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In Vitro Propagation of Blackberry (*Rubus fruticosus* L.)

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Abstract:

The success of mass micropropagation of fruit trees may be reached by using plant tissues culture techniques, since this has shown efficient results on seedling production with high quality and health. Preliminary experiments were carried out to define the constitution of culture medium that provides better results, in multiplication as well as *in vitro* rooting and acclimation methods of blackberry cv. 'Triple Crown' (*Rubus fruticosus* L.). The best survival percentage was 90% when both shoot tips and stem segments were treated with 40% sodium hypochlorite for 30 minutes. Murashige and Skoog (MS) medium supplemented with 2.0 mg BA+0.5 mg 2ip/l was the most efficient treatment on *in vitro* multiplication of blackberry inducing a higher number of shoots was 7.78 shoot/ explant. Also the combinations of MS medium with NAA and BA improved the multiplication of blackberry *in vitro,* the highest value was 4.42 shoot/explant that was observed in the presence of 1.0 mg BA combined with 0.1 mg NAA /l. The best rooting condition for explants of the blackberry was reached by keeping the explants in full strength MS medium enriched with 2.0 mg IBA+0.5 mg NAA/l which produced long roots with sub roots which were thick and involved in medium.

Keyword: *Rubus sp*., tissue culture, micropropagation, acclimatization.

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Introduction:

At present, the major commercial application of cell and tissue culture is plant propagation. Moreover, tissue culture offers additional opportunities for the rapid dissemination of new cultivars releases. Furthermore, plants can be produced during the entire year rather than during limited periods. Blackberries often termed "Brambles" are a diverse group of species and hybrids in the genus *Rubus*. They belong to the family Rosaceae. *Rubus* is one of the most diverse genera of flowering plants in the world and are adapted to a wide range of environments. Blackberries are native to Asia, Europe, North and South America. However, blackberries grown in specific regions are largely derived from species indigenous to that region. Blackberries have been used for eating, medicinal purposes, and as hedges to keep out marauders. In the southeastern US, *Rubus sp.* has been used to confer low-chilling and disease resistance into cultivars, such as Brazos and later Tupy, Carter *et al* (2006).

Yazzetti and Clark (2001) demonstrated that cultivar differences in chilling requirement which ranged from near 300 to 900h of chilling hours below 7.2°C. Additionally, Carter *et al* (2006) reported that Fruiting blackberries (*Rubus* sp.) require a period of chilling during the dormant season to resume growth in the spring has been listed as 100 to 700h (hours below 7.2°C). In Egypt, Farag *et al* (2010) found that among the 14 Egyptian weather stations, winter chill in 2009 ranged from 510 to 995 Chilling Hours below 7.2°C.

In initiation phase, the culture medium used had, as components, Murashige & Skoog 1962 (MS) salts, Myo inositol-100 mg/l, Vitamin B1-1 mg/l, Vitamin B6 - 0.5 mg/l, Nicotinic Acid - 0.5 mg/l and 6-benzylaminopurine (BAP) at 0.7 mg/l (Clapa *et al*., 2011 & 2013 and Fira, 2013).

In the *in vitro* multiplication stage, several experimental treatments were applied in order to establish the optimal culture media, the suitable culture vessels for this stage, the adequate types of microcuttings, as well as the gelling agent that should provide economical proliferation rates and a high number of shoots suitable for acclimatization. For multiplication, generally, MS medium containing plant growth regulators were used, especially BAP at various concentrations, mainly 1-3 mg/l, auxins (IBA or NAA) at low concentrations and GA3. For *in vitro* rooting, medium supplemented with auxins (especially IBA) and with no plant growth regulators were tested, Arikan *et al* (2014); Clapa *et al*. (2011); Lepse and Laugale (2008); Najaf-Abadi and Hamidoghli (2009); Ružičand Lazić (2006) and Villa *et al*.(2006, 2009). In species *Rubus laciniatus*, cultivar 'Thornless Evergreen' BAP concentrations lower than 1mg/l were tested, without the use of auxins and very high multiplication rates were achieved, Fira *et al*. (2009, 2010).

Acclimatization is an important stage during *in vitro* plant propagation, because it deals with gradual transition from the artificial culture conditions to the natural living environment. In the acclimatization stage

it is necessary to ensure optimal culture conditions to obtain high survival rates. The usual methods of *in vivo* acclimatization use various types of containers filled with different types of potting mix (peat, perlite, soil, vermiculite individually or in optimized combination) as transplanting substrates as well as artificial culture areas (greenhouses, tunnels, growth rooms). For acclimatization, generally, plantlets rooted in vitro on media favorable for rooting are used, Arikan *et al* (2014); Clapa *et al* (2011; 2013); Fira *et al* (2009); Najaf-Abadi and Hamidoghli (2009) and Ružić and Lazić (2006). Moreover, in blackberry cultivars 'Thornless Evergreen' and 'Loch Ness' direct *ex vitro* rooting in hydroculture was achieved, by using tap water as rooting substrate Fira *et al*. (2010, 2011).

Direct *ex vitro* rooting gave results superior to *in vitro* rooting.in two experimental treatments: float hydroculture and floating perlite beds. Which ensured normal plantlet development, Fira *et al*. (2014) and Lepse and Laugale (2009).

The success of mass micropropagation of fruit trees may be reached by using plant tissues culture techniques, since this has showed efficient results on seedlings production with high quality and health. However, for the commercial viability of micropropagation application in the field of horticulture and how this might compete with traditional methods of propagation (cuttings, etc.), it is necessary to decrease production costs. In the present study, we tried to improve the micropropagation protocol for Triple Crown cultivar including, best condition, new culture media, growth regulators, new rooting and acclimation methods.

Materials and Methods:

Micropropagation of blackberry was achieved through the years 2011-2013 in the tissue culture laboratory at North Sinai Research station, Desert Research Center, Egypt. The blackberry cultivar to be studied is "Triple Crown" introduced jointly by the USDA-Beltsville, Maryland and the Pacific West Agricultural Research Service in 1996, the varietal name comes from its three major characteristics of flavor, productivity and vigor. This thornless, semi-erect blackberry cultivar will need 500-800 chill hours to attain its maximum level of fruiting productivity, Galletta *et al* (1998). Blackberry seedling plants were bought from Germany. Cuttings of the new flashes were collected from the grown plants and washed well with water. Annual shoots were used for the initiation of *in vitro* cultures, which were trimmed eliminating the internodes. The plant material was washed with tap water for one hour. For disinfection, explants were soaked at 30% and 40% (v/v) in Sodium hypochlorite (5.2 %) for 20 and 30 minutes duration to determine the best concentration of the disinfection reagent and then were washed 5 times with sterilized distilled water to remove Sodium hypochlorite traces from explants. The sterilized explants were then cultured on establishment medium (MS hormone free medium) to evaluate the survival percentage and to get an aseptic culture for the next steps of the micropropagation process.

Micropropagation process.

The aseptic explants were inoculated on modified Murashige and Skoog (MS) multiplication medium to determine the best media for vegetative growth. Different concentrations of growth hormones included BA (0.0, 0.5, 1.0 and 2.0 mg/l), 2ip (0.0, 0.5 and 1.0 mg/l) and NAA (0.0, 0.1, 0.5 and 1.0 mg/l) were tested. The growth hormones were added to the MS multiplication medium separately or in combinations.

The MS culture medium was prepared using macro- and microelements and vitamins according to Murashige and Skoog (1962). Sucrose (30.0 g/l) was added and the pH value adjusted at 5.7-5.8 using NaOH (0.1N) and HCl (0.1N) before distribution. The medium were distributed in 350 ml glass jars (50 ml medium / jar) with polypropylene caps and then were autoclaved at 121 C˚ for 20 minutes. Explants at 2 cm long micro cuttings (5 explants/jar) were used. Incubation was done in the growth room under artificial light provided by fluorescent tubes (120cm, 2400 Lux) for 16-hours photoperiod at the temperature of 24-26°C. The jars were positioned on only one level on the shelves. After 6 multiplication cycles of 2 months each, several characteristics were studied, i.e. multiplication rates/explant taking into consideration of the shoots and shoot fragments 2 cm in length.

Rooting of the multiplied plantlets:

The multiplied explants after that were divided into single or clusters of 2 or 3 shoots. Shoots 3-5 cm in length and cultured on Murashige and Skoog rooting medium full strength 1x, half strength (½ x) and one quarter (¼ x) of salt medium strength and supplemented with NAA at 0.5 mg/l and two IBA at 1.0 and 2.0 mg/l, the jars were incubated in the growth room until rooting.

Acclimatization:

The rooted plantlets were then transferred to hardening before acclimatization in green house. These plantlets were washed well with water to remove the gelling material from roots and then were treated with antifungal before transferring to culture bots *ex vitro* into a mixture of acid peat + perlite + sand 1:1:1 (as a volume) in plastic trays and moisture with water. The pH value of this mixture was adjusted with 1.0 N HCl or 1.0 N NaOH to 5.7 to 5.8. Plantlets were cultured in small boats and enveloped with polyethylene sacs and incubated in growth incubator with light for about 50 days and then transferred to greenhouse.

The experiment was set as a factorial experiment in a split plot design with five replicates. All obtained data was tabulated and statistically analysed according to Gomez and Gomez (1984) using the L.S.D test at 5% level for distinguishing the significance differences between various treatment means.

Results and Discussions:

The sterilized explants were cultured on MS medium and incubated in growth chamber to evaluate the survival percentage and their response to form shoots. Data in Table (1) and Figure (1) declared that the best survival percentage was 90 % when both shoot tips and stem segments were treated with 40% sodium hypochlorite for 30 minutes. Whereas, the highest formatted

shoots percentage of the cultured explants for shoot tip and stem segments were 100% and 87.5% due to use 40% sodium hypochlorite for 20 minutes, respectively.

Table (1): The effect of sterilization process on the survival percentage of blackberry shoots

Charact. / Treat.	Shoot tip %	Stem segment %	Formed shoots %	
			Shoot tip	Stem segment
Reagent				
30%	66.6	67.5	75.0	83.0
40%	75.0	85.0	94.4	84.9
F-test	*	*	*	N.S.
Reagent duration				
20 min	63.3	75.0	87.5	85.3
30 min	78.3	77.5	81.9	83.5
F-test	*	*	*	*
Reagent x duration				
30% x 20 min	66.6	70.0	75.0	83.0
30% x 30 min	66.6	65.0	75.0	84.6
40% x 20 min	60.0	80.0	100.0	87.5
40% x 30 min	90.0	90.0	88.8	82.3
L.S.D. 5%	6.45	3.22	8.74	4.21

Figure (1): Blackberry explant at the establishment stage.

Table (2) and Figure (2) showed the effect of cytokinins (BA and 2ip) on the multiplication of blackberry cv. 'Triple Crown' explants *in vitro*. Different concentrations of BA (0.0, 1.0 and 2.0 mg/l) were combined with 2ip at (0.0, 0.5 and 1.0 mg/l) to evaluate their enhancement for shoot proliferation. The best combination was 2.0 mg BA with 0.5 mg 2ip /l resulted in 7.78 shoot/ explant followed by the combination 1.0 mg BA with 0.5 mg 2ip /l which produced 7.11 shoot/ explant.

The highest shoot length was 16.2 cm followed by 8.05 cm and 6.86 cm as results of the treatments (2.0 mg/l BA), (2.0 mg BA+ 0.5 mg 2ip /l) and (2.0 mg BA+ 1.0 mg 2ip /l), respectively, while the untreated one (0.0 BA + 0.0 2ip, control) produced the lowest mean of shoot number and shoot length per explant.

Table (2): The effect of Benzyl adenine (BA) and Isopentenyl adenine (2ip) on the multiplication of blackberry shoot *in vitro*.

Treatment	Shoot number	Shoot length
BA (mg/l)		
0.0	2.94	2.22
1.0	5.10	5.09
2.0	6.09	10.37
L.S.D. 5%	0.27	0.33
2ip (mg/l)		
0.0	3.50	7.79
0.5	6.07	5.46
1.0	4.57	4.43
L.S.D. 5%	0.27	0.33
BA x 2ip		
0.0 x 0.0	2.50	1.84
0.0 x 0.5	3.33	2.50
0.0 x 1.0	3.00	2.33
1.0 x 0.0	3.00	5.33
1.0 x 0.5	7.11	5.84
1.0 x 1.0	5.20	4.11
2.0 x 0.0	5.00	16.20
2.0 x 0.5	7.78	8.05
2.0 x 1.0	5.50	6.86
L.S.D. 5%	0.47	0.57

Figure (2): The effects of BA and 2ip on multiplications of blackberry shoot *in vitro*.

These results were agreed with Lepse and Laugale (2008) and Fira *et al* (2010) and also Fira *et al* (2011) propagated blackberry using medium fortified with BA that produced the highest number of shoot per explant.

Data in Table (3) exhibits the effect of BA and NAA combinations on the multiplication and shoot length of blackberry *in vitro*. Regarding to the mean of shoot number/ explant, the highest mean value was 4.42 shoot/explant that was observed in the presence of 1.0 mg BA combined with 0.1 mg NAA, followed by 4.21 and 4.11 shoot/ explant resulted with (0.5 BA + 0.5 NAA mg/l) and (1.0 BA +0.5 NAA mg/l), respectively, while the lowest value was 1.71 shoot/explant resulted with 0.5 mg/l BA, free NAA medium.

Table (3): The effect of Benzyl adenine (BA) and Naphthalene acetic acid (NAA) on the multiplication of blackberry shoot *in vitro*.

Charact. / Treatment (mg/l)	Shoot/explant	Shoot length
BA		
0.5	2.46	4.76
1.0	3.56	5.56
F-test	*	*
NAA		
0.0	1.96	4.61
0.1	3.18	5.36
0.5	4.16	5.31
1.0	2.75	6.04
L.S.D. 5%	0.15	0.35
BA x NAA		
0.5 x 0.0	1.71	3.44
0.5 x 0.1	1.93	4.33
0.5 x 0.5	4.21	4.61
1.5 x 1.0	2.00	6.64
1.0 x 0.0	2.20	5.77
1.0 x 0.1	4.42	6.39
1.0 x 0.5	4.11	6.00
1.0 x 1.0	3.50	5.43
L.S.D. 5%	0.21	0.49

The highest shoot length was 6.64 cm/shoot which obtained on MS medium supplemented with 0.5 mg BA +1.0 mg NAA /l , followed by 6.39 cm and 6.0 cm in medium supplemented with (1.0 mg BA+0.1 mg NAA /l) and (1.0 mg BA+0.5 mg NAA /l), respectively.

Figure (3): The rooted plantlets before acclimatization process.

It is clear from data in table (3) that presence of NAA at 0.1 mg/l combined with 1.0 mg BA/l was more effective on the multiplication rate and the shoot length than the other concentrations. The multiplied shoot formed roots on these media after 60 days from culture.

The proliferated shoots were separated into singles or clusters of 2 or 3 shoots and then were cultured on rooting media through 45- 60 days of culture, as in Table (4). The rooting percentage was ranged from 43.75 to 87.50% and the full strength medium showed the highest response (75.00 & 87.50%). While the one quarter strength medium (68.75 & 68.75%) was more effective than the half strength medium that recorded (62.50 & 43.75%). The highest root number (5.0 roots/shoot) was observed in half strength and one quarter medium but the roots were thin while it was 3.75 & 4.38 roots/shoot and thick in full strength medium. Regarding to root length, the highest root length was 18.4 cm /root was noticed with full strength medium contained 0.5 NAA + 2.0 IBA mg /l. followed by 18.0 cm with 0.5 NAA+1.0 IBA mg /l.

Table (4): The effect of Indol 3-butyric acid (IBA) and Naphthalene acetic acid (NAA) on the rooting of blackberry shoot.

Charact. / Treatment)	Rooting %	No. of root/shoot	Root length (cm)
IBA + NAA (mg/L)			
1.0 + 0.5	68.75	3.98	15.20
2.0 + 0.5	66.67	4.79	14.38
F-test	N.S.	N.S.	N.S.
Media			
1 x	81.25	4.07	18.20
½ x	53.13	5.00	12.59
¼ x	68.75	4.09	13.59
L.S.D. 5%	3.38	0.32	0.93
IBA x media			
1.0 x 1 x	75.00	3.75	18.00
1.0 x ½ x	62.50	5.00	12.60
1.0 x ¼ x	68.75	3.18	15.00
2.0 x 1 x	87.50	4.38	18.40
2.0 x ½ x	43.75	5.00	12.57
2.0 x ¼ x	68.75	5.00	12.18
L.S.D. 5%	4.81	0.47	1.33

Despite the good effects of Naphthalene acetic acid and Indol-3-butyric acid combinations resulted in complete rooting of the cultured shoot of *Rubus fruticosus*, The full strength MS salts medium produced long roots with sub roots which were thick and involved in medium, while half strength and one-quarter strength of MS salts medium produced hairy roots and sub roots which were upper surface of medium and the rooting period was longer than it's in the full strength medium. Such results are

confirmed by early studies of Fira *et al*. (2010), Clapa *et al*. (2011) and Arikan *et al*. (2014).

Acclimatization in solid substrate was done in plastic trays with lids using a mixture of peat+ perlite+ sand in 1:1:1 (V: V: V). In this case, roots were hard and acclimatization percentage reached 80 %, as presented in Figure (4).

Figure (4): The acclimatization in peat+ perlite+ sand mixture.

Conclusion:

For the initiation and *in vitro* multiplication of blackberry Triple Crown cultivar we recommend the culture medium with the following components: Murashige & Skoog 1962 (MS) salts, and, as growth regulator, 6-benzylaminopurine at the concentration of 2.0 mg/l. The best rooting condition was reached by keeping shoots in full strength of MS medium enriched with 2.0 mg IBA+0.5 mg NAA /l

Blackberry cultivar 'Triple Crown' *Rubus fruticosus*, rooting *in vitro* was best in full strength medium in the presence of 0.5 mg NAA+2.0 mg IBA /l and acclimation can be carried out using, as plant material, rooted shoot resulted from the multiplication medium were cultured using a mixture of peat+ perlite+ sand in 1:1:1 as a volume. In this case, roots were hard and acclimation percentage reached 80 %.

Based on our observation, it can be safty concluded that blackberry cv. Triple Crown is appropriate for the ecological condition of Egypt especially in the North region. Further research should be done in Egypt to focus on releasing blackberry cultivars better adapted to our lower-chill climates.

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الإكثار المعملي للبلاك بيري (توت العليق)

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الملخص:

يعتبر Blackberry من فواكه المناطق المعتدلة التي تنجح في ظروف مصر المناخية. لذا يجب الاهتمام بطرق إكثارها. وتعتبر تقنية زراعة الأنسجة النباتية أحد أهم طرق الإنتاج الكمي لمثل هذه النباتات حيث تعطي نباتات متماثلة ذات خصائص جيدة. وتهدف هذه الدراسة إلي تحديد أفضل بروتوكول لإكثار Blackberry صنف Triple Crown حيث أجريت هذه الدراسة بمعمل زراعة الأنسجة بمحطة شمال سيناء البحثية – مركز بحوث الصحراء – مصر خلال الفترة من ٢٠١١ حتي ٢٠١٣.

وقد أوضحت النتائج:

- يعتبر التعقيم بمحلول ٤٠% هيبوكلورات الصوديوم لمدة ٣٠ دقيقة أفضل طرق التعقيم حيث وصلت النسبة المئوية للنبيتات الحية ٩٠%.
- أعطت بيئة موراشيجي وسكوج المزودة بمعدل ٢ مجم BA + ٠,٥ كجم 2ip/لتر أعلي معدل تفريع ٧,٨٧ فرع/نبات.
- أظهر إضافة NAA و BA إلي بيئة MS تحسن واضح لمعدل التفريع حيث سجلت ٤,٤٢ فرع / نبات.
- سجلت أفضل نسبة تجذير في البيئة كاملة القوة والمضاف إليها ٢ مجم IBA + ٠,٥مجم NAA/لتر حيث أعطت أطول الجذور وأسمكها.