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Original Article

Micropropagation of 'Chester thornless' Blackberry Cultivar using Axillary Bud Explants

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Abstract

Sexual propagation is not suitable for production of true to type plants for commercial cultivation of selected cultivars due to the having heterozygous characteristics. *Rubus* species are highly heterozygous and therefore when they are propagated by seeds the high plant variability is obtained. Micropropagation methods became very important and have many advantages compared to the classical propagation methods. In Turkey, blackberries are commonly propagated by hard and soft wood cuttings, by layering and bush division. Propagation by layers requires rather large area for a layered, and weed control among the layers is a problem. However, propagation by cuttings is connected with important problem in inadequate rooting, especially in 'Chester Thornless' cultivar, which is one of the most commercially grown cultivar. In addition, limited number of plants can be propagated using classical propagation method and thus demand of growers for the plants can not be satisfied. Optimized and highly efficient regeneration protocols are also important for genetic transformation for improving of new cultivars. In this study we focused on optimization of protocol for *in vitro* propagation of 'Chester Thornless' blackberry cultivar using nodal segments. For this purpose, explants were cultured on Woody Plant Medium (WPM) supplemented with combination of plant growth regulators BAP (1.5, 2, 3 mg/l) with NAA (0, 0.1, 0.2, 0.4 mg/l) to detect the best multiplication medium. According to the obtained results, the highest proliferation rate was obtained for cultivar 'Chester Thornless' that produced 9.66 shoots on a medium containing 2 mg/I BAP+0.2 mg/I IBA combination. GA_3 effects on multiplication rates were also studied and generally the highest rates obtained by while GA_3 were not used. For the rooting, different concentrations of IBA and NAA (0, 0.1, 0.2 and 0.4 mg/I) were compared. A concentration of 0.4 mg/l NAA gave the greatest number of roots and maximum root length.

Keywords: blackberry; plant growth regulators; propagation; rooting; tissue culture

Introduction

Horticulture plants including fruits, vegetables, grapes and ornamentals are a frequent and extremely important element of man's environment. They are not merely structural elements of the environment, but they also imbue it with life and tinge it with beauty. They enhance the general quality of the environment and also indirectly contribute to an increase in the value of other components of the environment (Kravanja, 2006; Kamiloglu *et al*., 2009; Tosun *et al*., 2009; Dogan *et al*., 2014; Caliskan *et al*., 2017). They propagated in general vegetative methods including cutting, grafting and micro propagation (Ercisli *et al*., 2003; Cuce and Sokmen, 2017).

Rubus fruticosus L. is a shrub famous for its fruits called blackberries. It is commercially grown due to berry delicious taste, pleasant flavor and nutritional profile. The shrub is believed to have its origin in *Rubus armeniacus*, and is now distributed throughout Europe, Asia, Oceania and North and South America (Hummer and Janick, 2007). Blackberry belongs to the Rosaceae family, *Rubus* (Tourn) L. genus and Eubatus subgenus, a shrubby tree with erect, semi erect or creep grown habit, and most cultivars have thorny stems.

Turkey has rich genetic resources of *Rubus* species. In recent years, blackberry consumption becomes very important due to the including bioactive compounds and nutritive values for human diet and health. Due to the understanding health benefits of its fruits, blackberry became one of the most valuable commercial fruit in the world and in our country its production and consumption increases year by year (Diaconeasa *et al*., 2014; Akin *et al*., 2016).

Thornless blackberries are commercially propagated by tip layers or stem cuttings (Caldwell, 1984). *In vitro* techniques are important tools for modern plant improvement programs for introduction of new traits into selected plants, multiplication of elite selections and for

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development of suitable cultivars in a very short time (Taji *et al*., 2002). Thornless blackberries are usually reproduced by *in vitro* culture using axillary shoots by initiation of existing meristems (Caldwell, 1984). The ability to regenerate plants is crucial to the successful application of *in vitro* methods (Cao and Hammerschlag, 2000). Micropropagation techniques have been widely used especially in Europe and USA (Boxus, 1989). Adventitious shoot regeneration is also prerequisite for any plant improvement programme aimed at improving plants by *in vitro* techniques and genetic engineering. Plant biotechnology and genetic engineering technologies are particularly useful for fruit trees since these technologies have the potential to reduce the time needed for traditional breeding programme (Petri and Burgos, 2005).

In vitro propagation through the development of axillary buds and adventitious shoots eliminates the seasonal limitations encountered with these methods and needs a small quantity of explant. The *in vitro* plants propagated in this way in many species have proved to be healthy and true to type (Shen *et al*., 1990; Figas *et al*., 2016). Micropropagation techniques are capable of producing many plants in a short period of time also (Guney *et al*., 2016). In recent years, *in vitro* propagation techniques became very important since the plant material obtained through these techniques guaranties quality and safety compared to the traditional production which involves the transmission and contamination with pests and diseases (Jadan *et al*., 2015). Application of tissue culture techniques such as adventitous organogenesis and cryopreservation *in vitro* has been studied in different genotypes of genus *Rubus*, including blackberry, raspberry and their hybrids (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, 2004). However, micropropagation *in vitro* techniques requires a great deal of experimental work on optimization of the medium and plant growth regulators and their combinations. Nevertheless, as far as we know, very limited studies on *in vitro* propagation of blackberries using nodal segments as an explant sources were previously published. The aim of this study was to optimize protocol using various plant growth regulators and their combinations for *in vitro* propagation of 'Chester Thornless' blackberry cultivar.

Materials and Methods

Biological material

Explants were collected from actively growing shoots on August in Research and Implementation Area of Horticulture Department of Cukurova University, Turkey. Nodal segments of 'Chester Thornless' cultivar were excised from field-grown plants, immediately transferred to the tissue culture laboratory and cut into single-nod segments which were used as primary explants. Explants were washed in running tap water and then washed again thoroughly by adding a few drops of Tween-20. Surface sterilization was done using 6% sodium hypochlorite for 10 min. and then rinsed several times in sterile double distilled water inside the Laminar Air Flow Chamber.

Experimental procedures

Nodal segments of 'Chester Thornless' were placed on initiation medium containing major and minor salts as in basal medium (McCown's Woody Plants) and including vitamins supplemented with 2 mg/I BAP, 20 g/l sucrose and 7.5 g/l agar. All media were adjusted to pH 5.8 before autoclaving. All cultures were kept at 25 °C under 16 h light and 8 h dark cycle. Two weeks after initiation, the shoots were transferred to multiplication medium. 4 subcultures were done at 3-4 week intervals and for each subculture 75 explants.

To identify optimum multiplication and rooting conditions, various plant growth regulators were tested. To identify the best shoot multiplication rate various concentration and combination of BAP (1, 2, 3 mg/l), NAA (0, 0.1, 0.2, 0.4 mg/l) and IBA (0, 0.1, 0.2, 0.4 mg/l) were compared. According to the experimental results effects of various levels of GA_3 (0, 0.25, 0.50 mg/l) on multiplication rate and shoot length were also studied using the best combinations. In addition, effects of various auxins (IBA and NAA; 0, 0.1, 0.2 and 0.4 mg/L) application on the number of roots and length were also compared (Fig. 1). After rooting the plantlets were transferred to a plastic container containing a mixture of sterilized peat and perlite (v/v:1/1) under "mist" system (Sahin *et al*., 2002) (Fig. 2).

Statistical analysis

Data were taken from three replicate experiments and analysed a completely randomized factorial design. Variance analysis were applied all recorded data using JMP soft pack programme and means were compared by using Tukey test at 5% level of significance.

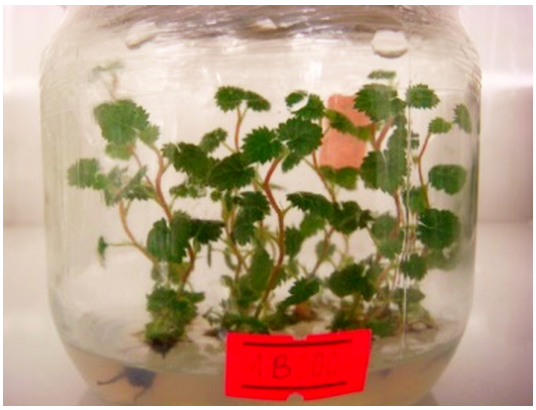

Fig. 1. Rooted plants before transplanting to the greenhouse

Fig. 2. Rooted plants under the "mist" system

Results

The results of various BAP and IBA combinations on multiplication rate of 'Chester Thornless' variety were given in Table 1. The interaction of BAP and IBA had a statistically significant effect on the number of shoots produced. The highest average multiplication rate in three subculture was obtained on medium containing 2 mg/l BAP + 0.2 mg/l IBA (9.66), whereas the lowest on medium with 3 mg/l BAP + 0.1 and 0.4 mg/l IBA (3.33). The lowest multiplication rates obtained mostly 3 mg/l BAP combinations while the least when 0.1 and 0.4 mg/l IBA used (Table 1).

The results of average length of shoots in the three subcultures of 'Chester Thornless' on medium with various BAP and IBA combinations were given in Table 2. As seen in Table 2, the interaction of BAP and IBA had a statistically significant effect on the length of shoots in the first and second subcultures while non-significant in the third subculture. The highest values were obtained with 2 mg/l BAP + 0.2 mg/l IBA whereas the lowest with 2 mg/I BAP + 0.1 mg/I IBA combinations. Positive influence of culture duration on shoot length was observed during third subculture in comparison with the first and second ones what can be connected with adaptation of plant material on culture conditions. Average shoot length was detected as 1.34 cm in the first subculture, 1.60 cm in the second subculture and 1.89 cm in the third subculture.

The results of average multiplication of shoots in the three subcultures of BAP and NAA combinations were given in Table 3. The interaction of BAP and NAA had a statistically significant effect on the multiplication rate in the first, second and three subcultures and the highest multiplication rate in only 2 mg/I BAP without NAA application. The average longest shoots obtained 2 mg/l BAP and 0 mg/l NAA concentration in all three subculture and average rate was 6.33 (Table 4). Also, shoot length increased within creasing the number of subcultures. Comparing to IBA and NAA combinations, NAA showed lower influence on shoot growth and multiplication rates.

It was known that GA_3 can synergistically act with auxins (Stowe, 1957). Effects of various GA_3 applications on multiplication rate of 'Chester Thornless' was also studied using the best combinations of BAP + IBA and BAP + NAA (Table 5). Addition of GA_3 in the tested concentrations negatively affect multiplication rate and differences were found statistically significant. However, positive effect obtained shoot length of plants and increased parallel to the subculture. The effects of various GA_3 applications on shoot length during three subculture medium were given in Table 6. The average longest shoots was obtained from 1.5 mg l/1 BAP with 0 mg l/1 NAA with 0.5 mg l/1 GA_3 combinations. Whereas the lowest 2 mg l/1 BAP with 0.2 mg l/1 IBA with 0 mg l/1 GA_3 combinations. Also, shoot length increased parallel to the increasing the number of subcultures (Table 6).

Najaf-Abadi and Hamidoghli (2009) reported best condition for *in vitro* propagation of Thornless trailing blackberry (*Rubus* sp.), medium containing 2 mg/I BA and 0.5 mg/l GA_3 and the same authors implied also the maximum shoot length in the same medium. A similar study was conducted by Wu *et al*. (2009) on 32 varieties of blackberry and the interaction of various BA concentrations with 0.1 mg/l IBA had a significant effect on the number of shoots produced and shoot length in the tested *Rubus* selections. Similar results were obtained in our study with BAP but addition of IBA gave the better results compare to the previous results. Bobrowski *et al*. (1996) published 5 various types of berries and the highest rates of proliferation 'Guarany' and 'Ebano' varieties had found. Researchers reported that the proliferation coefficient varied among the cultivars. In another study, Villa *et al*. (2007), compared effects on different basal MS medium (0, 50, 100, 150 and 200%) with different concentrations of BAP (0, 0.5, 1.0, 2.0 and 4.0 mg/l) using nodal segments as explants of 'Jumbo' cultivar. The same authors reported the longest shoots from 150% MS medium containing only 1 mg/l BAP.

Villa *et al*. (2007) reported that the great numbers of shoots were produced with 1 mg/I BA in WPM basal medium while Najaf-Abadi and Hamidoghli (2009) reported on best condition for *in vitro* propagation of Thornless trailing blackberry (*Rubus* sp.) and the maximum shoot length was produced in medium containing 2 mg/I BA in MS basal medium. Bobrowski *et al* (1996) used 'Ebano', 'Tupi', 'Guarany' blackberry cultivars, and the highest shoot number was obtained from 1 and 2 mg/1 BAP medium and when GA_3 and NAA added to the media a multiplication rate was decreased. The results of this study are similar with our results in this study.

The results of treatments of IBA and NAA combinations on average number of roots and root length in the three subculture of 'Chester Thornless' were given in Table 7. As seen in Table 7, the highest roots numbers in three subcultures was obtained from 0.4 mg/I IBA and 0.4 mg l/ NAA combinations. Najaf-Abadi and Hamidoghli (2009) reported the best rooting medium 2 mg l/I IBA application for some cultivars of Thornless blackberry (*Rubus* sp). These differences may be due to the using different genotypes and can be changed from genotype to genotype. As seen in Table 7, the longest roots were obtained from 0 mg l-1 IBA with 0 mg l/1 NAA and 0.2 mg l/1 NAA combinations. After transferring rooted plants in pots in the greenhouse environment (acclimatization under the "mist" system) survival rates was 98%.

Conclusions

As a conclusion, the highest average multiplication rate and length of shoots were obtained from 2 mg/I BAP + 0.2 mg/I IBA (9.66), and he lowest 3 mg/l BAP + 0.1 and 0.4 mg/l IBA (3.33) while the highest multiplication rate in only 2 mg/I BAP without NAA application (6.33) and the lowest in 1.5 mg/I BAP + 0.4 mg/l NAA (2.21) combinations. According to the obtained results BAP was found to be more effective than NAA in blackberry *in vitro* multiplication. GA_3 applications on multiplication of 'Chester Thornless' were affected negatively and differences found statistically significant. However, positive effect on shoot length of plants and the longest shoots was obtained from 1.5 mg/1 BAP with 0 mg/1 NAA with 0.5 mg/1 GA_3 combinations. Whereas the lowest 2 mg/1 BAP with 0.2 mg/1 IBA with 0 mg l/1 GA_3 combinations. As for the rooting, NAA was found to be more effective compare to the IBA and the highest roots numbers and length was obtained from 0.4 mg/I NAA. At the acclimatization stage about 95% of rooted micro cuttings survived in the field for three weeks after transplanting.

Table 1. The multiplication rate in three subcultures of the shoots derived from nodal segments of 'Chester Thornless' using various BAP and IBA combinations

TN	Combinations (mg/l)		Multiplication rates in three subcultures			
	BAP	IBA	1	2	3	Average
1	1.5	0.0	5.00bcd	6.33bc	5.00cd*	4.77
2	1.5	0.1	4.66cd	6.33bc	5.00cd	5.33
3	1.5	0.2	6.66b	6.00bcd	5.33bc	5.99
4	1.5	0.4	5.33bcd	4.00ef	4.00def	4.44
5	2.0	0.0	5.00bcd	5.00cde	4.33cde	4.77
6	2.0	0.1	6.33b	7.00b	6.33b	5.66
7	2.0	0.2	8.66a	11.66a	8.66a	9.66
8	2.0	0.4	5.33bcd	5.66b.f	3.66ef	4.88
9	3.0	0.0	5.33bcd	4.66c.f	3.66ef	4.55
10	3.0	0.1	2.00e	4.33def	3.66ef	3.33
11	3.0	0.2	4.33d	4.33def	3.33ef	3.99
12	3.0	0.4	4.00d	3.00f	3.00f	3.33
	$LSD_{0.05}$		1.79	1.77	1.15	
	Average		5.21	5.69	4.66	

*: Means in same column with different small letters are significantly different at p<0.05. TN: Treatment numbers

Table 2. The average shoot length in three subcultures of the shoots derived from nodal segment of 'Chester Thornless' using various BAP and IBA combinations

TN	Combinations (mg/l)		Average shoot length (cm) in three subcultures			
	BAP	IBA	1	2	3	Average
1	1.5	0.0	1.46abc*	1.33c	1.96^{NS}	1.58
2	1.5	0.1	1.23bc	1.46bc	2.00	1.56
3	1.5	0.2	1.60a	1.50bc	2.00	1.70
4	1.5	0.4	1.33abc	1.53bc	1.96	1.60
5	2.0	0.0	1.43abc	1.56bc	1.93	1.64
6	2.0	0.1	1.26bc	1.53bc	1.80	1.53
7	2.0	0.2	1.53ab	1.86a	1.90	1.76
8	2.0	0.4	1.36abc	1.70ab	1.83	1.63
9	3.0	0.0	1.33abc	1.70ab	1.83	1.62
10	3.0	0.1	1.20 c	1.66ab	1.86	1.57
11	3.0	0.2	1.20 c	1.73ab	1.86	1.59
12	3.0	0.4	1.20 c	1.70ab	1.80	1.56
	$LSD_{0.05}$		0.32	0.22	0.18	
	Average		1.34	1.60	1.89	

*: Means in same column with different small letters are significantly different at p<0.05. TN: Treatment numbers; NS: Non significant

Table 3. The multiplication rate in three subcultures of the shoots derived from nodal segments of 'Chester Thornless' using various BAP and NAA combinations

TN	Combinations (mg/l)		Multiplication rates in three subcultures			
	BAP	NAA	1	2	3	Average
1	1.5	0.0	5.66a	5.66b	5.00a	5.44
2	1.5	0.1	3.00cd	3.00e	2.66bc	2.88
3	1.5	0.2	2.00cd	3.33de	3.00bc	2.77
4	1.5	0.4	1.66d	2.66e	2.33bc	2.21
5	2.0	0.0	6.33a	6.66a	6.00a	6.33
6	2.0	0.1	4.00bc	4.33c	3.33bc	3.88
7	2.0	0.2	2.33cd	4.00cd	3.33bc	3.22
8	2.0	0.4	2.00cd	2.66e	2.66bc	2.44
9	3.0	0.0	5.66ab	4.00cd	3.66bc	4.44
10	3.0	0.1	2.66cd	3.00e	2.66bc	2.77
11	3.0	0.2	2.00cd	3.00e	2.33c	2.44
12	3.0	0.4	1.33d	3.00e	3.00bc	2.44
	$LSD_{0.05}$		2.08	0.97	1.25	
	Average		3.38	3.94	3.49	

*: Means in same column with different small letters are significantly different at p<0.05. TN: Treatment numbers

Table 4. The average shoot length in three subcultures of the shoots derived from nodal segment of 'Chester Thornless' using various BAP and NAA combinations

TN	Combinations (mg/l)		Average shoot length (cm) in three subcultures			
	BAP	NAA	1	2	3	Average
1	1.5	0.0	1.33abc*	1.63b	1.76c	1.57
2	1.5	0.1	1.36abc	2.13a	1.90bc	1.79
3	1.5	0.2	1.20bc	1.66b	1.83bc	1.56
4	1.5	0.4	1.30bc	1.66b	1.90bc	1.62
5	2.0	0.0	1.33abc	1.63b	1.86bc	1.60
6	2.0	0.1	1.56a	2.20a	2.00ab	1.92
7	2.0	0.2	1.20bc	2.00ab	2.03ab	1.74
8	2.0	0.4	1.43ab	1.93ab	2.00ab	1.78
9	3.0	0.0	1.40bc	2.16a	2.13a	1.89
10	3.0	0.1	1.23bc	2.23a	2.16a	1.87
11	3.0	0.2	1.30bc	1.70b	1.90bc	1.63
12	3.0	0.4	1.16e	1.66b	1.90bc	1.57
	$LSD_{0.05}$		0.25	0.36	0.21	
	Average		1.31	1.88	1.94	

*: Means in same column with different small letters are significantly different at p<0.05. TN: Treatment numbers

Table 5. Effects of various GA_3 treatments on multiplication rate of 'Chester Thornless'

Combinations (mg/l)	Multiplication rates in three subcultures			
	1	2	3	Average
2 BAP+ 0.20 IBA+ 0 GA_3	6.66a	8.66a	7.00a	7.44
2 BAP+0.20 IBA+ 0.25 GA_3	4.33b	4.00b	4.66b	4.33
2 BAP+0.20 IBA+ 0.50 GA_3	4.33b	4.00b	4.00b	4.11
Average	5.10	5.55	5.22	5.29
$LSD_{0.05}$	1.15	1.33	1.76	
1.5 BAP+0 GA_3	5.33a	7.00a	5.33a	5.88
1.5 BAP+0.25 GA_3	4.33b	4.00b	4.33b	4.22
1.5 BAP+0.50 GA_3	4.33b	4.00b	3.66b	3.99
Average	4.66	5.00	4.44	4.7
$LSD_{0.05}$	1.15	1.10	1.15	
2 BAP+0 GA_3	6.00a	7.33a	5.66a	6.33
2 BAP+ 0.25 GA_3	4.33a	4.33b	4.33ab	4.33
2 BAP+ 0.50 GA_3	4.00a	4.00b	3.66b	3.88
Average	4.77	5.22	4.55	4.84
$LSD_{0.05}$	1.33	0.94	1.15	

*: Means in same column with different small letters are significantly different at p<0.05.

Table 6. Effects of various GA_3 treatments on shoot length of 'Chester Thornless'

Combinations (mg/l)	Shoot length in three subcultures			
	1	2	3	Average
2 BAP+ 0.20 IBA+ 0 GA_3	1.80c	1.86c	2.06c	1.90
2 BAP+0.20 IBA+ 0.25 GA_3	2.56b	2.60b	2.66b	2.61
2 BAP+0.20 IBA+ 0.50 GA_3	3.43a	3.56a	3.76a	3.58
Average	2.59	2.67	2.82	2.69
$LSD_{0.05}$	0.35	0.31	0.38	
1.5 BAP+0 GA_3	1.90c	2.00c	2.06c	1.98
1.5 BAP+0.25 GA_3	2.66b	2.53b	2.66b	2.61
1.5 BAP+0.50 GA_3	3.96a	4.00a	4.33a	4.09
Average	2.84	2.84	3.01	2.89
$LSD_{0.05}$	0.27	0.35	0.34	
2 BAP+0 GA_3	1.93c	1.86c	2.00c	1.93
2 BAP+ 0.25 GA_3	2.53b	2.50b	2.63 b	2.55
2 BAP+ 0.50 GA_3	3.96a	4.03a	3.96 a	3.98
Average	2.16	2.79	2.86	2.60
$LSD_{0.05}$	0.32	0.42	0.35	

*: Means in same column with different small letters are significantly different at p<0.05.

Table 7. The average number of root number and root length (cm) in three subcultures of 'Chester Thornless' using various IBA and NAA combinations

Treatments	Root number in three subcultures				Root length in three subcultures			
(mg/l)	1	2	3	Average	1	2	3	Average
0	3.66d	3.66d	3.33d	3.55	3.86a	3.83a	3.73ab	3.80
0.1 NAA	4.66cd	5.00bc	4.33c	4.66	2.83c	2.70c	2.76c	2.76
0.2 NAA	6.33b	5.66b	5.66b	5.88	3.30b	3.46b	3.63b	3.46
0.4 NAA	8.66a	7.66a	7.00a	7.77	3.60a	3.73ab	3.96a	3.76
0.1 IBA	4.66cd	4.66c	4.33c	4.55	2.83c	2.73c	2.73c	2.76
0.2 IBA	5.33bc	5.33bc	5.66b	5.44	2.86c	2.76c	2.73c	2.78
0.4 IBA	6.33b	5.66b	6.00b	5.99	2.60c	2.50c	2.40d	2.50
Average	5.66	5.37	5.18		3.12	3.10	3.13	
LSD $_{0.05}$	0.85	0.93	1.20		0.29	0.27	0.25	
0	3.66d	3.66d	3.33d	3.55	3.86a	3.83a	3.73ab	3.80

*: Means in same column with different small letters are significantly different at $p<0.05$.

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