Asparagus (*Asparagus officinalis* L.) micropropagation

Hatice Büşra YAVUZ[1] **Nuray ÇÖMLEKÇİOĞLU**[2*]
[1]Eskişehir Osmangazi University, Institute of Science, Eskişehir, Türkiye
[2]Eskişehir Osmangazi University, Faculty of Agriculture, Department of Horticulture, Eskişehir,Türkiye
* **Correspondence:** ncomlekcioglu@ogu.edu.tr

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Abstract
Asparagus (*Asparagus officinalis* L.) is a dioecious species in the Asparagaceae family. Male plants are more productive than female plants. More efficient plant propagation can be followed by focusing on the production of male plants with tissue culture techniques. The aim of this study is to determine the micro-propagation effects of shoot tip and lateral bud explants taken from donor plants of different ages (3 and 5 years). Jersey Knight F1 was used in the experiment. To obtain shoots from explants, 0.2 mg l^{-1} NAA (Naphtalen acetic acid), 0.2 mg l^{-1} BAP (benzyl amino purine), 0.2 mg l^{-1} kinetin, 30 g l^{-1} sucrose and 7 g l^{-1} agar containing MS (Murashige & Skoog, 1962) medium was used. The shoots were transferred to proliferation MS medium containing 0.5 mg l^{-1} BAP and 0.2 mg l^{-1} IBA (indole butyric acid), and two subcultures were made. MS medium containing 0.5 mg l^{-1} IBA was used for rooting of the shoots. Shoot development was obtained from all cultured explants. In the first subculture, the average shoot numbers per explant obtained from 3 and 5 years old plants were determined as 7.0 and 7.18, respectively. While an average 9.43 shoots were obtained from shoot tip explant, 4.75 shoots from the lateral bud explant. In the second subculture, an average of 9.13 shoots from 3-year-old plants and 9.55 shoots from 5-year-old plants were obtained. While 10.53 shoots were obtained from the shoot tip, 8.15 shoots were detected from the lateral bud. The proliferation coefficient of the shoot tip explant in the first subculture differed significantly compared to the lateral bud explant. In contrast, the difference in proliferation coefficients decreased in the second subculture. 48.0% and 58.0% rooting rate were obtained from the explants of the 3 and 5 years old plants respectively. The average rooting were 65.0% in the shoot tip explant and 41.0% in the lateral bud explant.

Key words
Asparagus officinalis, micropropagation, donor age, shoot tip, axillary bud

Introduction
Asparagus (*Asparagus officinalis* L.) is an herbaceous and perennial monocot plant species. It's economic and nutritional value is quite high. It is a product that is harvested in the early season especially in the spring (Rasad et al., 2019). It can be grown worldwide and there are approximately 300 species (Kubota et al., 2012). While nearly 100 species grow in Anatolia and Europe, it is known that 12 species naturally grow in Turkey. These wild species are *Asparagus acutifolius* and *Asparagus verticillatus* (Altunel, 2021).
Asparagus (2n=20) is a dioecious plant. Male plants are more productive than female plants. Many breeding programs around the world focus on the production of such male hybrids (Desjardins, 1992). When a 100% male hybrid with superior characteristics is obtained, tissue culture propagation of the parents is required for large-scale seed production. Propagation of asparagus from seed is not efficient due to the low germination rate of this plant. Propagation by dividing the rhizome of the plant is a time-consuming process. 2-4 new plants are obtained from a plant under optimum conditions and within a year (Sarabi & Almasi, 2010). At the same time, production depends on the season. Tissue culture techniques allow vegetative propagation of male plants. Since microclonal reproduction is not dependent on the season, production can be made throughout the year. It allows the reproduction of healthy, high-yielding and high-quality male plants preferred in production with the selection of donor plants.
Although micropropagation has been practiced for a long time in asparagus, low propagation coefficient, weak roots or lack of root formation are important problems. The success of tissue culture is influenced by many factors that originate from the donor plant from which the explant is taken or are related to the conditions during the application of the culture technique. To improve asparagus micropropagation, genotype (Conner et al., 1992; Fortes et al., 1997), explant type (Inagaki et al., 1981; Harada & Yakuwa 1983; Maung et al. 2019), culture system, type and concentration of basal medium, carbohydrate sources (Harada and Yakuwa 1983; Levi and Sink 1991; Bojnauth et al. 2003; Pontaroli and Camadro 2005), growth regulators (Azad and Amin 2017), and growth retardants (Khunachak et al. 1987) should be considered.
The aim of this study is to determine the micropropagation effects of shoot tip and lateral bud explants taken from donor plants of different ages (3 and 5 years) in asparagus.

Material and Methods
This research was carried out in the tissue culture laboratory of Eskişehir Osmangazi University, Faculty of Agriculture, Department of Horticulture (Eskişehir, Turkey). 3 and 5 years old plants of Jersey Knight F1 asparagus variety obtained from Nomad Tarım A.Ş. were used as donors. Shoot tip and lateral (axillary) buds were used as explants. All tissue culture applications were made under aseptic conditions and a laminar air flow sterile cabinet was used.

Disinfection of explants
For disinfection of plant material, the shoots were cut into 3-4 cm lengths containing the apical meristem and lateral buds (Figure 1). After shaking for 20-30 seconds in 70% ethyl alcohol in separate groups, they were kept in 10% commercial bleach solution containing sodium hypochlorite (5%) for 15 minutes. Then rinsed 3-4 times with sterile distilled water.

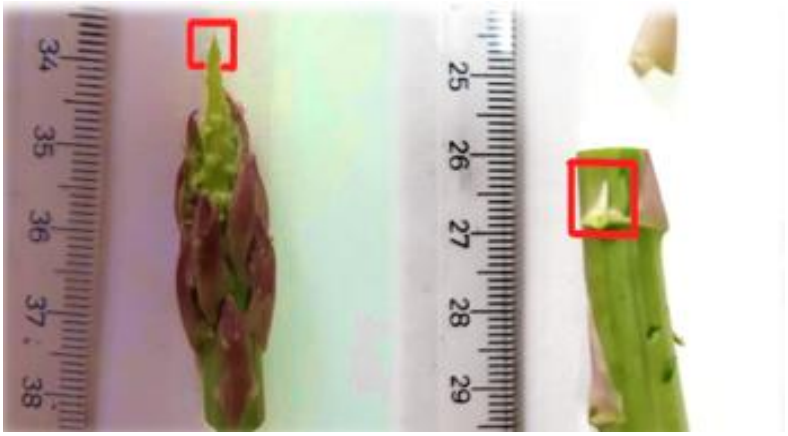


Figure 1. Cutting shoot tip and lateral bud explants

Nutrient Media

MS (Murashige & Skoog 1962) nutrient medium containing 30 g l^{-1} sucrose and 7 g l^{-1} agar was used as the basic medium. The pH of the nutrient media was adjusted to 5.9. To obtain shoots from explants, 0.2 mg l^{-1} NAA, 0.2 mg l^{-1} BAP and 0.2 mg l^{-1} kinetin were added. In order to proliferate the developed shoots, they were transferred to the multiplication (propagation) medium containing 0.5 mg l^{-1} BAP and 0.2 mg l^{-1} IBA. The shoots obtained after the second subculture were transferred to a rooting medium containing 0.5 mg l^{-1} IBA (Table 1).

Sterilization of the nutrient media was done by autoclaving at 121 °C for 15 minutes. Shoot tips and lateral buds from sterilized shoots were cut aseptically in a laminar air flow sterile cabinet and used as explants. The explants, which were cut with the help of scalpel and forceps, were placed on the nutrient medium in contact with the medium and without being immersed in the medium. 100 ml glass jars were used for planting. 5 explants were planted in each jar.

Table 1. Nutrient media and contents

	Nutrient media and contents
Shoot Induction Medium	MS + 0.2 mg l^{-1} NAA + 0.2 mg l^{-1} BAP + 0.2 mg l^{-1} kinetin + 30 g l^{-1} sucrose + 7 g l^{-1} agar
Shoot Multiplication Medium	MS + 0.5 mg l^{-1} BAP + 0.2 mg l^{-1} IBA + 30 g l^{-1} sucrose + 7 g l^{-1} agar
Root Induction Medium	MS + 0.5 mg l^{-1} IBA + 30 g l^{-1} sucrose + 7 g l^{-1} agar

Incubation

The glass jars, whose explant planting was completed, were taken to the climate room adjusted to 25 ºC temperature and 16/8 hour photoperiodic arrangement.

Experiment Design and Statistical Analyses

The study was carried out in four replications, with 25 explants in each replication (5 jars and 5 explants in each jar), using a total of 100 explants for each application. The results were analyzed by analysis of variance (ANOVA) using the Tarist Statistics Program (Açıkgöz et al., 2004). Biplot (principal component method) was made with Minitab 17 statistical program (Anderson, 1984). Means were compared with Least Significant Different (LSD).

Result and Discussion

In the experiment, shoot tip and lateral bud explant types were used from 3 and 5 years old donor asparagus plants. Shoot induction rate, the number of shoots per explant in the first and second subcultures (proliferation coefficient), and rooting rates were determined.

Shoot Induction

Shoot development started within 2 - 3 weeks after the shoot tip and lateral bud explants were planted (Figure 2). All explants taken in all of the studied donor age and explant types formed 100% shoots.

Figure 2. Shoots obtained in the shoot induction medium

In the experiment, the shoot formation of all explants is in agreement with the study of Paudel et al. (2018) that the combination of auxin and kinetin used in MS medium had a positive effect on shoot growth and development *in vitro* propagation of *Asparagus racemosus* Wild. Likewise, Sallam (2019) stated that 100% shoot was obtained from shoot tip and single node (axillary) explants.

Shoot Multiplication

The shoots that developed to 5-6 cm long in the shoot induction medium were transferred to the multiplication medium. Two subcultures were made in the study. Shoot multiplication coefficient data were obtained after 5 weeks of culture (Figure 3). In Table 1, the variance analysis table of the shoot numbers obtained per explant in the first subculture is presented. In the first subculture, it was determined that the donor plant age was not important in the number of shoots obtained, but showed significant differences ($P \leq 0.01$) according to the explant type. The interaction of donor plant age and explant type was found to be not significant.

Table 1. Table of variance analysis of donor plant age (A) and explant types (B) on average shoot numbers per explant in the first subculture

Variation source	Degrees of freedom	Sum of Squares	Mean Square	F Value	Table Value %5	Table Value %1
Repeat	3	2.273	0.758	1.949ns	3.860	6.990
Donor Plant Age (A)	1	0.123	0.123	0.315ns	5.120	10.560
Explant Type (B)	1	87.422	87.422	224.961**	5.120	10.560
A*B	1	0.063	0.063	0.161ns	5.120	10.560
Error	9	3.498	0.389			
Total	15	93.378	6.225			

ns = not significant, * = significant at alfa level %5, ** significant at alfa level %1

In the first subculture, the average number of shoots obtained from the explants of 3 and 5 years old donor plants were determined as 7.0 and 7.18 shoots, respectively. The difference between the means was found to be not significant. An average of 9.43 shoots were obtained from the shoot tip explant, while an average of 4.75 shoots were obtained from the lateral bud explant. The explant type was found to be significant ($P \leq 0.01$) in terms of the number of shoots obtained.

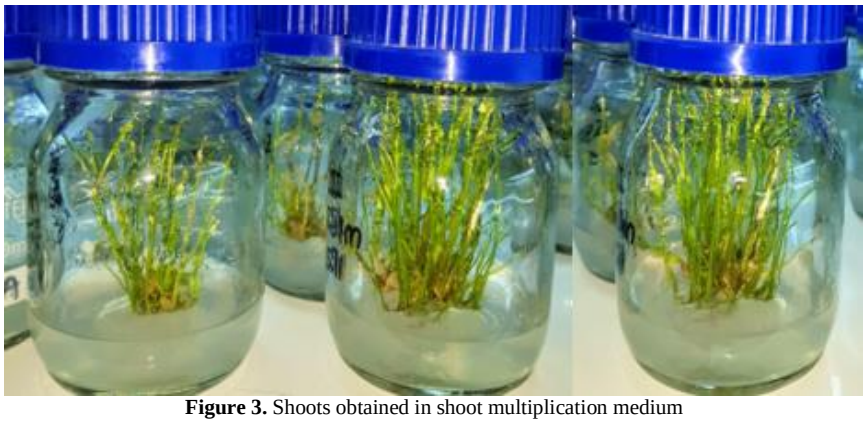


Figure 3. Shoots obtained in shoot multiplication medium

In the second subculture, effects of donor plant age ($P \leq 0.05$) and explant type ($P \leq 0.01$) were significant on the number of shoots obtained per explant. It was determined that the interaction of plant age and explant type was insignificant in the second subculture (Table 2).

Table 2. Table of variance analysis of donor plant age (A) and explant types (B) on average shoot numbers per explant in the second subculture

Variation source	Degrees of freedom	Sum of Squares	Mean Square	F Value	Table Value %5	Table Value %1
Repeat	3	0.613	0.204	2.882ns	3.860	6.990
Donor Plant Age (A)	1	0.723	0.723	10.200*	5.120	10.560
Explant Type (B)	1	22.563	22.563	318.529**	5.120	10.560
A*B	1	0.062	0.062	0.882ns	5.120	10.560
Error	9	0.637	0.071			
Total	15	24.597	1.640			

ns = not significant, * = significant at alfa level %5, ** significant at alfa level %1

In the second subculture, an average of 9.13 shoots were obtained from 3-year-old donor plants, while an average of 9.55 shoots were obtained from 5-year-old donor plants. Compared to the first subculture, 3-year-old donor plants produced more shoots than 5-year-old donors. When the effect of explant type in the second subculture is examined; 10.53 shoots were obtained from the shoot tip explant, while 8.15 shoots were detected from the lateral bud explant (Table 3). In the second subculture the shoot numbers per explant were increased for both explant types compared to the first subculture (Figure 4).

Generally, it was determined that the shoot tip explant was more successful than the lateral bud explant. According to the results obtained, the shoot tip explant gave the highest shoot regeneration rate at the same duration and medium. According to the first subculture data, the shoot tip explant showed a significant difference when compared to the lateral bud explant. On the other hand, as a result of the second subculture, the difference between the proliferation coefficients decreased according to explant types.

Table 3. Average number of shoots per explant according to donor plant age and explant type in the second subculture

Donor plant age	Number of shoot	Explant type	Number of shoot
3-year-old	9.13 **b**	Shoot tip	10.53 **a**
5-year-old	9.55 **a**	Lateral bud	8.15 **b**
LSD (%5)	0.301	**LSD(%1)**	0.432

The column having a different letter(s) are statistically significant

It has been reported by many researchers that the explant type and the optimum hormone concentrations are important in the number of shoots obtained. Rasad et al. (2019) obtained shoots from shoot piece and root explants in different culture media within 2 weeks. They reported that the highest shoot regeneration rate was obtained in the shoot piece explant (6.2 shoot explant^{-1}). Maung et al. (2019) found that apical bud (8.9 shoot explant^{-1}) and lateral bud

explants have micropropagation potential compared to shoot piece explant in micro-propagation. According to the studies, it was determined that the type of explant affects the regeneration capacity.

Figure 4. Shoots proliferation in the first subculture (A) and second subculture (B) medium

In this study, it was determined that the explant type effects on shoot regeneration was more significant than the effects of donor plant age. Shoot tip explants produced more shoots per explant than lateral bud explants. This may be due to different cell division rates at different explant. The shoot tips used as explants are located in the apical meristem and sub-apical meristem region. It has been reported that most of the cell division in asparagus occurs in the apical meristem of the shoot tip (Culpupper & Moon, 1939).

Root Induction

The shoots obtained in the shoot multiplication medium were separated as cluster shoots (containing 4-7 shoots) after 5 weeks. The shoot clusters were transferred to root induction medium containing 0.5 mg l^{-1} IBA for root initiation. Due to the low rooting rate after 8 weeks, these cluster of shoot were retransferred to MS medium containing 1.0 mg l^{-1} IBA (Figure 5). Rooting data were determined after 4 weeks (12 weeks in total)

The variance analysis table of rooting rates is presented in Table 4. It was determined that donor plant age ($P \leq 0.05$) and explant type ($P \leq 0.01$) had significant effects on rooting rates of shoots.

Table 4. Analysis of variance of rooting rates of donor plant age (A) and explant types (B)

Variation source	Degrees of freedom	Sum of Squares	Mean Square	F Value	Table Value %5	Table Value %1
Repeat	3	18.500	6.167	1.762**ns**	3.860	6.990
Donor Plant Age (A)	1	25.000	25.000	7.143*****	5.120	10.560
Explant Type (B)	1	144.000	144.000	41.143******	5.120	10.560
A*B	1	4.000	4.000	1.143**ns**	5.120	10.560
Error	9	31.500	3.500			
Total	15	223.000	14.867			

ns = not significant, * = significant at alfa level %5, ** significant at alfa level %1

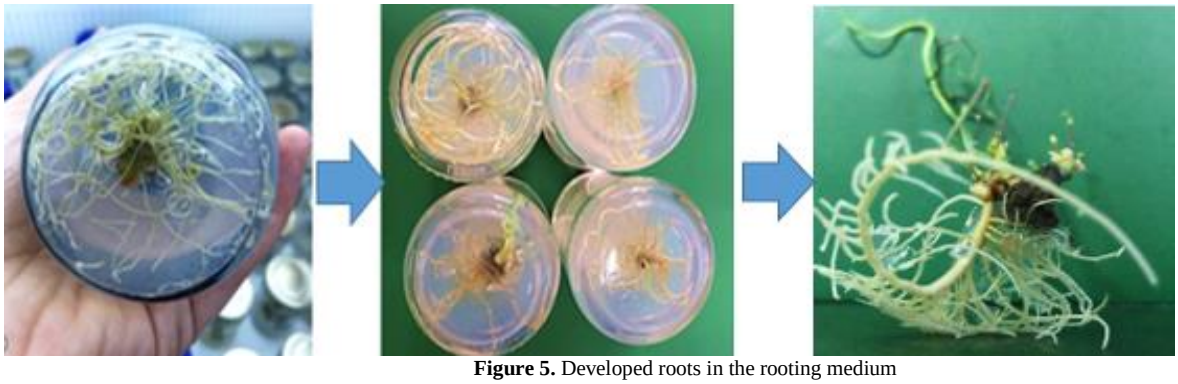


Figure 5. Developed roots in the rooting medium

While a rooting rate 48.0% was obtained in 3-year-old donor plant explants, a rooting rate of 58.0% was obtained from 5-year-old donor plant explants. It was determined that the explant type had a more significant effect on the rooting rate compared to the age of the donor plant. As an average value, 65.0% rooting was determined in the shoot tip explant, while 41.0% rooting was determined from the lateral bud explant. The shoot tip explant formed more roots than the lateral bud explant (Table 5).

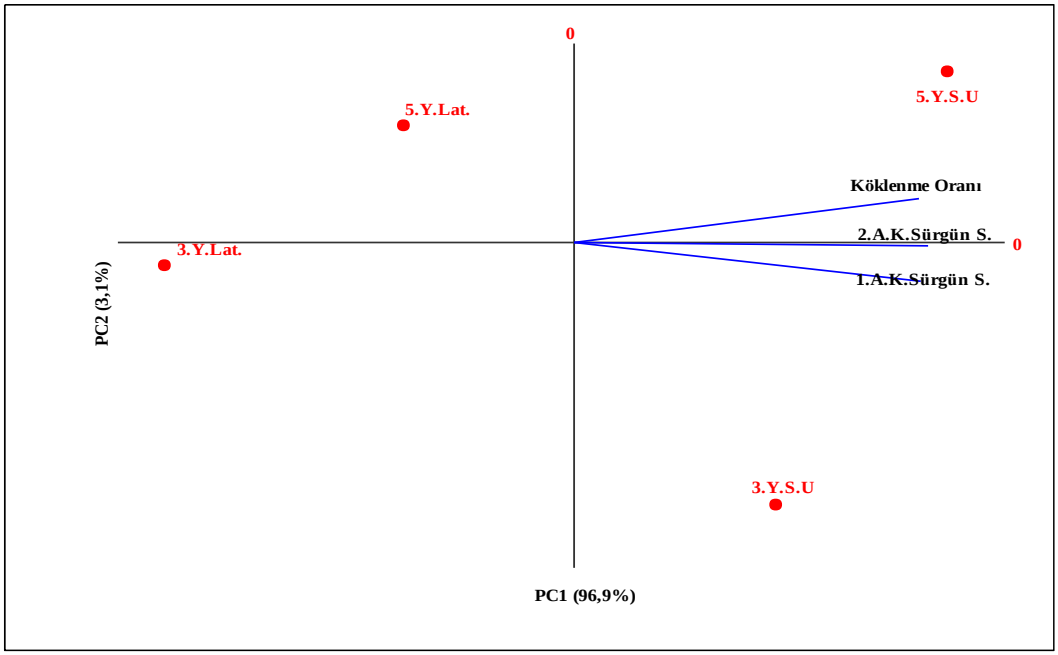
Table 5. Rooting rates (%) by donor plant age and explant type

Donor plant age	Rooting rate	Explant type	Rooting rate
3 year-old	48.0**b**	Shoot tip	65.0**a**
5 year-old	58.0**a**	Lateral bud	41.0**b**
LSD(%5)	2.117	**LSD(%1)**	3.040

The column having a different letter(s) are statistically significant

The rooting rates presented in Table 5, are belong to cluster shoots. The rooting rate obtained was found to be compatible with the study of Maung et al. (2019) in which cluster shoots achieved higher root rate than single shoots. Hormone type, concentration and rooting time effect are also very important in *in vitro* rooting of asparagus. In previous studies, the effects of different auxin hormones have resulted in different results. The reason for this difference may be the effect of many factors, but the most important factor may be the response of the genotype. It has been stated that the most suitable auxin hormone for *in vitro* rooting of asparagus is IBA (Azad & Amin 2017; Maung et al., 2019; Sallam, 2019). Certain metabolic products can accumulate in the nutrient medium during shoot development in *in vitro* cultures. At the same time, some components in the medium may be consumed. Even if the plants are capable of rooting, one or both of the conditions may inhibit root formation. It has been stated that reculturing the plantlets in the culture medium, can remove root inhibitors or it is possible to use a new root promoter (Slabbert et al., 1990).

Figure 5 shows the biplot plot of shoot numbers and rooting rate obtained in the first and second subcultures of the donor plant age and explant type. The principal components biplot analyzes of the examined traits and the variance of donor plant age and explant type were explained at a rate of 100%. The shoot numbers obtained per explant in the first and second subcultures showed higher performance in the shoot tip explant. Likewise, it was determined that the rooting rate showed high performance and high stability. Shoot tip explants were more successful than the lateral explant type in both subcultures.



3.Y.Lat= 3 years lateral, 3.Y.S.U=3 years shoot tips, 5.Y.Lat= 5 years lateral, 5.Y.S.U=5 years shoot tips

Figure 5. Biplot graph of the relationship between shoot numbers and rooting rates per explant in first and second subcultures.

In general, the advantages of micropropagation in artificial nutrient media and in obtaining a new plant under aseptic conditions are at the forefront. By associating *in vitro* culture techniques with asparagus breeding; it is important for many reasons such as the difficulties in creation of homozygous parent lines due to the dioecious nature of asparagus, the preference of male plants due to their higher performance, the difficulties of propagation by seed and the low vegetative (rhizome root) reproduction coefficient. Micropropagation offers the easiest, the fastest and the most reliable method in asparagus breeding and propagation.

As a result, it was determined that the effects of explant type on micropropagation was more important than the plant age of donor plant. It was concluded that harvesting the donor plant in the season could affect the success of the study positively due to active cell growth and development in the explant used. It has been determined that efficient mass micropropagation is possible with both explant types.

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Statement of Conflict of Interest

The author(s) declare no conflict of interest for this study.

Author's Contributions

The contribution of the authors is equal

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