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Micropropagation and Biomass Production of *Rubus fruticosus* L. (Blackberry) plant

Hwida M. Fathy[1], Omneya F. Abou El-Leel[2] and Mona A. Amin[1]

[1]*Timber Trees Research Department Horticulture Research Institute (HRI), Agricultural Research Centre (ARC), Giza, Egypt.*
[2]*Medicinal and Aromatic Plants Research Department, Horticulture Research Institute (HRI), Agricultural Research Centre (ARC), Giza, Egypt.*

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ABSTRACT

Blackberry is considered one of the most industrial plants, while a large amount of its fruits is sold as fresh another has multiplied biomass production, such as flavonoids, vitamin C, polyphenolic and antioxidants which inter in medicinal and food processes. Therefore, this investigation has been carried out to study the micropropagation of *Rubus fruticosus* shootlets, induction and growth of callus and enhancement of previous bioproduct content in both of shootlets and calli. The results revealed that free MS medium gave the highest significant values in survival (91.67%), shootlet numbers (1.83), leaves numbers/explant (7.33) and shootlet length (31.67mm) in the establishment stage compared with WPM or B5 media. Multiplication medium provide with BA at 0.6 mg/l recorded the highest survival (100%) and greatest number of shootlet /explant (3.4) after the first subculture compared to other treatments. Adding activated charcoal at 2g/l to quarter strength of MS or the culture on half MS strength gave the maximum rooting (100%). In acclimatization stage, transplanting the plantlets in green house on mixture soil of peatmoss only or of (1peatmoss:1sand /v/v) was the most suitable for raising the survival of plantlets to (100%). For callus induction, the increase in concentration of 2, 4-D from 0.5 to 1.0 mg/l on callus medium plus 0.5 mg/l NAA raised the rate of callus formation by (100%). The combination between 0.5mg/l NAA and 1.0mg/l 2, 4-D gave the highest value of callus fresh weight across three subcultures. The highest content of bioactive compounds obtained from shoots represented as chlorophyll a, chlorophyll b, total carotenoids, total phenols and total antioxidant (112.26, 46.57, 127.72, 239.45 and 948.61 mg/100g), respectively were recorded as result of using 0.4 mg/l BA. While, using 0.2mg/l BA in the culture medium resulted the highest amount of total flavonoids and vitamin C (28.50 and 222.30 mg/100g FW), respectively.

***Keywords*:** Rubus –Micropropagation- callus –flavonoids-antioxidant

Introduction

Rubus fruticosus L. (Rosaceae) is a shrub famous for its fruits, which is called blackberry, that is traded globally due to its delicious taste, pleasant flavor and nutritional profile. The shrub is believed to have its origin in Armenia, and is now distributed throughout Europe, Asia, Oceania and North and South America (Hummer and Janick, 2007). It grows wild in the Northern areas of Pakistan, where it is known by local names *Baganrra* (Murad *et al.,* 2011). The *Rosacea* family is the19th largest family of plants. The genus *Rubus*, with almost 700 species, is the largest genus of this family. *Rubus* comprises 12 subgenera, with few domesticated species (Marulanda *et al.,* 2007).

Blackberry bushes can prevent soil erosion from infertile, disturbed sites. The ancient Britons used thorny stems as a boundary or barrier in the way modern people use barbed wire (Allen and Hackney, 2010). A purple to dull blue dye is obtained from the fruit (Huxley, 1992). A fiber is obtained from the stem and used to make twine (Freethy, 1985).

The fruit has medicinal, cosmetic and nutritive values. It is a concentrated source of valuable nutrients, as well as bioactive constituents of therapeutic interest highlighting its importance as a functional food (Eyduran *et al*., 2008). It is also used as ingredients in cooked dishes, salads and bakery products like jams, snacks, dessert, and fruit. *Rubus fruticosus* contains vitamins, steroids and lipids in seed oil (Radocaj *et al*., 2014) and minerals. Berry fruits are rich in phenolic compounds such as phenolic acids, anthocyanins, flavonols, ellagitannins, gallotannins and proanthocyanidins (Reyes-

Corresponding Author: Hwida M. Fathy, Timber Trees Research Department Horticulture Research Institute (HRI), Agricultural Research Centre (ARC), Giza, Egypt.
E-mail: fhwida@hotmail.com

Carmona *et al.,*2005), which demonstrated considerable antioxidant properties. Flavonoids and phenolic compounds in the berries are anti-carcinogens and have antineurodegenerative and anti-inflammatory effect (Muhammad *et al*., 2014). Successful micropropagation protocols were already developed for different *Rubus* species, planting blackberry material of cultivars such as Marion, Black Satin, Thornless Evergreen, Loch Ness and Cacanska bestrna being produced on large scale using in vitro propagation. In most of the cases, high quantities of new blackberry plants can be produced easily, in a short period of time (Ruzic and Lazic, 2006). Callus induction and plant regeneration from adventitious buds can be easily obtained from several species, such as sugar beet (Zhang *et al*., 2004). Lower regeneration rates were observed in woody tree species (Caboni *et al*., 1999). Recent studies have focused on the identification of the phenolic components of *Rubus* leaves as well as determination of their antioxidant activity (Dall'Acqua *et al*., 2008), these compounds have been confirmed with significant inhibitory activities on oxidants and bacteria, suggesting potential in food protection (Barbieri *et al*., 2017; Oz and Kafkas, 2017). Flavonoids are polyphenolic compounds that constitute a large group of secondary plant metabolites.

The aim of this study is to obtain a reliable micropropagation protocol in order to achieve a fast multiplication rate and high amounts of qualitative planting material and its secondary products. A callus induction protocol was also aimed for further breeding purposes based on somaclonal variability.

Materials and Methods

These investigations were executed during the period of 2016 to 2018 on *Rubus fruticosus* L. at Tissue Culture Technique and Biotechnology Laboratories , Horticulture Research Institute, Agricultural Research Centre- Giza to examine the effect of some factors affecting the behaviors of in vitro consecutive micropropagation stages including those of culture establishment, shootlets multiplication, rooting ,acclimatization stage , callus establishment ,callus growth and some biomass production of in vitro shootlets and callogenesis .

1-Explants source:

The source of the explant was kindly obtained from Plant Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt. The starting explants used were nodal segments of approximately 1.0 cm length, containing two axillary buds.

2- Culture establishment stage.

In this stage nodal explants were cultured on three types of media MS- basal medium (Murashige and Skoog, 1962), WPM (Llyod and McCown, 1980) and B5 (Gamborg, 1986) without any growth regulators used. Cultured explants were incubated for 4 weeks under controlled conditions. Explant survival %, number of shootlets, shootlet length (mm) and number of leaves per explant were calculated .

3-Shootlets multiplication stage.

The experiment of this stage was designed to study the effect of different concentrations of Benzyl adenine (BA) at 0.2 , 0.4 and 0.6 mg/l separately or combined with Naphthalene acetic acid (NAA) at 0.2mg/l on shootlets multiplication through three successive subcultures. The experiment included 7 treatments in order to induce shootlets multiplication with 4weeks intervals. Cultures were subjected to three sequential subcultures with 4weeks intervals. Data of each subculture (shootlets survival percentage, number of shootlets /explant, shootlets length (mm) and number of leaves formed per shootlet) were recorded.

4- Rooting stage.

In this stage, a trial was conducted to study the influences of various MS medium strengths (1/4,1/2 and full strength) supplemented with 0.4mg/l Indol butyric acid (IBA) plus 2g/l activated charcoal on rooting behavior of grown shootlets. In vitro produced shootlets from multiplication stage were individually separated and incubated for 6 weeks. After that rooting %, number of initiated roots and length of the formed roots were recorded.

Culture media and incubation conditions.

During all in vitro stages, five replicates were considered, each replicate comprised 5 explants. Media employed in these investigations were supplemented with 25g/l sucrose and solidified by the addition of 0.7% agar, prior to autoclaving at 1.2Kg/cm² for 15 min. The PH of the culture medium was adjusted to 5.7± 0.05. Medium was dispended into 200ml ca. glass jars each contained 35 ml of medium and were incubated under controlled conditions in the growth chamber temperature at 24± 2°C. The photoperiod was 16 h light/8 h darkness, controlled automatically and provided by 3klux light intensity from cool fluorescent lamps (120cm long,40 watts).

5- Ex vitro acclimatization stage.

An experiment was applied to study the effect of five different soil mixtures on ex vitro produced shoots survival ability at green house. The mixture was peat or sand or clay or (1 peat :1 sand, v/v) and (1peat:1clay, v/v). Plantlets (10±2cm) produced from in vitro rooting stage were washed from ager and transferred into plastic pots (0.2 liter) containing soil mixture after saturation with 0.2% Topsin-M70 fungicide in 10 replicates each replicate consisted of 6 plantlets. The culture pots were covered by transparent polyethylene bags and maintained under green house. After two weeks from culture, one pore per polyethylene bag was performed, and after another two weeks the bags were gradually removed. Acclimatized plantlets were water irrigated twice a week for four weeks before transplanting outdoor. At the end of this experiment survival percentage were recorded.

6- Callus establishment and formation.

When in vitro shoots reached 6-8 cm long after four weeks of culture the leaves were cut under sterile conditions in laminar air- flow cabinet at about 4-5mm and cultured on MS-medium supplemented with different concentration of naphthalene acetic acid (NAA) and/or 2,4-dichlorophenoxyacetic acid (2,4-D) were used at nine combination as follows:

Free MS(control), MS provided with (0.5 or 1.0 mg/l NAA) ,(0.5 or 1.0 mg/l 2,4-D), (0.5mg/l NAA +0.5 mg/l 2, 4-D), (0.5mg /l NAA +1.0mg/l 2, 4-D), (1.0 mg /l NAA +0.5mg/l 2, 4-D) and (1.0 mg/l NAA +1.0 mg/l 2, 4-D) .

Leaves explants were cultured under aseptic conditions in 100 ml jars containing 25ml of solidified basal MS-medium with the previous supplements. Callus was formed after 28 days, and maintained by sub culturing every four weeks on the best callus formation medium to obtain a large amount of stock calli for following experiments. Callus induction (%) was calculated as the number of callus induced divided by the number of cultured explant as follows:

$$\text{Callus induction \%} = \frac{\text{Number of callus induced}}{\text{Number of cultured explants}} \text{ X } 100$$

The fresh weight of callus was determined after each subculture in order to calculate the growth rate.

Growth dynamics:

For characterizing the growth of the obtained calli, average of fresh weight (g/jar) of calli obtained from leaves explant were determined. Growth rate of calli was based on fresh weight changes over 3 months. Growth rate (fresh weight callus) of blackberry cultures across three subcultures were determined as following:

$$\text{GR(g/jar)} = \frac{\text{Fwf} - \text{Fwi}}{\text{Fwi}}$$

Where, GR = Growth rate (g/jar), Fwf= Final fresh weight, Fwi= Initial fresh weight.

7. Chemical analysis of fresh callus and shoots:

Sample preparation and extraction

For methanolic extract, the extraction process was carried out by grounding (2 g) green leaves or calli in a pestle with 20 ml of 80% methanol. The homogenate was filtered to obtain methanolic

extraction colorless.

Spectrophotometric measurements

The spectrophotometric measurements were performed by using an ultraviolet-visible spectrophotometer (model MA9523-SPEKOL 211, ISKRA, Horjul, Slovenia).

a-Total phenols

The total phenolics content of methanolic extract was determined according to the method described by (Singleton *et al*., 1999**)** by folin-ciocalteu reagent. The absorbance was recorded at 725nm.

b-Total flavonoids

Total flavonoids were estimated by using the method of (Woisky and Salation, 1998**)** using Aluminum chloride, the absorbance was measured at 420 nm.

c-Total antioxidant capacity

The total antioxidant capacity of the extracts was evaluated by the phosphomolybdenum method described by (Prieto *et al*., 1999). The absorbance of the solution was measured at 695 nm with a spectrophotometer against methanol as the blank. Ascorbic acid (AA) was used as the standard.

d- Ascorbic acid content (vitamin c)

Ascorbic acid was determined according to the method of (Klein and Perry, 1982). The absorbance was recorded at 515nm.

e- Plant pigments content (Chlorophylls (a, b) and carotenoids) determination

The protocol devised by Nagata and Yamashta, (1992) was followed to determine chlorophyll a, b and total carotenoids contents. Blackberry leaves or calli sample (0.2 g) were ground in 10 mL of 80% acetone and filtered through Whatman No. 1 filter papers. The filtered extract was transferred in a cuvette and absorbance was noted at 663, 645, 505 and 453 nm by using UV-spectrophotometer.
The following formulae were used to calculate chlorophyll a, chlorophyll b and total carotenoids contents.
Chlorophyll a = 0.999 A663 – 0.0989 A645
Chlorophyll b = -0.328 A663 + 1.77 A645
Carotenoids = (0.216 A663 – 1.22 A645) – (0.304 A505 + 0.452 A453).

Statistical analysis

The lay–out of the experiment was statistically analyzed assuming a complete randomized design. A comparison among the means was done according to LSD multiple range test at 5%level according to Steel and Torrie (1980).

Results and Discussion

Culture establishment stage.

Data presented in Table (1) demonstrated the effect of three types of medium on establishment of *Rubus fruticosus* L. explants. The highest significant value of explant survival (91.67%) was achieved when explants cultured on either MS or WPM media. The greatest number (1.83)of shootlets formed per explant ,the longest shootlest (31.67) mm and the highest number of leaves

(7.33/shootlets) were recorded when explant were cultured on MS (Fig.1) comparing with the other two media under investigation. However, te lowest significant value of survival (33.33%), number of shootlets formed per explant (1.0), the lowest length of shootlets (8.30mm) and leaves formed per shootlets (3.33) were recorded by using B5 medium.

These results confirmed with those obtained by Benamar (2017) on *Pistacia vera* who stated that the most suitable medium for culture initiation was MS medium and Darwesh *et al*. (2017) on *Khaya senegalensis* who, indicated that culturing of explant on MS medium increased shootlets proliferation rate higher than the culture on WPM and B5 medium.

Table 1: Effect of different medium type (MS, WPM and B5) on establishment proliferation of *Rubus fruticosus* L. explants after four weeks of culture.

Parameters / Medium type	Survival % of explants	Number of shootletes /explant	Shootlets length(mm)	Leaves number /explant
MS	91.67	1.83	31.67	7.33
WPM	91.67	1.00	8.33	3.33
B5	33.33	1.00	8.30	3.33
LSD	14.59	0.29	9.39	1.75

Fig. 1: Effect of MS medium on establishment of *Rubus fruticosus* L. explants after four weeks of culture.

Multiplication capability

The tabulated data in Table (2) and Fig. (2) indicate to the in vitro shootlets behavior during multiplication stage of *Rubus sp*. under different BA concentrations (0.0,0.2,0.4and 0.6mg/l) each alone or combined with 0.2mg/l NAA for three repeated subculture. Results revealed the promoting effect of supplementing MS medium with BA (0.2, 0.4 and 0.6mg/l) recorded the highest significant values (83.3, 97.0 and 91.4%) of shootlets survival, respectively compared with (52.7%)for control treatment. However, providing culture medium with NAA at (0.2mg/l) caused the lowest value (47.2%) of shootlets survival when combined with BA at (0.2 or 0.6 mg/l). Increasing the number of subcultures to three times gave raising in survival from (65.4, 66.4 to 73.7%), respectively, however with no significant difference. The interaction between growth regulators treatments and rebated subculture recorded the maximum significant value (100%) of explant survival on the first subculture when cultured media was provided with 0.4 or 0.6mg/l BA only, and after the third subcultures at 0.4mg/l BA.

The result of shootlets number formed /explant behaved as the same trend as shown in Table (2). The highest shootlets number (2.3 and 2.2 /explant) were recorded due to supplement of BA at 0.4 and 0.6mg/l only compared to (1.0 shootlets/explant) for control treatment. Raising subculture number from one to three, resulted insignificant decreases on number of new formed shootlets from (1.8,1.5 to1.3 shootlets/explant), respectively. Also culture medium provided with BA at 0.6 mg/l gave the highest significant value (3.4 shootlet/explant) after the first subculture compared with (1.0 shootlets/explant) for control treatment.

On the other hand, control treatment gave the longest shootlets (22.2mm), the third subculture resulted in longer shootlets (17.9 mm) over the first and second subculture. Moreover, the longest shootlet (33.3 mm) were recorded as well as due to control treatment after the first subculture. Besides

the greatest number (8.7) of leaves /shootlets were formed when medium was treated with BA at 0.6 mg/l plus 0.2mg/l NAA after the first subculture.

Supporting results were obtained by, Parasharami *et al.,* (2003) on *Alnus nepalensis,* Gad (2011) on *Populus alba* and Darwesh *et al.,*(2017) on *Khaya senegalensis,* as they stated the positive effect of BA supplemented multiplication medium on shootlets proliferation characteristics .This positive result could be explained on basis of the assumption that the externally applied cytokinin (BA) changed the internal hormonal balance that might induce the highest cell differentiation into vegetative organ. George and Sherrington (1984) concluded that cytokinins have been shown to activate RNA synthesis and to stimulate protein synthesis and enzyme activity.

Table 2: Effect of different concentrations of BA and NAA on shootlets multiplication capability of *Rubus fruticosus* L. after three subculture.

Parameters / Treatments	Survival%				Shootlets number /explant			
	SUB 1	SUB 2	SUB 3	Mean (A) Treatments	SUB 1	SUB 2	SUB 3	Mean(A) Treatments
Control	41.7	50.0	66.0	52.7	1.0	1.0	1.0	1.0
0.2mg/l BA	75.0	83.3	91.7	83.3	1.5	1.5	1.4	1.5
0.4 mg/l BA	100.0	91.0	100.0	97.0	3.2	1.8	1.8	2.3
0.6 mg/l BA	100.0	91.0	83.3	91.4	3.4	2.2	1.0	2.2
0.2mg/l BA + 0.2mg/l NAA	41.7	50.0	50.0	47.2	1.2	1.1	1.0	1.1
0.4mg/l BA + 0.2mg/l NAA	58.0	58.0	66.7	60.9	1.4	1.4	1.4	1.4
0.6mg/l BA + 0.2mg/l NAA	41.7	41.7	58.3	47.2	1.2	1.2	1.2	1.2
Mean subculture (B)	65.4	66.4	73.7		1.8	1.5	1.3	
LSD(0.5)	A=18.5	B=NS	AB=31.9		A=0.3	B=0.2	AB=0.5	
Parameters	**Shootlets length (mm)**				**Number of leaves /explant**			
Control	33.3	16.0	17.3	22.2	8.3	7.7	7.3	7.8
0.2mg/l BA	9.6	14.7	27.0	17.1	6.3	6.3	7.7	6.8
0.4 mg/l BA	14.1	12.7	11.4	12.7	6.0	5.0	6.3	5.8
0.6 mg/l BA	16.7	16.0	20.0	17.6	7.7	7.3	7.7	7.6
0.2mg/l BA + 0.2mg/l NAA	11.5	11.7	20.0	14.4	6.7	6.7	5.3	6.2
0.4mg/l BA + 0.2mg/l NAA	15.3	16.4	16.7	16.1	7.7	6.3	6.3	6.8
0.6mg/l BA + 0.2mg/l NAA	16.7	12.5	12.9	14.0	8.7	7.7	6.7	7.7
Mean subculture (B)	16.7	14.3	17.9		7.3	6.7	6.8	
LSD (0.5)	A=3.6	B=2.3	AB=6.2		A=1.1	B=NS	AB=1.9	

*SUB. = subculture.

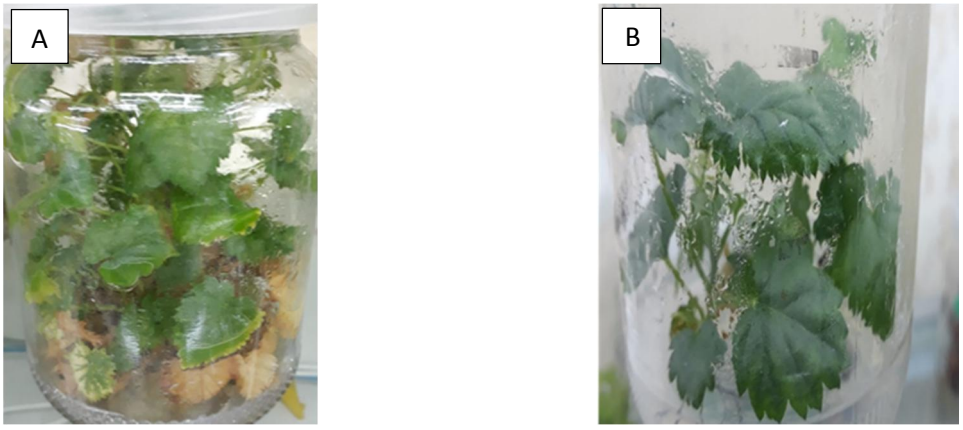

Fig. 2: Multiplied shootlets of *Rubus fruticosus* L. at the first (A) and the third (B) subculture during multiplication stage.

Rooting stage.

Data presented in Table (3) demonstrated the effect of different MS medium strength (quarter,half and full)without or with (2g/l) activated charcoal (AC) on survival % of shootlets, shootlet length(mm), leaves number /shootlet, rooting %, number of root/shootlet and root length (mm).

Generally, results revealed that provided rooting medium with AC raised shootlets survival%, shootlets length, rooting% and root length recorded (68.7%, 24.0 mm, 85.1% and 42.9 mm) compared

to the absence of AC. On the other hand, the highest number of leaves (9.8) and the greatest number of roots (1.8)/ shootlet were produced when the medium was AC free.

Concerning the interaction between strength medium and the presence of AC, it is quite clear from data that quarter strength of MS medium with 2g/l AC gave the maximum survival of shootlets (100%), the longest shootlets (31.7mm) and the maximum rooting percent (100%) as well as half strength of MS medium without AC. The greatest number of leaves formed /shootlet (12.0) and the longest roots (53.3mm) were obtained due to half MS medium strength without AC, while the greatest root number (2.5) /shootlet formed on quarter strength MS medium without AC.

The results are in harmony with, Alexandru *et al*., (2011) on Blackberry cultivar'' Loch Ness'' and Nitishkumer and Reddy (2011), Darwesh *et al*.,(2017) on *Khaya sp*. They mentioned that the reduction in the salt strength of MS medium has enhanced rooting behavior, since high concentration of salts may inhibit root growth, even it the presence of auxins in culture media. Also, supporting results in the promoting effect of activated charcoal on rooting behavior were stated by Somika *et al.,*(2002) on *Morus indica* , Gad (2011) on *Populus alba* and Sami *et al.,*(2016) on *Hibiscus syriacus*, since illustrated that the improvement effect of AC on rooting morphogenesis perhaps due to, irreversible adsorption of in useful compounds, darkening and aeration of culture medium and gradual raise of substances naturally present in AC, then they become available to plant.

Table 3: Effect of different MS-medium salt strength and activated charcoal (AC) at 2g/l on rooting behaviors of *Rubus fruticosus* L. in vitro cultured.

	Shootlets Survival %				**Shootlets length (mm)**				**Leaves number/explant**			
Medium Strength	**Quarter**	**Half**	**Full**	**Mean AC**	**Quarter**	**Half**	**Full**	**Mean AC**	**Quarter**	**Half**	**Full**	**Mean AC**
MS	20.0 c	40.0bc	41.7bc	33.9B	22.3ab	28.3a	17.3ab	22.6A	9.0 bc	12.0a	8.3 bc	9.8A
MS+2g/l AC	100.0 a	46.0bc	60.0b	68.7A	31.7a	26.7ab	14.3b	24.0A	9.7 ab	10.3ab	7.0 c	9.0A
Mean Strength	60.0 A	43.3A	50.9A		27.5A	27.5A	15.8A		9.3 B	11.2A	7.7 C	
	Rooting %				**Numbers of roots/shootlet**				**Root length (mm)**			
MS	60.0 b	100a	33.3c	64.4B	2.5a	1.3 b	1.7ab	1.8 A	26.7a	53.3a	43.3a	41.1A
MS+2g/l AC	100.0a	77.8b	77.8b	85.1A	2.0ab	1.3 b	1.0 b	1.4 A	46.9 a	35.0a	46.7 a	42.9A
Mean Strength	80.0A	88.9A	55.8B		2.3 A	1.3 B	1.3 B		36.7 A	44.2A	45.0A	

*Digits followed by the same letter are not significantly different. **AC.= activated charcoal

Ex vitro acclimatization stage.

The effect of five soil mixtures on survival percentage of *Rubus fruticosus* plantlets of ex vitro acclimatization stage are shown in Figs. (3) and (4, A, B and C)). The data indicated that the maximum value for plant survival percent after the end of acclimatization period (100%) recorded in either the mixture of peat moss only or peat moss combined with sand at (1:1) v/v. On contrast, the lowest survival value (20 %) was produced by using sand soil only. Plantlet formed fruit after two years at this stage (Fig.4 C).

These results are agreement with those observed by Chan *et al., (*2009) on *Gynura procumbens*, Taha *et al., (*2008) on *Paulownia kowakamii* and Gad (2011) on *poupuls alba* .They found that the acclimatization mixture of peat moss and sand with a ratio of (1:1) might had improving effect on acclimatization of plantlets growth behavior especially survival percent ,while the sand has high penetration effect and good aeration for root, peat moss has both high water and nutrient holding characteristics.

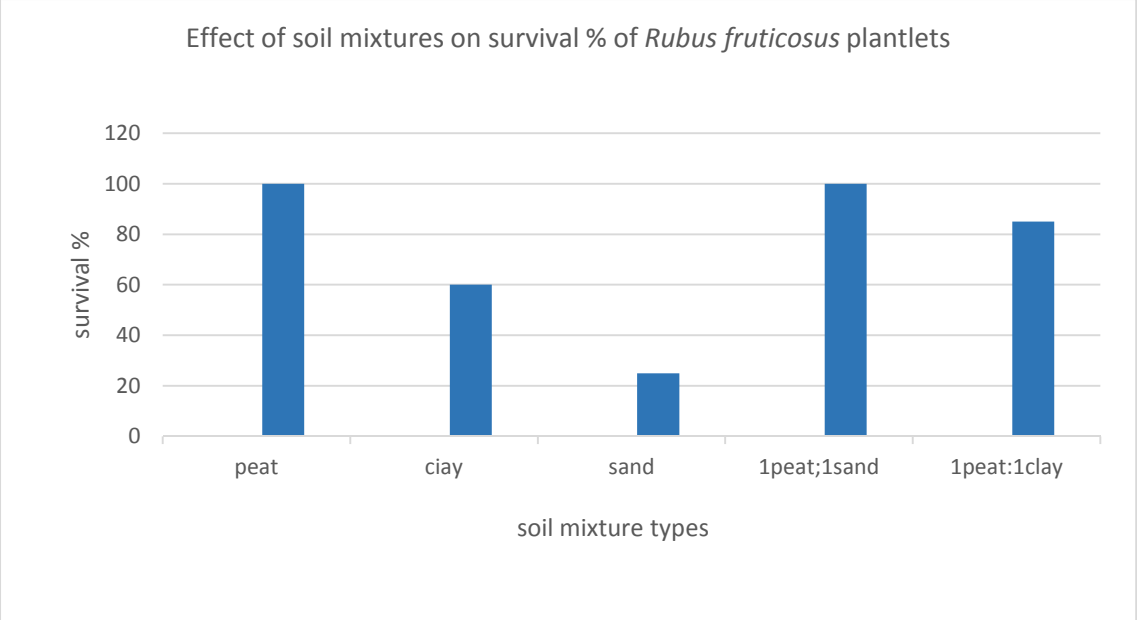

Fig. 3: Effect of soil mixtures on survival % of *Rubus fruticosus* plantlet during acclimatization stage.

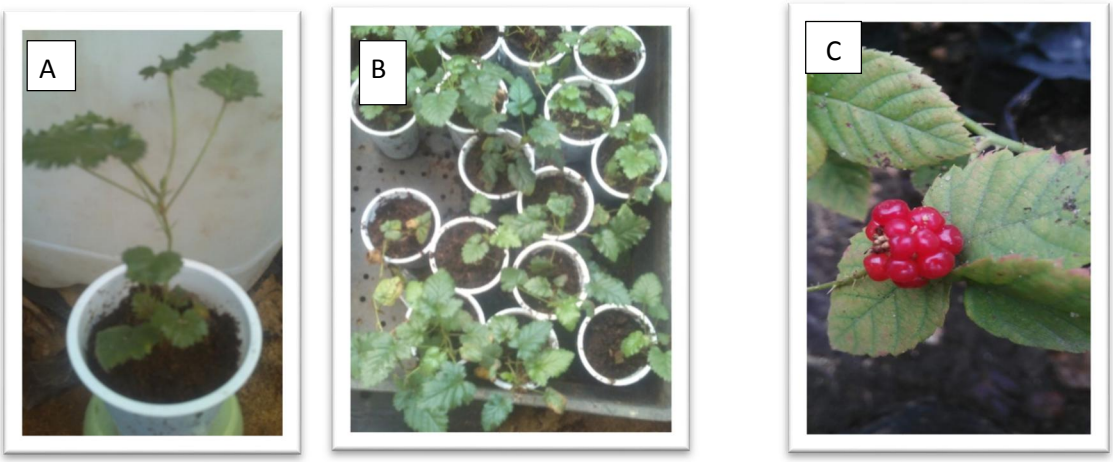

Fig. 4 : Acclimatization of *Rubus fruticosus* L. Plantlets on mixture of peat (A) and (1peat:1sand) (B) at ex vitro stage. (C) plantlet formed a fruit after two years of acclimatization

Callus induction

Data represented in Fig. (5) indicated that no callus formation was observed on control medium. Callus formation (%) from leaves after 28 days of culture (100%) due to 2,4-D supplement at any concentration (0.5 or1.0) mg/l, in presence or absence of NAA. Medium containing NAA (0.5 or 1.0) mg/l only showed lower callus formation (96%).

Appearance and quantity of the formed calli depended on the type and concentration of applied auxins(Fig.7). Namely, calli were dark green and firm on medium containing NAA. Adding 2,4-D into regeneration medium brought about the formation of loose, yellow callus mass reached to light yellow at (1.0 mg/L 2, 4-D alone). The data revealed that large amount of proliferating callus was on the medium with 2, 4-D, this result is on line with (Afify, *et al*., 2011) in fennel and (Saranga and Janick, 1991) on celery**.**

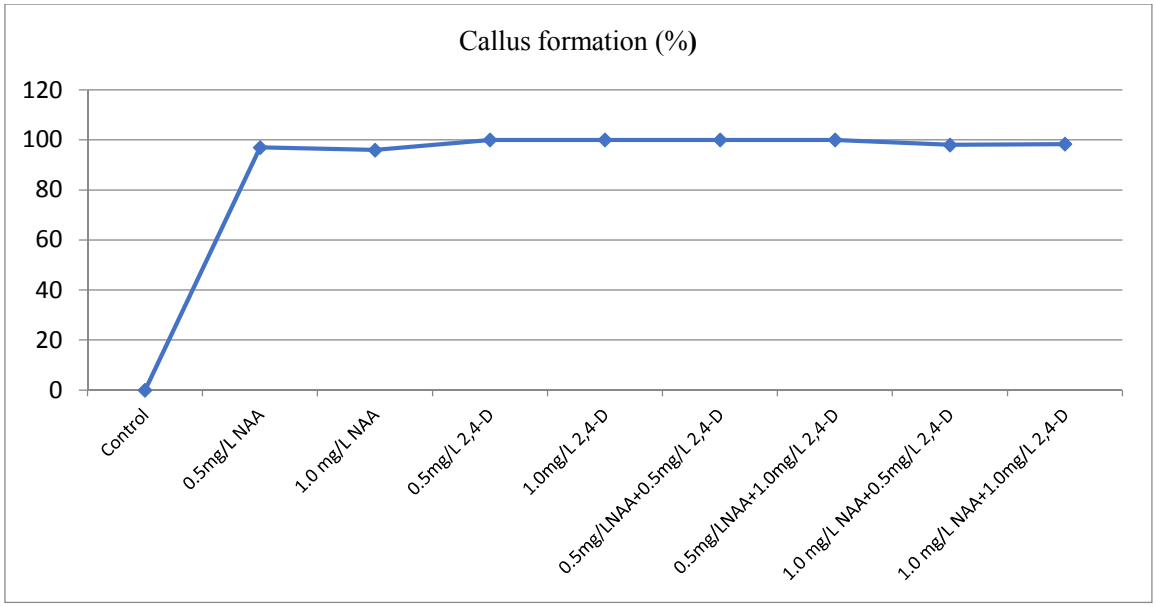

Fig. 5: The effect of different concentrations of NAA and 2, 4-D on callus formation (%) of Blackberry (*Rubus fruticosus* L.) from leaves after 28 days of culture.

Growth dynamics:

Growth curves of calli were based on fresh weights changes over 12 weeks as shown in Figs. (6and7). In general, fresh weight increased and growth rate (GR) of calli recorded the highest values on MS medium supplemented with 0.5mlNAA+1.0ml 2,4-D and 1.0 ml NAA+1.0ml 2,4-D, while GR was increased slowly in callus formed on medium supplemented with 0.5ml NAA. Growth rate was in increasing order from the 4th to the 12th weeks. The calli presented growth curves with lag, exponential, linear and stationary phases. The adaptive period (lag phase), in the fresh weight of calli increases slowly, occurred up to the 4th week. According to (Scraggy and Allan, 1993) the lag phase is considered an energy producer period. The fast growth phases (exponential and linear phases) occurred between the 4th and the 9th weeks, it disagreed with (Ahmed *et al*., 2000) who observed the highest callus growth rate at the fourth week of cultivation. The stationary phase occurred between the 9th and 12th weeks. In this phase, the rate of cellular division is gradually reduced and then remains constant. According to (Smith, 2000) in the stationary phase occurs deprivation of nutrients in culture medium and a reduction of the O2 amounts inside the cells. At the contrast of that, our results found that callus growth rate increased continuously until the end of 12th weeks.

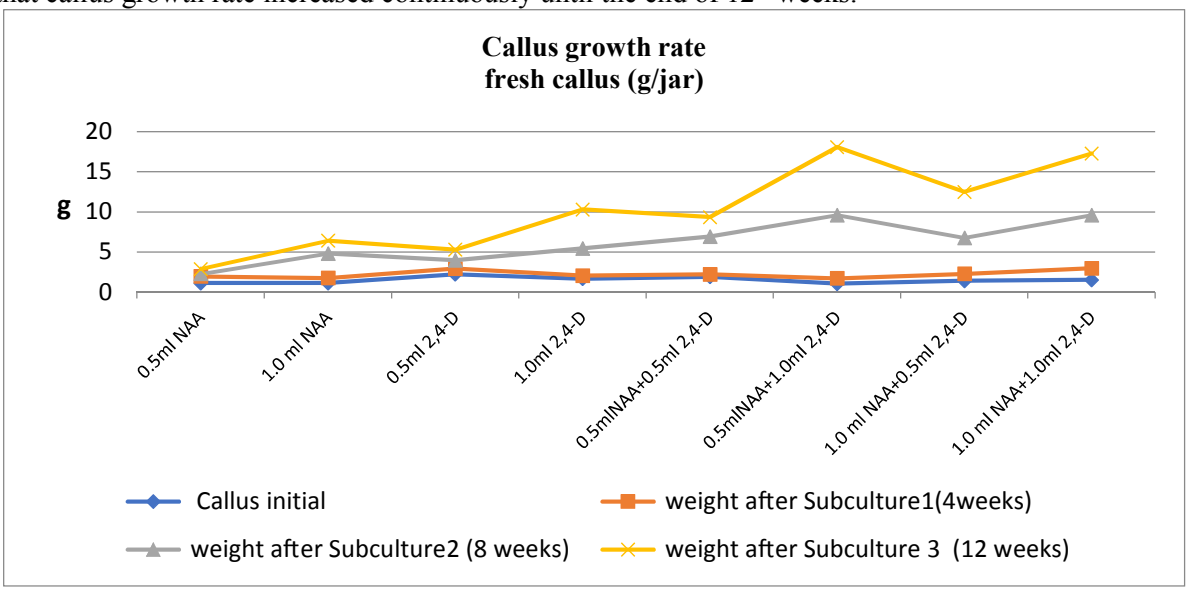

Fig. 6: The effect of different concentrations of NAA and 2, 4-D on callus growth rate (fresh callus g) of Blackberry (*Rubus fruticosus* L.) from leaves.

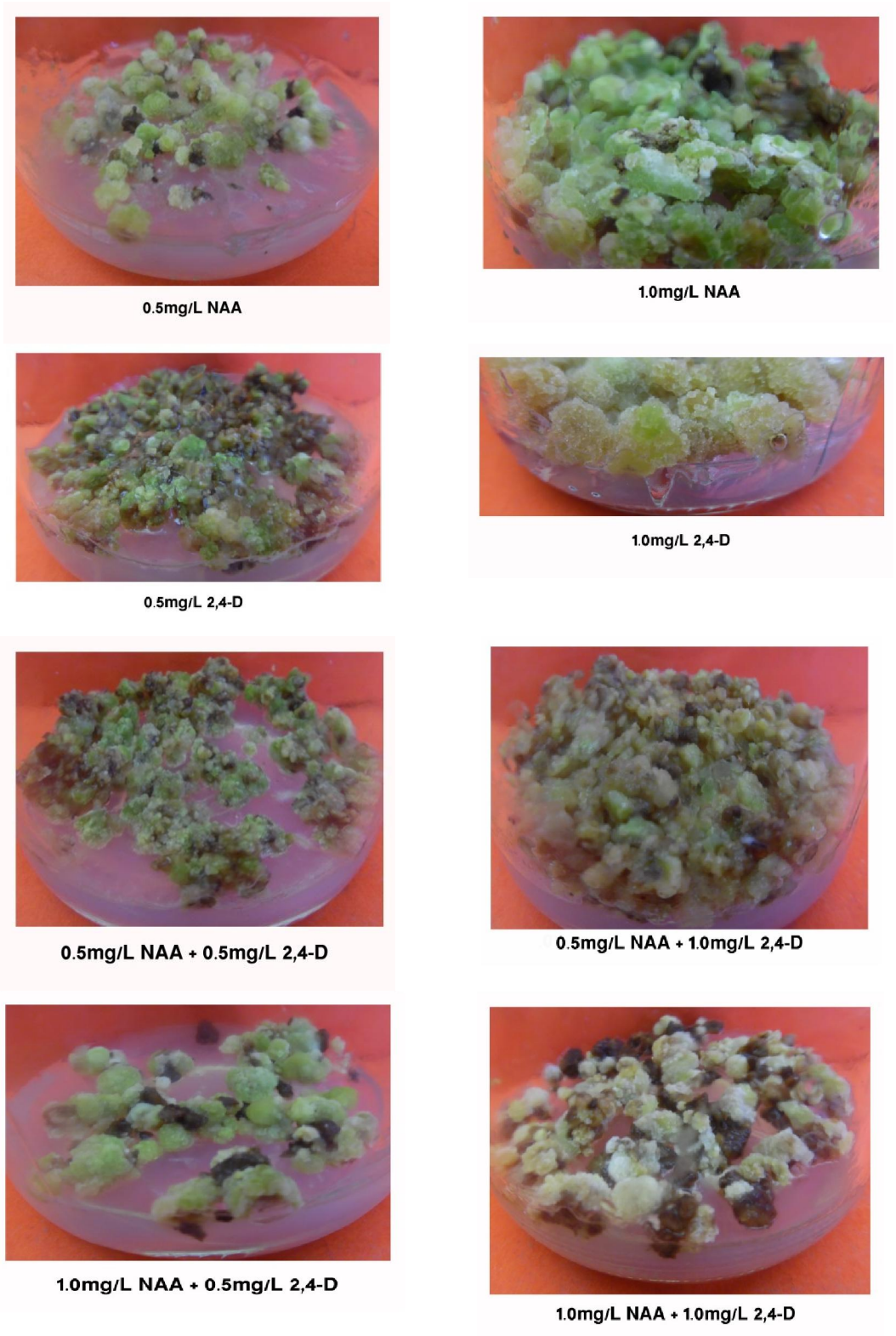

Fig. 7: The effect of different concentrations of NAA and 2, 4-D on callus formation of Blackberry (*Rubus fruticosus* L.) from leaves**.**

Chemical analysis of fresh shootlets and callus:

Data presented in Table (4) indicated that the highest content of chlorophyll a, chlorophyll b, carotenoids, total phenols and total antioxidant (112.26, 46.57, 127.72, 239.45 and 948.61 mg/100g

fw), respectively were recorded due to the supplement of in case of using 0.4 mg/l BA. While, using 0.2mg/l BA. in the culture medium resulted the highest value of total flavonoids and vit.C (28.50 and 222.30 mg/100g) respectively. These results manifested the correlation between the efficiency of antioxidant activity and the accumulation of phenolic compounds. In this consistent with our results, the relationship between antioxidant activity and phenolic content has been also evaluated (Sun and Ho, 2005 and Guo *et al*., 2008) where a positive correlation between antioxidant activity and phenolic content were observed.

Table 4: Chemical composition (%) of in vitro shootlets Blackberry (*Rubus fruticosus* L.) treated with different concentrations of BA and /or NAA.

Treatments for shootlest	Chlorophyll a (mg/100g)	Chlorophyll b (mg/100g)	Carotenoids (mg/100g)	Total Phenols (mg/100g)	Total Flavonoids (mg/100 mg	Total antioxidant (mg/100g	Vit.c (mg/100g)
Control	39.94	9.69	56.90	136.80	5.96	584.81	18.61
0.2mg/l BA	90.67	12.31	103.90	230.09	28.5	732.33	222.30
0.4 mg/l BA	112.26	46.57	127.72	239.45	15.93	948.61	195.83
0.6 mg/l BA	68.79	0.72	83.29	174.75	9.34	376.69	190.40
0.2 mg/l BA +0.2mg/l NAA	91.34	28.92	116.71	117.68	6..33	188.31	169.76
0.4mg/ l BA +0.2mg/l NAA	67.27	8.12	80.74	192.92	4.32	444.61	163.52
0.6mg/l BA +0.2mg/l NAA	38.82	3.51	51.18	196.70	1.65	626.22	169.37
LSD0.5%	0.60	1.79	7.00	0.71	6.67	4.45	9.64

Moreover, results in Table (5) demonstrated that the highest content of chlorophyll a, chlorophyll b, total phenols, total flavonoids and vit.C (35.55% ,41.83 % , 112.25%, 6.33% and 14.03%), respectively were obtained from callus formed on medium modified with 0.5ml /l 2,4-D. On the other hand, the highest content of carotenoids (24.23%) obtained from callus formed on medium contained 1.0 ml/l NAA+1.0ml/l 2,4-D and total antioxidant was the highest to (214.42%) in the callus formed in medium contain 0.5 ml/l NAA. The results clearly showed that the highest total phenolics level obtained from callus grown on culture medium contained (0.5ml/l 2, 4-D).

These results disagree with (Bela and Shetty, 1999) who investigated that total phenolic levels of developing embryos in anise in the absence of 2, 4-D were higher than in the control. Palacio *et al.* (2012) reported that production in vitro of secondary compounds from medicinal plants is possible due to the variation of culture conditions, including changes in type and concentration of plant growth regulators. According to **(**Coenen and Lomax, 1997), the accumulation of secondary compounds correlates negatively with cell growth, which is also found in this study, since callus with higher concentrations of phenolic compounds showed lower fresh and dry weight and compact consistency (Table 5and Fig. 6). The oxidative action of phenolic compounds is a limiting factor for growth of in vitro cultures (Arunyanart and Chaitrayagun, 2005).

Table 5: Chemical composition (%) of callus formed from Blackberry (*Rubus fruticosus* L.) leaves treated with different concentrations of NAA and /or 2, 4-D.

Treatments for callus	Chlorophyll a (mg/100g)	Chlorophyll b (mg/100g)	Carotenoids (mg/100g)	Total Phenols (mg/100g)	Total Flavonoids (mg/100 mg	Total antioxidant (mg/100g	Vit.c (mg/100g)
0.5ml /l NAA	0.94	4.37	5.14	15.25	0.38	214.42	12.93
1.0 ml /l NAA	18.28	32.26	12.87	86.95	2.95	121.09	1.07
0.5ml /l 2,4-D	35.55	41.83	16.33	112.25	6.33	117.44	14.03
1.0ml / l 2,4-D	14.62	27.79	12.22	57.12	2.12	32.18	13.45
0.5ml /l NAA+0.5ml/l 2,4-D	11.55	13.53	20.68	89.95	4.16	66.26	11.96
0.5ml /l NAA+1.0ml/l 2,4-D	3.74	1.18	20.45	106.71	4.14	93.86	12.20
1.0 ml/l NAA+0.5ml/l 2,4-D	2.74	11.18	14.52	107.33	3.12	130.43	12.39
1.0 ml/l NAA+1.0ml/l 2,4-D	11.33	0.07	24.23	107.23	2.00	85.92	11.77
LSD 0.5%	1.94	6.01	0.63	3.97	0.30	2.68	0.65

Conclusion

This study showed that, MS medium is the most effective multiplication for mass production of *Rubus fruitcosus* L. plant especially when provided with 0.6mg/l BA. Decreasing the strength of medium had a promoting effect on rooting plantlets. The optimum number of acclimatized plants can

be obtained on peat moss mixture. Generally, bio products produced from in vitro shootlets (total carotenoids, total phenol, total antioxidant, total flavonoids and vit.C) were two times to more than ten times compared to that produced by in vitro callus.

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