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Original article

Optimal Propagation and Rooting Mediums in *Rubus* spp. by in Vitro Micropropagation

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Abstract

Rubus spp. is a shrub-form plant known for its fruits called blackberries. Blackberries are plants with high commercial value, delicious taste, nice aroma, and high nutritional value. Turkey has wealthy genetic origins of *Rubus* species. Conventionally, the trading propagation of *Rubus* plants is done as vegetatively, utilizing truncation, rooting, or stratuming. However, these traditional methods are time-consuming and inefficient in virus-free plant production. Cloning of plant grown in the tissue culture also enables to obtain virus-free plants and to provide fast replicating high standard plants. *Rubus* obtained by micropropagation is used for the formation of commercial fruit plantations as well as source plant formation. In this work, the aim is the development of in vitro micropropagation process of the wild *Rubus* in the Trakya Region. Proliferation from axillary buds was made by adding BAP (6-Benzylaminopurine), NAA (Naphthalinacetic acid) and GA3 (Gibberellic acid) in various combinations and concentrations to the MS medium. Rooting was successfully realized with 83.3% rooted plants in 1 IBA medium. No roots were seen in 0 MS. The survival rate of plants transferred to ex vitro conditions was 100%.

Keywords: Micropropagation, In Vitro, Apical Buds, Shoot, Root, Ex Vitro.

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INTRODUCTION

The Rosaceae family comprises more than 100 genera, which in turn contain about 3000 species, being the third most important family of plants economically in the major temperate regions of the world (Zarei et al., 2017). One of the most representative genera of this family, *Rubus*, has about 750 species, some of which are of ecological and some of the economic value, especially for their use as a fruit and ornamental species (Espinosa et al., 2016). This genus has a worldwide distribution where most of its constituent species are installed in mild regions of the Northern Hemisphere and only a few species originate in the tropics and/or Southern Hemisphere. (Huang and Hu, 2009). As a cultivable species, it has not acquired the desired degree of development, mainly due to the limited technological offers that this crop has, among which the poor propagation system stands out (Cancino-Escalante et al., 2011). *Rubus* is a bush tree that grows upright, semi-steep, or creeping. Most cultivars have spiny stems, and barbless Rubus are spread traditionally with shoot tip or cutting steel (Broome and Zımmerman, 1978; Caldwell, 1984). Due to the low fertility of the seeds and the long germination and development period of the seeds, the natural propagation of blackberry only by seed makes it very difficult to achieve an increase in production capacity (Aguilera-Arango, 2019). Similarly, asexual reproduction, in addition to the loss of genetic variability, has some problems, such as the spread of diseases endogenously found in the parent plant (Vaca and Landázuri, 2013).

Rubus spp. is a plant famous for its fruits named blackberries in the shape of bushes. It is commercially bred for its berry tasty taste, nice flavor and nutritive value. Turkey has wealthy genetic origins of *Rubus* species. *Rubus* fruits, which are widely consumed in Turkey, are used in traditional products such as mulberry molasses and mulberry pulp. Although the fruits are consumed fresh, they are used in fruit juices, natural dyes, marmalades, and cosmetics industry (Ercişli and Orhan, 2005). Selecting precious individuals from *Rubus* plants that have big varieties in different regions and provinces of Anatolia will also attributed to the improvement of breeding studies (Ercişli, 2014). In last times, blackberry using up happens very significant to the bearing bioactive compositions and alimentary worths for human diet and good physical condition. Owing to the comprehension health efficacy from the fruits, blackberry happens worthy trading fruit in the globe, and we nation its generation and using up rises from year to year (Diaconeasa et al., 2014; Jadan et al., 2015; Akin et al., 2016).

Conventionally, the trading distribution of *Rubus* plants is done vegetatively, utilizing truncation, stem suckers, or stratuming (Dziedzic and Jagła, 2013). But these traditional methods are time-exhausting durations and do not efficient virus-free planting samples. Clonal by growing plantlets in tissue culture lets also the elimination of viruses and the founding of quickly reproducing identical high-standard plants (Martin, 2002). Raspberry obtained by micropropagation is utilized for the formation of commercial fruit plantations as well as mother plant formation. Researchers have indicated that

micropropagated raspberry plants under area circumstances are observable characteristics of an individual like those reproduced by conventional methods, and even show ahead of winter resistance, fruit quality, and heaviness (Bite and Petrevica, 2002; Georgieva et al. 2009). Additionally, the different analyses showed that being without genetic modifies in plant growth via in vitro culture and their relevance for trading utilization (Vujovic, 2017; Kefayati et al. 2019). Nevertheless, it does not have standard security for the reproduction and breeding of conventional planting substances. Here, mediums such as in vitro replication have been utilized to find a way out of this trouble. In addition, the influence of some agents, like the in vitro propagation medium, is still mysterious.

Blackberries are generally propagated by micropropagation utilizing axillary shoots of present meristems (Caldwell, 1984). The skill to regrow plants is very important to the accomplished, practice of in vitro techniques (Cao and Hammerschlag, 2000). In vitro propagation methods are widely used in Europe and the USA (Boxus, 1989). Adventitious shoot formation, along with micropropagation methods and genetic engineering, has been used for many plant breeding improvement goals in plants. Biotechnology and genetic engineering applications are especially beneficial for plants because these applications have the capacity to decrease the time necessary for conventional breeding study (Petri and Burgos, 2005).

The apps of many tissue culture methods such as adventitious organogenesis have been examined in a varied variety of the *Rubus* genus, including blackberry, raspberry, and their hybrids, in addition to its application in many plants (Orlikowska, 1984; Wainwright and Flegmann, 1986; Fiola et al., 1990; McNicol and Graham, 1990; Swartz et al., 1990; Cousineau and Donelly, 1991; Turk et al., 1994; Graham et al., 1997; Mezzetti et al., 1997; Meng et al., 2004; Zawadzka and Kusharenko, 2006). Nevertheless, available in vitro propagation methods very quite investigation on most effective use of the medium and plant growth regulators and their compounds. However, as is known from former publications, there are quite restricted practices for micropropagation of nodal segments in blackberry as a material source.

The aim of this study is to determine the optimum reproduction and rooting medium by in vitro propagation and rooting experiments by obtaining plantlets in the shoot propagation medium of wild *Rubus* spp. in the Trakya region. As a result of the trials established in our laboratory, the growth and rooting of plants were investigated in media containing different plant growth regulators. To our knowledge, an in vitro micropropagation study of naturally grown blackberry varieties found in this region has not been previously reported.

MATERIALS and METHODS

Explant Retrieval and Surface Sterilization

The starting material used in the study was taken from the Marble village of Tekirdağ. The explants cut as a single node were washed under running tap water for 15 minutes for pre-sterilization and then washed with 2-3 drops of commercial detergent on a magnetic stirrer for 5 minutes and left under running tap water for about 30 minutes. The explants, whose pre-sterilization were completed, were taken into a sterile cabinet and first kept in 70% ethyl alcohol solution for 2 minutes. Then, it was kept in a magnetic stirrer for 10 minutes in 0.1% HgCl2 solution. Finally, it was passed through autoclaved distilled water 3 times for 5 minutes. Obtained sterile explants were prepared for In vitro culture media in nutrient media containing plant growth regulators in different doses and combinations to improve shoot regeneration.

Medium and Culture Condition

In this study, naturally grown blackberry varieties in the Trakya region were used. MS basal culture medium including macroelements and microelements, vitamin, ethylenediamine di-2-hydroxyphenyl acetate ferric (Fe-EDDHA), and varied integrations of plant growth regulators were utilized for shoot initiation, multiplication, and rooting of *Rubus* spp. The micropropagation medium was carried out as MS medium (Murashige and Skoog, 1962) containing diverse concentrations of BAP, GA, NAA, as well as mineral salts and vitamins for shoot development. The whole nutrient medium comprised 30 g/L sucrose and 8 g/L agar. After adding plant growth regulators, the pH of the medium was adjusted to 5.7 using 0.1 N NaOH or 0.1 N HCl. The prepared medium was autoclaved at 121 °C and 15 psi pressure for 15 minutes. All cultured plants were incubated at 24 ± 2 °C for a 16/8 hour photoperiod at 3000 lux light intensity. Explants were transferred to the propagation medium after 3 weeks. Subcultures were made every 3-4 weeks.

Shoot Proliferation and Root Induction

Nodal buds of sterilized shoots were cut about 0.5-1.0 cm and nodal segments of plant material were accommodated on the beginning medium including an essential component in basal medium (MS) and containing vitamins added with 0.2 mg/I BAP, 30 g/l sucrose, and 8.3 g/l agar. After about 3 weeks taken to MS medium including five diverse contents of BAP (0, 0,5, 1, 2, 3 mg L–1) for subculture. The whole the medium included 0.3 GA and 0.1 NAA mg L–1. The growing shoots were subcultured every four weeks (Figure 1a). The shoots later reaching a length of 2–3 cm was inoculated into the rooting medium. In addition, combinations of different plant growth regulators and MS medium containing 10 mL of L–1 Fe-EDDHA were used for rooting: 0, 1, 2, 3, and 4 IBA. Rooted plantlets were gradually taken from the glass jars 4 weeks later (Figure 1b, c) and taken to the acclimatization greenhouses and

then to the outside environment. Just before the plantlets were taken to the acclimatization greenhouses, the number of roots and root lengths were counted and transferred to the soil (Figure 1d, e).

Statistical Analysis

Experiments were designed in an entirely randomized block pattern with five replications and at least five explants per duplicate. Statistical evaluation of the analyzed data was made with the Duncan test, one of the One-Way ANOVA post hoc tests of the SPSS ver 22 statistical program (Snedecor and Cochran, 1967).

Figure 1: a, A view of micropropagation of *Rubus* spp. under in vitro conditions, b, c: rooting, d, e: explants transferred to the soil.

RESULTS and DISCUSSION

Considering the combined use of plant growth regulators (BAP + GA3; BAP + IAA or BAP + NAA) for species of the genus *Rubus*, it is possible to achieve maximum shoot size and quantity in materials (Sigarroa-Rieche and García-Delgado, 2011; Marulanda, et al., 2000). In this study, we studied the in vitro micropropagation, rooting, and acclimatization of wild *Rubus* spp. at different concentrations of phytohormones. The outcomes of different BAP compositions on the proliferation rate of *Rubus* spp. were given in Table 1. The number of shoots propagated with a low and high concentration of BAP has a statistically important action. The maximum growth rate in six subcultures was attained in the medium

including 3 mg/l BAP + 0.3 mg/l GA + 0.1 mg/l NAA (23.00±1.51a), while the minimum rate was get in the medium comprising 0 MS (1.60±0.24c) (Table 1).

Medium factors were very effective in vitro propagation and regeneration studies (Wei et al. 1992). Najaf-Abadi and Hamidoghli (2009) also observed the top condition for micropropagation of Thornless trailing blackberry (*Rubus* spp.), media including 2 mg/I BA and 0.5 mg/l GA3, and the identical researchers attained also the most shoot length in the identical condition. Parallel outcomes were attained in our study with BAP, however the extra GA and NAA were provided better ends compared to prior outcomes. Kefayeti et al. (2019), on the other hand, they obtained better results when IBA was added to the medium containing BAP. Villa et al. (2007), using the "Jumbo" nodal segments in their study using the "Jumbo" variety, with diverse BAP contents (0, 0.5, 1.0, 2.0 and 4.0 mg/l) and different MS medium (0, 50, 100, 150 and 200%) checked their effects. In the identical study, they obtained the tallest shoots from 150% MS media including just 1 mg/l BAP. In our study, the best shoot development was obtained from the medium including 1 mg/l, 2 mg/l, and 3 mg/l BAP, and maximum shoot length was obtained from the medium containing 3 mg/l BAP. The reason for obtaining the best results with high BAP in our study may be the difference in the cultivar and MS concentration. According to Kefayati et al. (2019) investigated the number of shoots taken by the combination of BAP and IBA using the "Chester thornless" cultivar. The highest proliferation rate was acquired from the media including 2 mg/l BAP + 0.2 mg/l IBA, while the lowest number was obtained from the media containing 3 mg/l BAP + 0.1 and 0.4 mg/l IBA. The lowest proliferation was usually obtained with combinations of 3 mg/l BAP, when at minimum 0.1 and 0.4 mg/l IBA were utilized. In our study, BAP combinations and GA and NAA were used together. Bobrowski et al. (1996) using 'Ebano', 'Tupi' 'Guarany' blackberry varieties determined that the highest shoot number was taken from 1 and 2 mg/1 BAP media and when GA3 and NAA were extra-joined the medium, the proliferation rate diminished. The results of this study have parallel results with our study. Munoz et al (2021) evaluated shoot growth of plants in the 5th subculture using MS medium to which 2 mg L-1 BAP was added. Shoots were taken into MS medium containing 20 g L-1 sucrose and 2 mg L-1 BAP assessing 0, 0.1, and 0.5 mg L-1 NAA every 30 days. Researchers have shown that the presence of cytokinin (BAP) in the shoot propagation medium has the highest effect on the growth rate at 1.4 mg L-1 BAP in *Rubus* spp. Wu et al. (2009) also noted that the utilization of cytokinin for the shoot multiplication phase was decisive in *Rubus* and was better at 1.0 mg L-1. In this study, I showed the effect of cytokinin at different concentrations in wild *Rubus* spp. In this study, mean shoot size results in five subcultures at different BAP concentrations are dedicated in Table 2. As shown in Table 2, different concentrations of BAP had a statistically important impact on shoot length in the first three subcultures, but not after the fourth subculture. While the maximum shoot length was attained with 3 mg/l BAP + 0.3 mg/l GA + 0.1 mg/l NAA (2.80±0.13a), the minimum shoot length was get with 0 MS (0.8±0.07d). If I associate the plant material taken into the medium with the medium conditions, it was seen that the number of subcultures and the length of the

shoot were inversely proportional. It was found that while the shoot length was 2.80±0.13a cm in the medium where it was maximum, it was 0.8±0.07d cm in the medium where it was minimum.

In addition, while Debnath (2004) observed frequent callus formation in blackberry plants in his study, I did not encounter callus formation in the study. It is thought that this may be due to the performance of a maximum of six 21-day subcultures during the propagation stage and the use of low doses of growth regulators (Oliveira et al., 2006).

Table 1. The shoot development in six subcultures of the shoots derived from nodal segments of *Rubus* spp. using various BAP concentrations.

Shoot development	0 MS	0,5 BAP+ 0,3 GA+ 0,1 NAA	1 BAP+ 0,3 GA+0,1 NAA	2 BAP+ 0,3 GA+0,1 NAA	3 BAP+ 0,3 GA+0,1 NAA
	2	14	14	16	24
	1	10	28	19	23
	2	9	13	21	21
	1	12	20	24	19
	2	11	16	18	28
	2	7	15	19	27
	1,60±0,2c	11,20±0,86b	18,20±2,72a	19,60±1,36a	23,00±1,51a

p=0,01

Table 2. The shoot length in six subcultures of the shoots derived from nodal segments of *Rubus* spp. using various BAP concentrations.

Shoot length	0 MS	0,5 BAP+ 0,3 GA+ 0,1 NAA	1 BAP+ 0,3 GA+0,1 NAA	2 BAP+ 0,3 GA+0,1 NAA	3 BAP+ 0,3 GA+0,1 NAA
	1	1,2	1,5	2,5	3,2
	0,9	1,3	2	2,4	2,8
	0,7	1,8	1,8	2,3	2,9
	0,8	0,9	1,7	2,1	2,4
	0,6	1,3	1,6	1,9	2,7
	0,8±0,07d	1,30±0,14b	1,72±0,08c	2,24±0,10b	2,80±0,13a

p=0,01

I investigated the effects of different IBA concentrations on rooting rate with the best IBA combinations. *Rubus* spp. the results of the applications of 0, 1, 2, 3, and 4 IBA combinations in terms of root number and root length are given in Table 3. The highest root number and mid-root length were obtained from 1 mg/IBA (1.06±0.15a, and 6.20±1.56a, respectively). As a result of the statistical evaluation of the root lengths of the plants in the rooting medium after 4 weeks, the medium with the highest rooting percentage was determined as 1 IBA (83.33%). Failed to get rooting at 0 MS. On the other hand, Olivera et al. (2009) showed satisfactory root growth in almost 100% of plants subjected to in vitro rooting. A similar result was found by Raeva-Bogoslovskaya et al. (2021), in his study with 4

different *Rubus* cultivars, which determined that the rooting percentages of the media containing 1 IBA differed from each other and *'Cacanska Bestrna'* had the highest rooting percentage (100%) in the medium containing 1.0 mg L-1 IAA and the rooting of the raspberry-blackberry hybrids was auxin type. Determined that there were significant differences depending on the very close values (90-95%) were obtained by Debnath (2004). Wild *Rubus* spp. I can state that rooting rates differ slightly from previous studies because of the variety used. Leitzke et al. (2009), and Sigarroa and Garcia (2011) do not recommend MS medium for elongation and rooting, while they counted 2 roots per shoot planted in MS medium. Muñoz-Concha et al. (2021), obtained results similar to the results of my study and found the survival rate of plants after acclimatization to be 88.2%. Najaf-Abadi and Hamidoghli (2009) found that better rooting media for some Thornless blackberry (*Rubus* spp.) cultivars was 2 mg l/l IBA. Maximum long root was obtained from 0 mg l-1 IBA with combinations of 0 mg l/1 NAA and 0.2 mg l/1 NAA. In this study, the maximum mean root number (6.20±1.56a) and mean root length (83.33%) were taken from the medium comprising 1 mg l/l IBA. He stated that the reason for this variation may be due to the use of different varieties and may vary from genotype to genotype. Najaf-Abadi and Hamidoghli (2009) transferred the root crop to the container under greenhouse conditions (air circulation under a "fog" system), and they determined the survival rate to be 98% after acclimatization. In this study, the transfer of rooted plants to the controlled greenhouse environment, the survival rate was found to be 100% (Figure 1d, e).

Table 3. The root length and root number average in six subcultures of the shoots derived from nodal segments of *Rubus* spp. using various IBA concentrations.

Rooting		Root length				
Trial medium	Rooting ratio (4 week)	0 MS	1 IBA	2 IBA	3 IBA	4 IBA
0 MS	0	0	0,8	0,3	0,4	0,6
1 IBA	83,33%	0	0,6	0,4	0,6	0,8
2 IBA	75%	0	1,3	0,7	0,9	0,5
3 IBA	62,50%	0	1,2	0,6	1	0,4
4 IBA	50%	0	1,4	0,8	1,1	0,7
		0,0±0,00c	1,06±0,15a	0,56±0,09b	0,8±0,13ab	0,6±0,07b
		Root number average				
		0	6	4	2	2
		0	4	2	3	4
		0	12	3	2	1
		0	3	4	1	1
		0	6	5	4	3
		0,0±0,00c	6,20±1,56a	3,60±0,50ab	2,40±0,50bc	2,20±0,58bc

p=0,01

Seasonal elements highly influenced in vitro propagation and regeneration studies (Wei et al. 1992). For this reason, in vitro propagation studies have increased in many plants in recent years, as well as in *Rubus* species (Andrade et al. 2021; Raeva-Bogoslovskaya et al. 2021; Muñoz-Concha et al. 2021; Kefayati et al. 2019; Borodulina et al. 2019). Many problems can be solved with biotechnological methods in plant reproduction (Muñoz-Concha et al. 2021). Optimization of in vitro conditions provides access to large numbers of plant explants at some time of the year (Galletta et al. 1998). The advantages of in vitro propagation enjoyed by the breeding production in very plant types are not well advanced for blackberries, together with wild *Rubus* spp., which may be material for future breeding studies. This study aimed to discover the optimum medium for micropropagation of wild *Rubus* spp. in vitro shoot development and rooting stages of wild *Rubus* spp.

CONCLUSION

For in vitro propagation and rooting stage, the optimum culture media; the best shoot development was obtained from the medium containing 1 mg/l, 2 mg/l, and 3 mg/l BAP, and maximum shoot length were obtained from the medium containing 3 mg/l BAP. The highest root number and mid-root length were obtained from 1 mg/IBA (1,06±0.15a and 6.20±1.56a, respectively). As a result of the statistical evaluation of the root lengths of the plants in the rooting medium after 4 weeks, the medium with the highest rooting percentage was determined as 1 IBA (83.33%). In ex-vitro adaptation, it has passed to the air-conditioning and greenhouse stages by using a mixture of perlite: peat (3:1). The perlite: peat used and controlled environmental conditions contributed to the survival levels and seedling height. Taking into account, that there are few recorded studies on wild *Rubus* species, the methodology offered here creates a base on advancing in vitro propagation durations of species chosen below accent concerned chiefly with a rating of threat, the problem of proliferation under conventional process, population ease of access plant material presence and powered for the ability to continue utilization.

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