MICORPROPAGATION OF *VIBURNUM OPULUS* (ROSEUM) BY USING SINGLE NODES

Suaad A. Yaseen
Assist. Prof.
Dept. Hortic. Coll. Agric. Engin. Sci, Duhok University, Kurdistan Region, Iraq.
Email: suaad.yaseen@uod.ac

ABSTRACT

The present study was carried out to investigate the micropropagation of *viburnum opulus* (Roseum) by using single nodes with axillary buds excised from soft cuttings using full strength MS salts, 30g.l^{-1} sucrose, 7g.l^{-1} Agar and different concentrations of plant growth regulators in culture medium. Results of the experiment at initiation stage revealed that the culture of single nodes of viburnum on a medium containing 4 mg.l^{-1} BA gave the highest number of shoots (2.21 shoots/explant). Concerning the interaction, a nutrient medium containing (2 mg.l^{-1} BA+ 0.4 mg.l^{-1} IAA) gave the highest values of mean number of shoots, leaves and length of new shoots (1.61 shoots/explant, 2.01 leaves/explant, 1.61 cm respectively). At shoot multiplication stage, the addition of 2.0 mg.l^{-1} from both BA and kinetin gave the highest number of shoots per explant estimated at 2.65 and 3.31 shoots/explant respectively at root formation stage; the interaction treatment of full MS salt strength with the use of 0 .5 mg.l^{-1} NAA gave the highest rooting percentage reaching 85.5 %. The highest number of roots (18 roots/ explant) was recorded as well from the combined treatment of full MS salt strength with the use of 0.5 mg.l^{-1} NAA. While the longest roots (6.65 cm) were recorded from the interaction treatment of half salt strength and 0.50 mg.l^{-1} IBA..

Key word: initiation stage, shoots, cytokinins, auxins, MS salts.

الاكثار الدقيق لشجيرة الطبة الثلج (*Viburnum Opulus*) باستخدام العقدة المفردة

سعاد عبدالحميد ياسين
استاذ مساعد
قسم البستة، كلية علوم الهندسة الزراعية، جامعة دهوك ،اقليم كوردستان/العراق

المستخلص

نفذت هذه التجربة في مختبر زراعة الانسجة النباتية– كلية علوم هندسة الزراعية جامعة دهوك للمدة من 2014 الى 2015 وذلك لاكثار شجيرة طبة الثلج خارج جسم كائن الحي باستعمال عقدة مفردة حاوية على براعم ابطية ووسط كامل القوة مجهز بتراكيز مختلفة من الاوكسينات والسايتوكاينينات. اظهرت النتائج في مرحلة النشوء ان اعلى معدل لعدد الفروع كان عند زراعة على وسط MS المجهز ب 5 ملغم/لتر BA. وتم الحصول على اعلى معدل لعدد الفروع والاوراق واطوال النموات عند الزراعة على وسط MS مزود ب 0.4 ملغم/لتر IAA. بالنسبة للتداخلات كان افضل هذه التداخلات في اعطاء نموات الخضرية هي الاوساط المجهز ب0.4 ملغم/لترIAA+ 2 ملغم/لترBA. حيث تم الحصول على اكبر معدل لعدد الفروع والاوراق واطوال النموات .اما بالنسبة لمرحلة التضاعف الخضرية فان اعلى معدل عدد الفروع واطوال النبات تم حصول عليه في وسط مجهز ب 2 ملغم/لتر من كلا BA و Kinetin اعطى اعلى عدد الفروع والي قدرت ب 3.33 , 2.66 فرع / جزء نباتي بالتتابع ز كان التداخل بين وسط MS كامل القوة مع اضافة 0.5 ملغم/لتر من NAA اعطى اعلى نسبة للجذور والتي وصل ل(85.5%). واعلى عدد الجذور (18 جذر/ جزء النباتى) حصلت عليه في وسطMS كامل القوة والمجهزبـ 0.5 ملغم/لترNAA.

الكلمات المفتاحية: افرع شجيرة طبة الثلج، السايتوكاينينات، الاوكسينات، وسط الاملاح.

Received:23/4/2021, Accepted:25/7/2021

INTRODUCTION

Viburnum opulus **(**Roseum) belongs to the family *Adoxaceae*, sometimes included in the monotypic family *Viburnaceae*, formerly also for the *Caprifoliaceae*. It is known as guelder rose, European guelder, European cranberry bush, water elder, rose elder, Rose Ebru, cherry-wood, crampbark, snowball tree, and as gilaburu in Turkey. Is a deciduous shrub is found in natural habitats in Europe, Russia, and some regions in North Africa and North Asia (23 and 3), covers more than over 70 shrubs species, and its growing to height 3-3.7 m (10-12 ft.) as a medium rate .it is hardy to zone and not frost tender, is grown as an ornamental plant for its flowers, and the more commonly type called the Snowball or RoseumTree, this cultivar has only sterile type of flowers that give the appearance of snowballs, there are no fruits to follow and sterile. The flowers bloom in late spring, and are pollinated by insects (9).The leaves, a fresh green color through the spring and summer, turn purple in the autumn. Viburnums are very popular landscaping shrubs uses as border, massing, screen, specimen, and hedge. They are so forgiving and adaptable as well as beautiful. Is a hardly vigor native shrub suited to growing in wood land garden or a shrub border (43 and 20). Viburnum shrub are usually propagated through several vegetative ways like softwood and semi-hardwood cuttings, And cant propagated by Sexual propagation because flowers are sterile and not produced seed. So it is propagating through vegetative and is the only traditional ways to propagate viburnum shrub. In propagation by cuttings only 20-30% of the cuttings are usually survive due to poor rooting (33). The number of woody plants that can be propagated using *in vitro* culture has increased significantly in the last few years and includes rhododendrons (6, 27), birch (30), mountain laurel (29), roses (12, 18 and 41) and lilac (19). *In vitro* techniques of micropropagation generally involve the culture of shoot tips or isolated buds on cytokinin rich media and the production of more shoots through the outgrowth of axillary meristems. These shoots can then be used either to produce more shoots, or induced to form adventitious roots *in vitro,* or rooted as softwood cuttings under mist. Propagation through cuttings is another option for getting true to type plants. The use of hardwood cuttings is one of the least expensive and easiest methods of vegetative propagation (17). As a result of the common use of classical vegetative multiplication methods to propagate *Viburnum opulus* (11 and 16), few macropropagation protocols are available. The most efficient procedures for initiation, multiplication, elongation, rooting, and acclimatization of these opulus varied with respect to medium composition, and other culture conditions (22, 34 and 40). The use of tissue culture techniques offers new prospects for fast multiplication of many plant species. Recently, studies on *in vitro* propagation of woody plants have found that these techniques may be suitable for rapid propagation of selected trees (21 and 22).Plant tissue culture technique has advantages of mass production, giving plantlets whenever needed. The usage of this technique is advantageous in production of qualified disease free plants, true to type plants production independent on seasonal and other environment conditions in a smaller space (13). This study was aimed to investigat development a reliable and successful *in vitro* culture protocol for *viburnum opulus* plants to overcome the conventional propagation of such kinds of shrub and developing a procedure for mass production for the micropropagated plantlets after testing various growth regulators at different concentrations.

MATERIALS AND METHODS

This experiment was carried out at the laboratory of plant tissue culture of Horticulture Department, College of Agriculture, University of Duhok between 2014 and 2015.

Plant Materials [Source of Explants and Explant Preparation]:

Explants were removed from actively growing shoot tips and node segments of *Viburnum. opulus* 'snowball' were collected from plants grown in the garden of the College of Agriculture at University of Duhok. Immediately after collection, explants including single nodes with axillary bud were washed under tap water for 1 hour, followed by tap water and liquid soap for 20 minutes, after washed with distilled water two times.

Then surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 10 min. explants then rinsed with distilled water three times.

In initiation stage: Explants were cultured on solidified MS culture medium with 30 $g.l^{-1}$ sucrose, agar (7$g.l^{-1}$) and its pH was adjusted 5.7+ 0.1 before autoclaving at 121 ºC for 20 min. on refreshing of the culture media, explants were cultured on Murashge and Skoog (32) medium supplemented with different concentration of :

1. BA (0, 2.0, 3.0 and 4.0) $mg.l^{-1}$
2. IAA (0, 0.2, 0.4 and 0.6) $mg.l^{-1}$
3. BA (0, 2.0, 3.0 and 4.0) $mg.l^{-1}$. In combination with IAA (0.0.2, 0.4 and 0.6 $mg.l^{-1}$.

Four explants were cultured in each jar (10 × 5 cm) containing 30 ml MS

Medium were placed, vertically. Each treatment was replicated 5 times. The jars were enclosed with autoclaved resistant polyethylene after culturing, which were tied with rubber band. For initiation stage, the data were recorded

1. Number of shoots per explant
2. Shoot length (cm).
3. Number of leaves per shoot

All the other parameters were taken after eight weeks interval.

Shoot multiplication stage: On the basis of stage I results, produced shoots from the treatments were moved to MS medium (multiplication stage medium) Explants were cultured on solidified MS culture medium with 30 $g.l^{-1}$ sucrose, agar (7 $g.l^{-1}$) and its pH was adjusted 5.7+ 0.1 before autoclaving at 121 ºC for 20 min. on refreshing of the culture media, explants were cultured on Murashge and Skoog (32) medium supplemented with different concentration of:

1. BA (0, 1.0, 2.0, 3.0 and 4.0) $mg.l^{-1}$
2. Kinetin (0, 2, 4, 6 and 8.0) $mg.l^{-1}$

Four explants were cultured in each jar (10 × 5 cm) containing 30 ml MS medium were placed, vertically. Each treatment was replicated 5 times. The jars were enclosed with autoclaved resistant polyethylene after culturing, which were tied with rubber band. For shoot multiplication, the data were recorded

1. Number of shoots per explant
2. Shoot length (cm).
3. Number of leaves per shoot

All the other parameters were taken after eight weeks interval.

Rooting Stage: For rooting, micro-shoots raised were harvested after 8 weeks and each shoot was transferred to a test tube containing 15 ml of (½ and ¼) strength MS media supplemented with different levels of NAA and IBA. NAA and IBA were used at 0, 0.5, 0.10, and 1.50 $mg.l^{-1}$ with the use of different MS salts concentrations including ¼, ½ and full strengths

All cultured tubes were incubated in 16 h daily light, of Philip white tubes florescent, with concentrated 1000 lux, and 25+1ºC temperature. Data were recorded for different rooting characterized including:

1. Root percentage
2. Number of roots per explants
3. Root length (cm).

Acclimatization Stage: After 6-8 weeks from *viburnum* shoots rooting, several plantlets were selected from those that formed good vegetative and seedy growth. They were washed under tap water to remove agar from the roots which might be a source of contamination. Care was taken to avoid cutting of any part of the roots during washing. They were then put in Benlate fungicide solution 0.1% and then planted in plastic pots filled with a sterilized mixture of peat moss and river soil (1:1). In order to maintain high humidity in the culture environment, the pots were covered with a light plastic cover which permits light passing and contains many openings to permit air circulation. Plants were watered and given a solution containing MS salts with 0.25 of it is original power. The plastic cover was removed periodically after two weeks from planting. After four weeks; the plastic cover was removed after the transplants being sprayed with Benlate fungicide (0.1%).

Statistical Analysis: The **e**xperiments were analysed using Complete Randomized Design (CRD) with 10 replicates. Significant differences among mean values were designated using Duncan multiple range tests at $P \leq 0.05$ (39).

RESULTS AND DISCUSSION

Initiation stage: Table 1 shows the effect of different concentrations of BA and IAA and

their interactions on the average number of shoots, average number of leaves and length of new shoots at initiation stage. Using high concentrations of BA (4.0 mg.l^{-1}) led to obtain the highest number of shoots (2.1 shoots/explant) (Figure 1). This may be attributed to cytokinins deficiency in lateral bud as compared with apical bud (42). Whereas concerning number of leaves and shoot length, the lowest concentration of BA (2.0 mg.l^{-1}) recorded the highest values (1.8 leaves/explant and 1.4 cm respectively). These results are in agreement with those found by Kyoichi *et al*. (26).Whereas the effect of IAA did not differ significantly from the other treatments concerning number of shoots and leaves, but concerning the shoot length, the concentration of IAA (0.4 mg.l^{-1}) gave the highest values (2.0 cm) as compared with the other concentrations (0.2 and 0.6 mg.l^{-1})IAA. Concerning the interaction between BA and IAA concentrations, it is clear that the treatment of 2 mg.l^{-1} BA+ 0.4 mg.l^{-1} . IAA and 4 mg.l^{-1} BA+ 0.6 mg.l^{-1} IAA gave the highest values of number of shoots (1.61 shoots/ explant). The treatment of 2 mg.l^{-1} BA + 0.4 mg.l^{-1} IAA gave the highest value of leaves number (2 leaves/explant) in which significantly differed from the control treatment. However control treatment gave the lowest response in which significantly differed with the concentration used. These result are in agreement with that had been found by Pontikis and Spoutzaki (36) and Pasqual and Audo (35) that using of cytokinins and auxin in this category is very important and the role of cytokinins at this stage is to help the meristems grow and form a vegetative shoot by balancing with auxins auto produced by the meristem.

Table 1. The effect of BA, IAA concentrations and their interactions on the average number of new shoots, number of leaves and length of new shoots at initiation stage

Treatments	mg.l^{-1}	Average number of shoot	Average number of leaves	Length of new shoots (cm)
Control		1.00 b	0.61 b	0.77 b
	2	1.80 ab	1.08 ab	1.40 ab
BA	3	1.80 ab	1.61 ab	1.00 b
	4	2.21 a	1.01 ab	1.23 ab
	0.2	1.41 ab	1.01 ab	1.00 b
IAA	0.4	1.75 ab	1.02 ab	2.00 a
	0.6	1.81 ab	1.01 ab	1.21 ab
	2+0.2	1.21 b	1.41 ab	1.41 ab
	2+0.4	1.61 ab	2.01 a	1.61 ab
	2+0.6	1.41 ab	1.41 ab	1.41 ab
	3+0.2	1.21 b	1.61 ab	1.21 ab
BA + IAA	3+0.4	1.22 b	1.21 ab	1.41 ab
	3+0.6	1.41 ab	1.21 ab	1.22 ab
	4+0.2	1.40 ab	1.00 ab	1.41 ab
	4+0.4	1.40 ab	0.81ab	1.40 ab
	4+0.6	1.60 ab	1.41 ab	1.60 ab

Different letters within each comparison represent significant differences according to Duncan´s multiple range test at 5% level.

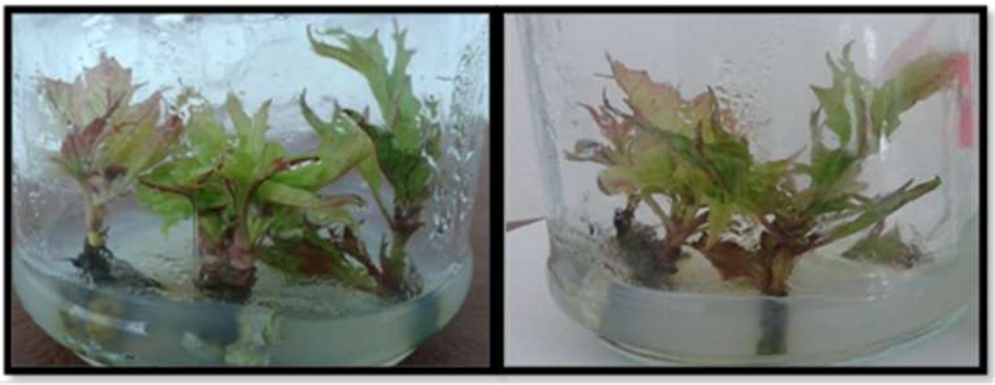

Figure (1): Shoots initiation of Viburnum on MS media supplemented with BA + IAA at different concentrations after eight weeks of culture.

Multiplication stage: Table 2 shows the effects of BA and Kinetin on shoot multiplication stage of viburnum shrub. It can be noticed that the addition of 2.0 mgl-1 gave the highest number of shoots per explant estimated at 2.65 and 3.31 shoots/ explant for both BA and Kinetin respectively. The addition of high BA and kinetin concentrations (4.0 mg.l^{-1} BA and 8.0 mg.l^{-1} kinetin) reduced the number of shoots per explant even when compared with the control by giving only 1.51 and 1.64 shoots per explant respectively. The addition of 1.0 m.gl^{-1} BA gave the longest shoots (3.52 cm). Whereas, the highest number of leaves (9.00 leaves/ explant) was achieved by the addition of 2.0 mg.l^{-1} kinetin which was the superior treatment significantly except when compared to 4.0 mg.l^{-1} which gave 8.0 leaves per explant (Figure 2). The reasons behind the positive role of cytokinins on the multiplication stage might be due to their profound role in releasing lateral buds from the dominance of terminal buds without the need to remove the apical bud by promoting formation of xylem tissues of buds which facilitate the transformation of water and nutrient leading to lateral bud growth (31). Nevertheless, the important role of cytokinins in the increasing the synthesis of RNA, protein and metabolic activity inside the cell which enhance bud growth as well (5). The effectiveness of BA was proved to be superior upon kinetin. MS medium containing BAP was satisfactory for several species and cultivars (7 ,21). On the other hand, Karim *et al*., (24) agreed the superiority of BA more than Kinetin in regeneration of shoots. Karim *et al*., (25) reported that between various concentrations used, better response towards shoot multiplication from nodal and shoot apex explant was obtained on MS medium containing 1 mg.l^{-1} BA. Also similar results were obtained by (4) they found that the shoots multiplicity on different concentrations of BA were higher compared to the once multiplicated on kinetin media.

Table 2. Effect of different BA and Kinetin concentrations on the shoot multiplication of Viburnum after eight weeks in culture

Control	**0**	**1.30 ef**	**2.70 a-c**	**5.66 cd**
BA	**1**	**2.64 a-c**	**3.52 a**	**7.31 ab**
	2	**2.65 a-c**	**2.52 bc**	**5 cd**
	3	**2.32 a-e**	**2.63 a-c**	**5.33 b-d**
	4	**1.51 d-f**	**2.74 a-c**	**4.50 cd**
Kinetin	**2**	**3.31 a**	**3.23 ab**	**9.00 a**
	4	**2.64 a-c**	**3.33 ab**	**8.00 a**
	6	**2.50 a-d**	**3.08 a-c**	**5.50 bc**
	8	**1.64 c-f**	**2.64 a-c**	**4.00 cd**

Different letters within each comparison represent significant differences according to Duncan´s multiple range test at 5% level.

Figure (2): Viburnum responded to cytokine at shoot multiplication stage after eight weeks in culture (A- BA at 1 mg.l^{-1} B- Kinetin 2 mg.l^{-1}).

Effect of different concentrations of NAA and IBA on rooting of viburnum shrub after eight weeks in culture: At rooting stage, two auxins including NAA and IBA were tested at 0,0.50, 1.00 and 1.50 mg.l^{-1} with the use of different MS salts strength quarter, half and full viburnum. Table 3 shows that the addition of NAA at 1 mg.l^{-1} gave the highest rooting percentage (44.87%) and the longest roots (4.00 cm), whereas the addition of 0.5 mg.l^{-1} NAA gave the highest number of roots (11.44 roots/explant). On the other hand, the longest roots (4.00 cm) were obtained when 1.0 mgl-1 IBA was added to the culture medium. Concerning the effect of MS salts strength, the highest rooting percentage (51.76%); the highest number of roots (9.71 roots/explant) and the longest roots (3.25 cm) were recorded with the use of full strength MS salts. These parameters were significantly different from the two other MS salt strengths except the number of roots which did not differ from the number recorded with half salt strength (8.24 roots/ explant). The interaction treatment of full MS salt strength with the use of 0.5 mg.l^{-1} NAA gave the highest rooting percentage (85.50 %) which was significantly different from the rest of treatments except the treatment of full strength salts with 1.00 mg.l^{-1}IBA which gave 72.00%. The highest number of roots (18 roots/ explant) was recorded as well from the combined treatment of full MS salt strength with the use of 0.50 mg.l^{-1} NAA (Figure 3). The longest roots were recorded from the interaction treatment of half salt strength and 0.50 mgl-1 IBA reached to 6.65 cm which was significantly different from the rest combined treatments except the treatments of full salts strength with 1.0 mg.l^{-1} IBA. Treatment with IBA has been previously recorded in by (14),who achieved the highest percentage of plant rooting in MS media supplemented with IBA. These results proved that auxins have a role in rooting process since they promote adventitious roots initiation in the bases of cultured shoots (1 and 38). The differences in the potency of IBA and NAA in promoting rooting might be attributed to the structure of the auxins under study, the endogenous hormone level, as well as the genetic makeup of species under consideration (15).The superior effect of full salts strength upon the lower strengths might be due to the high level of endogenous auxin levels in the plant material used in culture which might compensate the need of a higher C/N ratio. A number of research workers including Rout (37). Liu and Gao (28) mentioned that ½ MS medium fortified with auxins as the most optimum media for root initiation and development.

Table 3. Effect of different concentrations of NAA and IBA on rooting of viburnum shrub after eight weeks in culture

MS Salt strength	Auxins concentrations on mg.l^{-1}	Rooting Percentage (%)	Mean of MS Salts for Rooting Percentage	Number of Roots/Explant	Mean of MS Salts for Number of Roots/Explants	Mean Length of Roots (cm)	Mean of MS Salts strength for Length of Roots
¼ MS Salts	0	0 f	2.75 c	0 e	0.54 b	0 g	0.17 c
	NAA 0.5	24 e		5.23 c-e		1.43 fg	
	NAA 1.0	0 f		0 e		0 g	
	NAA 1.5	0 f		0.1 e		0 g	
	IBA 0.5	0 f		0 e		0 g	
	IBA 1.0	0 f		0 e		0 g	
	IBA 1.5	0 f		0 e		0.1 g	
½ MS Salts	0	0 f	38.85 b	0 e	8.24 a	0 g	2.24 b
	NAA 0.5	49 cd		15.32 ab		1.5 fg	
	NAA 1.0	37 d		5.5 cd		1.45 fg	
	NAA 1.5	48 cd		13.32 ab		2 ef	
	IBA 0.5	62 bc		14.23 ab		6.65 a	
	IBA 1.0	61 bc		3 de		2 ef	
	IBA 1.5	47 cd		13.65 ab		4.23 bc	
1/1 MS Salts	0	0 f	51.76 a	0 e	9.71 a	0 g	3.25 a
	NAA 0.5	85.5 a		18 a		2.83 d-f	
	NAA 1.0	49 cd		16.65 a		2.5 ef	
	NAA 1.5	50 cd		14.65 ab		3.26 c-e	
	IBA 0.5	60.5 bc		10 bc		5.5 ab	
	IBA 1.0	72 ab		7.15 cd		4.43 bc	
	IBA 1.5	61 bc		6.74 cd		3.94 cd	

Mean of Auxins

Auxins Concentration mg.l^{-1}		Rooting Percentage (%)	Number of Roots/Explant	Mean Length of Roots (cm)
Control	0	0 d	0 e	0 f
NAA	0.5	41.65 ab	11.45 a	1.93 c-e
	1	29.16 c	8.75 ab	2.66 bc
	1.5	33.34 bc	7.53 b	1.30 e
IBA	0.5	41.65 ab	2.33 de	1.74 de
	1	44.87 a	8.5 ab	4.00 a
	1.5	37.65 a-c	5.88 cd	2.76 b

Different letters within each comparison represent significant differences according to Duncan´s multiple range test at 5% level.

Hardening: the rooted plantlet were cautiously removed and transferred to presoaked vermiculite for hardening initially inside a rectangular plastic box, maintained for 15 days in the culture room and then transferred to the mist chamber for further establishment .Castillo *et al*., (10) used peat-moss vermiculite for hardening and obtained satisfactory acclimatization. Good establishment of plants were seen after one week in the mist chamber.

Cconclusion, this investigation provides an optimize and reliable micropropagation protocol for in vitro mass production of viburnum opulus and can be used for reproduction and conservation. It is highly recommended to test more effective factors on *viburnum opulus* moicropropagation like use of 2iP, TDZ or other growth regulators at different concentrations and combinations.

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