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Influence of Plant Growth Regulators and Dark Pretreatment on Shoot Proliferation, Rooting and Acclimatization of Thorny Blackberry

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THIS study aimed to establish an efficient micropropagation protocol for blackberry. For shoot multiplication, shoots tips-derived plantlets were cultured on MS medium supplemented with 0.5 or 1.5 mg l^{-1} BA individual or in combined with 0.5 mg $l^{-1}GA_3$. The highest number of shoots/cluster, shoot height, shoot fresh weight and number of leaves were observed on MS+1.5 mg l^{-1} BA+0.5 mg l^{-1} GA_3. In a second proliferation experiment, explants were subjected to in vitro auxin pretreatments (0.5 and 1.0 mg l^{-1} IBA or NAA) for two weeks with presence of 1.5 mg l^{-1} BA. Results indicated significant increase in shoot fresh weight, shoots cluster height (9.75) of explants pretreated with 0.5 mg l^{-1} IBA under dark incubation condition but the roots number significantly increased (4 roots/cluster) under light incubation and MS+1.0 mg l^{-1} NAA. Rooting and growth were examined under dark versus light pretreatments, followed by further growth for four weeks on MS medium supplemented with different concentrations (0.5 and 1.0 mg l^{-1}) of auxin types (IBA and NAA). All auxins concentrations maximized the root length under light incubation condition with no significant difference among all values under light condition. Anyway, the highest root length (77.6.mm) was obtained on MS +0.5 mg l^{-1} IBA. While, the highest roots number (7.2 roots/plantlet) was recorded on MS+1.0 mg l^{-1} NAA, under light condition. On MS medium without auxin under dark pretreatment, plantlets possessed the highest shoot/root ratio. The effects of in vitro auxin treatments on subsequent ex vitro growth were studied. Plantlets previously grown on MS medium+1.0 mg l^{-1} NAA or IBA recorded the highest root fresh and dry weight and root number.

Keywords: *Rubus fruticosus*, Tissue Culture, Benzyl Adenine, Gibberellic Acid, Auxin, micropropagation, Acclimatization

Introduction

The blackberry (*Rubus fructicosus* L.) among other important berry fruits, *i.e*., raspberry and strawberry, belong to the family Rosaceae. The fruits have high nutritional values, rich in anthocyanin, phenolic substances and vitamins C and E, which improve human health. Blackberry plants are normally propagated by shoot tip, cuttings or layering that is costly, labor intensive, lacking rooting potential and allows disease, especially virus transition to the new propagules (Anderson, 1980). *Rubus spp* are also highly heterozygous and are not propagated by seeds because of the high variability (Clark and Perkins-Veazie 2011 and Kefayeti et al, 2018). Due to their known health benefits, plants of the *Rubus spp* are grown in several areas worldwide for fresh fruit consumption, processing and export. However, berry fruits, except strawberry, are recently-introduced to the Egyptian growers, and it is not yet planted in large-scale production, which could be due to the unavailability of certified planting stocks or the high costs of importing

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such materials. Therefore, the utilization of clonal propagation of elite Rubus verities through tissue culture may help overcoming these limitations.

Techniques of tissue culture allow the production of disease-free planting stock in a limited space and time and facilitate germplasm storage and exchange between nations. The early report of Swartz et al. (1983) indicated that micro propagated blackberry plants were phenotypically stable, or even better than traditionally propagated plants. The genetic stability of tissue culture derived raspberry and blackberry were proven by Vujović et al. (2017), who indicating their suitability for micropropagation by axillary branching. It was also reported that the in vitro performance vary, depending on the genotype (Graham et al., 1997) and explant type (Meng et al., 2004).

Successful micropropagation protocol requires specific medium composition and culture conditions during each stage. In this respect, several trials were conducted on Rubus plants starting from the early works of (Broome and Zimmerman, 1978, Anderson, 1980, Compton and Presrce, 1988 and George et al., 2008) to the later works of (Georgiva et al., 2016 and Kefayeti et al, 2018). In these studies, wide ranges of plant growth regulator (PGR) regimes were tested during the multiplication phase (1-2 mg l^{-1} BA, 0.1-1.0 mg l^{-1} of NAA or IBA). Further studies examined only the cytokinin types and concentrations (AbdAlla and Mostafa, 2015, Fathy et al, 2018, Munoz-Concha et al, 2021). Under high cytokinin level in shoot multiplication medium, proliferated shoot clusters are normally short, dense and compact, which could result in difficulty in dividing these clusters into single plantlet for subsequent rooting, indicating the importance of supplying the medium with shoot elongation stimulant, such as GA_3 (Pua et al., 1983, Najaf and Hamidoghali, 2009, Geng et al., 2016).

During the in vitro rooting stage, several types and concentrations of root promoting substances were tested using a combination of one or more auxin types, normally NAA, IBA and IAA (Anderson, 1980, AbdAlla and Mostafa, 2015, Kefayeti et al, 2018) while only few plantlets rooted without hormone supplementation (Georgievna et al., 2016). In the study of Raeva-Bogosaovskaya et al (2021), blackberry cultivars showed differences in rooting frequency, with noticeable effects of auxin type and levels, indicating the importance of tailoring the protocol of rooting for the cultivar under investigation, especially for those which were not examined before. Munoz-Concha et al (2021) found that rooting of the blackberry *cv.* Chester was better on WPM medium than MS, but they did not mention the addition of auxin to the medium.

The environmental conditions during plant micropropagation is important, although detailed research did not implement for blackberry. Results of Swartz et al (1990) on Rubus and Malus leaves, Pawlicki and Welander (1994) on apple, Mendi et al (2010) on snake melon have shown enhanced shoot organogenesis under dark pretreatment of explant, in contrast to the finding of Miguel (2022) on cucumber. Light spectral quality was also found to affect direct shoot regeneration in strawberry (Mohamed et al., 2015).

It is well recognized that the ex vitro acclimatization is the most important step for successful micropropagation of Rubus, among other plant species (Alabadí and Blázquez, 2008, Dziedzic et al., 2013 and Bach, 2018). However, limited research efforts were directed towards the effects of light versus dark conditions and the hormonal treatments during in vitro rooting stage on subsequent ex vitro growth performance during past acclimatization stage of blackberry. Lebedev et al. (2018) showed that the highest survival rate of raspberry plantlets ex vitro was obtained in those derived from a medium supplemented with low level of IBA (0.2 mg l^{-1}), and the micro shoot height in vitro did not correlate with subsequent growth during acclimatization. Therefore, the in vitro medium composition and environmental conditions effects on the subsequent ex vitro growth need further studies.

The aim of the current study was to examine optimum hormonal composition and light versus dark pretreatment on the in vitro shoot multiplication and rooting as well as the ex vitro growth in acclimatization of blackberry cv APF-45.

Materials and Methods

The present study was conducted at the Plant Tissue Culture Laboratory of the Department of Horticulture, Faculty of Agriculture, Suez Canal University, Ismailia, Egypt, during May 2021 to July 2022.

Mother plants of blackberry, cv. APF-45 (imported from USA), were kindly provided from a private research farm in Behaira governorate, Egypt. For culture initiation, shoot tip explants from greenhouse- grown mother plants were excised and moved to the tissue culture lab. They were washed in running tap water for 30 min., and then sterilized by 10% Clorox solution for three minutes, followed by three times washes by sterile distilled water each for 3 minutes. The explants were cultured in test tubes, each containing 10ml of Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) under aseptic conditions in a laminar air-flow hood. The culture medium pH was adjusted to 5.7 before being solidified with 7 g.l^{-1} agar. Cultures were incubated for 6 weeks in a growth room under 22±2 ºC and light period was 16/8 hr. with cool white fluorescent light.

Experimental protocol. The study included four experiments:

Exp. 1. *Effect of BA and GA_3 on shoot multiplication of blackberry*

Shoot tip-derived plantlets (2 cm in length with 1-2 leaves) were cultured on MS medium supplemented with 0.5 or 1.5 mg l^{-1} BA individual or in combination with 0.5 mg l^{-1} GA_3. For each treatment, five glass jars (300 ml^3), each containing three explants, were considered as replicates. The experimental design was CRD with 5 replicates. After 6-weeks incubation period, data were taken as number of shoots/cluster (NSC), shoot cluster height (SHT), fresh weight (SFW) and number of leaves (LN).

Exp. 2. *Effect of auxin and light or dark pretreatment on the in vitro shoot proliferation and growth of blackberry*

In this experiment, individual plantlets, resulted from dividing the clusters of Exp. 1 that pre-cultured on MS media supplemented with 1.5 mg l^{-1} BA, were implemented as explants for the current experiment (Exp. 2). Explants were cultivated on control (MS medium) or on MS medium with concentrations 0.5 or 1.0 mg l^{-1} of either IBA or NAA. Three explants were cultured per each jar (300ml^3) containing 30 ml medium. Dark pretreatments were imposed by incubating half of the above treatments under darkness for 2 weeks, and the second half under normal light conditions (16/8 hr. light period at irradiance of 45 µmol m^{-2} sec^{-1}) and 22±2 ºC. The experiment was a 2x5 factorial design with five replicates. After six weeks, clusters were removed from jars and data were taken on shoot fresh weight (SFW), NSC, LN and root number (RN).

Exp. 3. *Effect of auxin and light or dark pretreatment on the in vitro rooting and growth of blackberry.*

Single plantlets were excised from proliferated shoot cluster and examined for growth and rooting characters under the influence of auxin type, concentrations, and dark or light treatment. Explants were aseptically cultured on MS medium amended with 0, 0.5 and 1.0 mg l^{-1} of IBA or NAA, in addition to a control (MS without hormones). Five explants were cultured per culture jar (300 ml^3) in 10 replicates for each auxin treatment. Half of the cultures were incubated under darkness, while the second half were incubated under normal light conditions, as in Exp. 2 and arranged on the shelves of the growth room as 2x5 factorial design experiment. After 6 weeks from culture initiation, data were recorded on plantlet fresh weight (PFW), LN, plantlet height (PHT), RL and RN as well as shoot/root ratio (S/R).

Exp. 4. *Effect of in vitro auxin treatments during rooting stage on the ex vitro growth of blackberry.*

In vitro-derived plantlets were examined for different ex vitro growth attributes as affected by auxin treatments. Six week- old rooted plantlets were removed from the culture jars (previously incubated under light and auxin treatment from Exp. 3), washed in running tap water for 15 min to remove excess of agar, then transferred to the greenhouse for acclimatization. They were cultured on 50-cell black plastic trays, each cell contained 50 ml of moist peat moss + perlite (3:1 v/v) as one plantlet per cell. Plantlets from the 5 auxin treatments were represented in each tray as 10 plantlets per treatment, using 3 replicates (trays) in total. They were mist-irrigated twice/day and fertilized with solution of 1g. l^{-1}19-19-19 NPK fertilizer twice/week, and covered with clear plastic tunnel during Feb-April, 2022 until fully acclimatized. By the end of April, 2022, data were recorded on LN, SFW, root fresh weight (RWD), shoot dry weight (SDW) and the ratio of SDW/RDW (S/R).

Statistical analysis

Data of the 4 experiments were subjected to the analysis of variance (ANOVA) and analyzed using CoStat 6.400 statistical software. Means comparisons were performed using the least significant difference test at $p \leq 5\%$ level of significance.

Results

Culture initiation of blackberry shoot tip explants was accomplished on MS medium free of plant growth regulators after 4 weeks by forming single plantlets with 1-3 leaves suitable for sub-culture onto shoot multiplication experiment.

Exp. 1. Effect of BA and GA_3 on shoot multiplication of blackberry

Results in Table 1 indicated significant increase in number of shoot per cluster (NSC) using MS medium supplemented with BA individual at concentration 1.5 mg l^{-1} BA (12 shoots/explant).

The addition of GA_3 to 1.5 mg l^{-1} BA maximized the NSC (14 shoots/cluster), but, the difference was not significant (Fig. 1). While, GA_3 significantly improved NSC (8.3 shoots/explant), when GA_3 was added to MS with 0.5 mg l^{-1} BA. Results also showed the outperformances of MS medium supplemented with 1.5 mg l^{-1} BA + 0.5 mg l^{-1} GA3 on shoot height (SHT), shoot fresh weight (SFW), and number of leaves (LN) and recorded 34 mm, 0.312 g and 26 leaves/cluster, respectively.

TABLE 1. Effect of BA and GA3 on shoot multiplication of blackberry.

Treatments	Number of shoots/cluster	shoot cluster height (mm)	Shoot fresh weight (g)	Number of leaves
BA 0.5 mg l^{-1}	2.3 c	21 c	0.207 b	13 c
BA 1.5 mg l^{-1}	12 a	18 d	0.302 a	23 b
BA 0.5 mg l^{-1} + GA_3 0.5 mg l^{-1}	8.3 b	26 b	0.214 b	16 c
BA 1.5 mg l^{-1}+ GA_3 0.5 mg l^{-1}	14 a	34 a	0.312 a	26 a
LSD 0.05	2.6	2.2	0.01	2.8

Means with the same letter in each column are not significantly different, $P \leq 0.5$

Fig. 1. In vitro shoot multiplication of blackberry in response to BA and GA_3 concentrations.

Exp. 2: *Effect of auxin and light or dark pre-treatment on the in vitro shoot proliferation and growth of blackberry*

Results of the main and interaction effects of dark pretreatment versus light and different concentrations of auxin types on SFW, NSC, LN, PHT, and RN are shown in Table (2). The main effect of dark pretreatment demonstrated significant ($p \leq 5\%$) increase in SFW and shoot multiplication frequency (NSC) by 2-weeks dark pretreatment of growing explants, compared to those under normal light conditions. However, number of leaves and plantlets length were not affected by incubation under dark condition. On the other hand, shoot cluster under normal light conditions had initiated more roots than those exposed to dark pretreatment. With regard to the main effect of short exposure to auxin pretreatment, the obtained data (Table 2) indicated that pretreatment with 0.5 mg l^{-1} IBA, significantly increased SFW and SHT.

This treatment had also significantly increased NSC (8.6 shoot/cluster) compared with other auxin treatments while, it was insignificantly different from control (MS + 1.5 mg l^{-1} BA). The lowest values of NSC and LN were recorded in shoot clusters that exposed to 1.0 mg l^{-1} NAA. However, this later treatment had resulted in significant increase in root number/cluster compared to all other auxin pretreatments (Fig. 2).

The interaction between light or dark incubation and auxin effects, were significant for all studied growth and multiplication characters (Table 2). In this regard, a combination of 0.5 mg l^{-1} IBA and dark incubation pretreatment significantly outperformed the control treatment for SFW and PHT. This treatment had significantly increased NSC (9.8 shoot/cluster) over all other treatments combination, while was not different from control (9.5 shoot/cluster). On the other hand, the combination of pretreatment with 1.0 mg l^{-1} NAA under light condition resulted in the least shoot proliferation and leaves number, but the highest root number/shoot (4 roots) over all other pretreatment combinations (Fig. 2).

TABLE 2. Effect of auxin and light or dark pretreatment on the in vitro shoot proliferation and growth of blackberry.

Light and dark	Auxin (mg l^{-1})	Shoot fresh weight (g)	Number of shoots/cluster	Number of leaves/cluster	Plantlet height (mm)	Number of roots/shoot
Light		0.447 b	5.2 b	19.65 a	30.70 a	1.85 a
Dark		0.508 a	6.6 a	22.40 a	27.75 a	1.35 b
LSD 0.05		0.05	0.69	3.4	3.6	0.3
	0	0.365 c	8.3 a	26.38 a	23.13 c	1 b
	IBA 0.5	0.685 a	8.6 a	17.38 bc	34.75 a	1.25 b
	IBA 1.0	0.514 b	5.9 b	23.63 ab	29.50 abc	1.25 b
	NAA 0.5	0.436 c	4.5 c	22.13 ab	27.00 bc	1.25 b
	NAA 1.0	0.389 c	2.3 d	15.63 c	31.75 ab	3.25 a
LSD 0.05		0.07	1.09	5.4	5.7	0.5
Light	0	0.297 d	7.0 bc	22.25 ab	25.00 ab	1.00 c
Light	IBA 0.5	0.602 b	7.5 b	16.75 b	34.25 ab	1.25 c
Light	IBA 1.0	0.436 cd	5.3 cd	23.75 ab	33.25 ab	1.50 c
Light	NAA 0.5	0.421 cd	4.5 de	22.75 ab	27.50 ab	1.50 c
Light	NAA 1.0	0.479 bc	1.8 f	12.75 b	33.50 ab	4.00 a
Dark	0	0.433 cd	9.5 a	30.50 a	21.25 b	1.00 c
Dark	IBA 0.5	0.768 a	9.8 a	18.00 b	35.25 a	1.25 c
Dark	IBA 1.0	0.592 b	6.5 bcd	23.50 ab	25.75 ab	1.00 c
Dark	NAA 0.5	0.451 c	4.5 de	21.50 ab	26.50 ab	1.00 c
Dark	NAA 1.0	0.298 d	2.8 ef	18.50 b	30.00 ab	2.50 c
LSD 0.05		0.10	1.54	7.6	8.0	0.8

Means with the same letter in each column are not significantly different, $P \leq 0.5$.

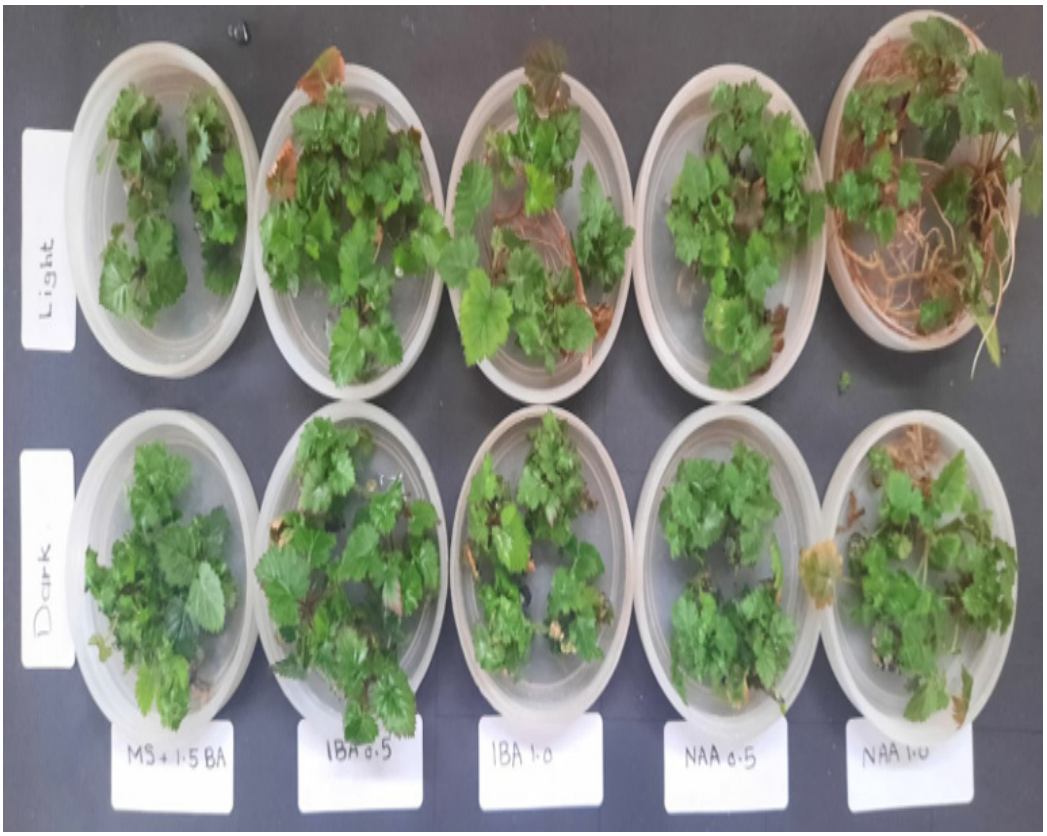

Fig. 2. Shoot proliferation of blackberry in response to pre-treatment with dark or light incubation and concentrations (0.5 and 1.0 mgl^{-1}) of auxin types (IBA and NAA) .

Exp. 3: *Effect of auxin and light or dark pretreatment on the in vitro rooting and growth of blackberry.*

Data presented in Table (3), indicated that blackberry plantlets grown under dark pretreatment or normal light conditions were not significantly different in fresh weight and number of leaves per plantlet. However, under light condition, the length and number of roots were significantly high under light incubation compared to dark pretreatment incubation (65 vs 52 mm in height and 4.68 vs 3.72 roots/plantlet, respectively). However, results showed that plantlets exposed to dark incubation pretreatment were significantly longer and had more shoot/root ratio than those under control.

With regard to the main effect of auxin treatments, the highest significant plantlet FW was achieved on MS medium supplemented with 1.0 mg l^{-1} NAA (0.878 g/plantlet), followed by IBA at 1.0 mg l^{-1} treatment (0.734 g/plantlet). Number of leaves was maximized on medium with 0.5 mg l^{-1} IBA, while increasing IBA concentration to 1.0 mg l^{-1}, significantly enhanced PHT over all other treatments. Rooting potential was best achieved with NAA treatment in terms of root length, using 0.5 mg l^{-1} or root number, using 1.0 mg l^{-1} NAA in the medium. The shoot/root ratio was significantly lower than the control for all IBA and NAA concentrations, and the least ratio was recorded when the medium was supplemented with high level of NAA (1.0 mg l^{-1}).

The interaction between auxin concentrations and dark pretreatments significantly influenced all measurements of rooting and growth characteristics of blackberry plantlets in vitro (Table 3 and Fig. 3). The highest PFW was recorded in medium supplemented with NAA at 1.0 mg l^{-1} in plantlets partially incubated in dark, and the increase was four times over the control.

TABLE 3. Effect of auxin and light or dark pretreatment on the in vitro rooting and growth of blackberry.

Light and dark	Auxin (mg l^{-1})	Plantlet fresh weight (g)		Number of leaves/plantlet		Plantlet height (mm)		Root length (mm)		Root number/ plantlet		Shoot/root ratio	
Light		0.629	B	7.1	a	38.6	b	65.0	a	4.68	a	0.68	b
Dark		0.594	B	6.8	a	47.2	a	52.0	b	3.72	b	1.11	a
LSD 0.05			41.1		0.6		5.01		8.28		0.43		0.1
	0	0.286	E	7.1	b	34.4	b	48.6	b	1.8	e	1.91	a
	IBA 0.5	0.525	D	8.5	a	41.9	ab	62.0	ab	2.8	d	0.83	b
	IBA 1.0	0.734	B	6.8	b	51.7	a	51.1	b	4.5	c	0.62	c
	NAA 0.5	0.634	C	5.9	b	44.6	ab	69.1	a	5.2	b	0.62	c
	NAA 1.0	0.878	A	6.4	b	41.9	ab	61.6	ab	6.7	a	0.48	c
LSD 0.05			65.1		1.0		7.92		13.1		0.68		0.16
Light	0	0.334	D	7.6	ab	36.8	bc	47.8	b	2.4	f	1.26	b
Light	IBA 0.5	0.536	C	9.2	a	37.6	bc	77.6	a	2.8	ef	0.86	c
Light	IBA 1.0	0.787	B	7.0	bc	44.4	abc	58.2	ab	5.2	bc	0.51	def
Light	NAA 0.5	0.692	B	6.4	bc	42.8	abc	74.0	ab	5.8	b	0.43	ef
Light	NAA 1.0	0.796	B	5.4	c	31.4	c	67.2	ab	7.2	a	0.32	f
Dark	0	0.239	E	6.6	bc	32.0	c	49.4	ab	1.2	g	2.56	a
Dark	IBA 0.5	0.513	C	7.8	ab	46.2	abc	46.4	b	2.8	ef	0.80	cd
Dark	IBA 1.0	0.682	B	6.6	bc	59.0	a	44.0	b	3.8	de	0.73	cd
Dark	NAA 0.5	0.575	C	5.4	c	46.4	abc	64.2	ab	4.6	cd	0.81	cd
Dark	NAA 1.0	0.961	A	7.4	abc	52.4	ab	56.0	ab	6.2	b	0.64	cde
LSD 0.05			0.09		1.4		11.2		18.5		0.97		0.22

Means with the same letter in each column are not significantly different, $P \leq 0.5$

Fig. 3. In vitro growth and rooting of blackberry in response to dark and light incubation conditions and concentrations of auxin types.

The highest significant LN was recorded with the application of IBA at 0.5 mg l^{-1} under light condition (9.2 leaves/plantlet) followed by the same IBA level under dark pretreatment (7.8 leaves/plantlet). In addition, plantlet height (PHT) recorded significant increase using IBA 1.0 mg l^{-1} under exposure to dark pretreatment (5.9 cm) compared to the control (3.2 cm). Considering rooting characteristics, the highest significant root length was obtained on MS media amended with IBA at 0.5 mg l^{-1} (77.6 mm), or NAA at 0.5 mg l^{-1} (74.0 mm) under light condition. However, number of roots was significantly the high (7.2 roots/plantlet) in the medium supplemented with 1.0 mg l^{-1} NAA under light condition, in comparison to the control (2.4 roots/plantlet) under the same condition of light (Fig. 4). Root number under dark pretreatment, had also recorded the highest values on the medium supplemented with 1.0 mg l^{-1} NAA (6.2 roots) compared to the control (1.2 roots). However, the control treatment (without auxin) recorded the highest S/R ratio (2.56) under dark pretreatment or under light (1.26) as shown in Table (3).

Exp. 4: *Effect of in vitro auxin treatments during rooting stage on the ex vitro growth of blackberry:*

The ex vitro growth of blackberry plants, after being fully acclimatized, was not significantly affected by the in vitro auxin treatment during rooting stage, in terms of SFW, PHT and RL (Table 4

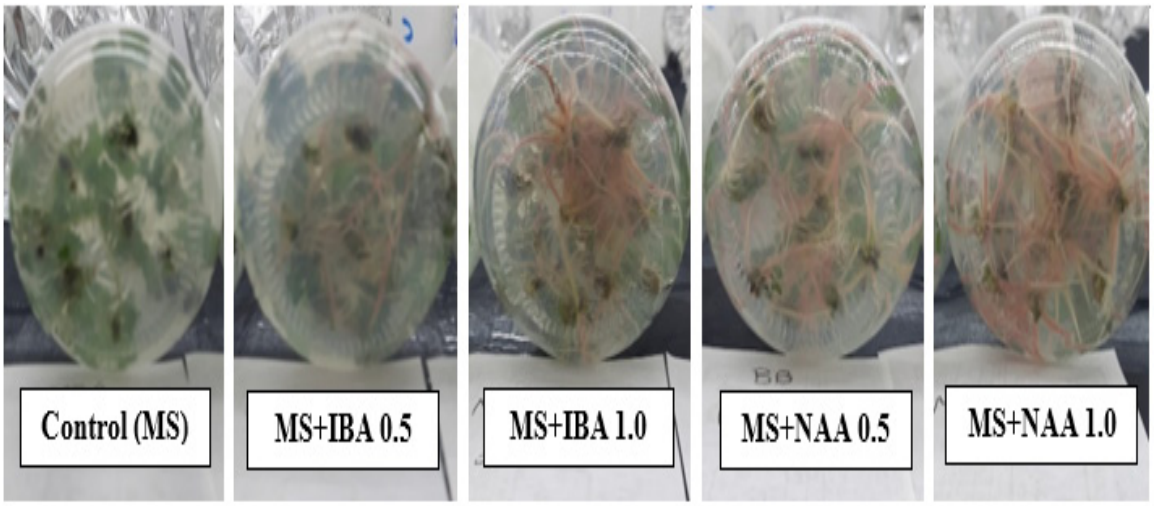

Fig.4. In vitro rooting pattern of blackberry (jar bottom) in response to auxin treatments under light incubation.

TABLE 4. Effect of in vitro auxin treatments during rooting stage on the ex vitro growth of blackberry.

Medium	Number of leaves/ plant	Shoot fresh weight (g)	Plantlet height (mm)	Root fresh weight (g)	Root length (mm)	Number of roots/ plant	Shoot dry weight (g)	Root dry weight (g)	Shoot/ root ratio
MS	8.3 a	0.458 a	104.7 a	0.609 c	75.7 a	15.0 b	0.097 c	0.084 c	1.15 a
IBA 0.5	8.0 a	0.570 a	115.0 a	0.671 b	87.7 a	16.7 b	0.134 b	0.092 c	1.46 a
IBA 1.0	9.0 a	0.645 a	127.7 a	0.835 a	84.3 a	16.0 b	0.127 b	0.111 b	1.14 a
NAA 0.5	9.3 a	0.661 a	116.0 a	0.843 a	85.0 a	17.0 b	0.154 a	0.114 b	1.35 a
NAA 1.0	8.7 a	0.651 a	120.0 a	0.802 a	68.7 a	26.0 a	0.164 a	0.139 a	1.18 a
LSD	2.3	0.228	19.9	0.057	13.3	2.7	0.01	0.014	0.3

Means with the same letter in each column are not significantly different, $P \leq 0.5$.

However, RFW was significantly high in plants derived from MS medium supplemented with 1.0 mg l^{-1} IBA, 0.5 mg l^{-1} NAA, or 1.0 mg l^{-1} NAA, compared to the control plants which recorded the least RFW. Root number/plant possessed the highest value in ex vitro plants previously grown on MS + 1.0 mg l^{-1} NAA with 26 roots compared to an average of 15 roots/plant in the control (Fig. 5). Generally, data revealed that shoot dry and fresh weights (SDW and RDW) were significantly the highest in plants derived from MS + NAA at either 0.5 or 1.0 mg l^{-1}, recording about 1.65 times increase over control. However, the S/R ratio did not significantly differ among plants previously exposed to the in vitro treatments (Table 4). Blackberry transplants were successfully acclimatized and transferred to larger pots (Fig. 6) for subsequent field growth and fruit production.

Discussion

To achieve the optimum in vitro multiplication in the thorny blackberry genotype, it was found that increasing BA from 0.5 to 1.5 mg l^{-1} had resulted in more than 5 folds of increase in NSC (12 shoots/explants) which was better than previous reports. AbdAlla and Mostafa (2015) used two types of cytokinin (2 mg l^{-1} BA + 0.5 mg l^{-1} 2ip) and obtained a maximum of 7.7 shoots/explants, while Fathy et al. (2018) used lower BA level (0.6 mg l^{-1}) and obtained only 3.4 shoots/explants. Munez-Concha et al. (2021) supplemented the medium with BA at 1.4 mg l^{-1}, almost similar to our study, and got an average of 5.2 shoots/explants. These differences in blackberry proliferation rate may be due to the difference among the genotypes examined, as indicated by Raeva-Bogoslovskaya et al. (2021). The thorny blackberry cultivar utilized in our study showed higher potential for shoot multiplication than thornless cultivars tested in many of the aforementioned reports. Results also showed enhanced NSC (14 shoots/explants) and other growth traits, especially shoot elongation, when GA_3 was supplemented to BA-amended medium, similar to the results reported by Georgieva et al. (2016) on strawberry and raspberry, Geng et al. (2016), Pau et al. (1983) on apple and Gonbad et al. (2014) on tea. In other study, the use of WPM medium amended with 2.0 mg l^{-1} BA + 0.5 mg l^{-1} GA_3 had no effect on shoot multiplication, but increased shoot elongation in Chester blackberry cultivar (Kefayeti et al., 2018) which could be due to a sufficient level of GA in the original explant. The role of GA_3 in cell division in tissue culture is proved and date back to the early work of Digby et al. (1964).

Fig. 5. Growth and rooting in acclimatization of blackberry ex vitro in response to in vitro auxin treatments.

Fig. 6. Successful acclimatization of blackberry.

The results in the second experiment (Exp. 2) indicated that the explants cultured on MS medium supplemented with 1.0 mg l^{-1} IBA under dark pretreatment showed a significant impact on increasing NSC, SFW and length compared to those growing on MS medium amended with 1.0 mg l^{-1} NAA under normal light condition. This may be explained by the relative increase in rooting of proliferated shoot cluster on NAA-amended medium under light at the expense of enhanced axillary branching. The stimulation of shoot multiplication of blackberry under the influence of cytokinin (BA) and auxin (IBA) was in agreement with the results reported by Kefayeti et al. (2018) and Raeva-Bogoslovskaya et al. (2021). The positive effect of dark pretreatment on shoot proliferation were previously mentioned in several plant species (Meng et al., 2004, Pawlicki and Welander, 1994). The increase in NSC and SFW was viewed clearly between light and dark treatment while there was no significant difference between 0.5 mg l-1 IBA treatment and control. In addition, the increase of IBA concentrations or the use of NAA in culture media lead to decrease in NSC that could be due to the opposite effect of auxins on proliferation compared to the linear growth.

Results of the rooting experiment (Exp. 3) indicated that NAA followed by IBA increased plantlet FW, RL and LR, subsequently reduced S/R ratio, while in MS medium without auxin (control), this ratio was increased. Although both NAA and IBA had positive effect on the blackberry growth and rooting compared to the control, NAA seems to be more effective, in accordance with the finding of Kefayeti et al. (2018). The increase in S/R ratio in medium without auxin may be due to the observed decrease in root weight under this condition, in contrast to the medium amended with auxin, possibly where more nutrients were diverted to support growth. The stimulation

of rooting potentials under light versus dark conditions was clear during the proliferation (Exp. 2) and rooting (Exp. 3) stages. This finding is in parallel with the result of Bertazza et al. (1995) on pear cultivars and Yu et al. (2019) on cotton when in vitro root extension and numbers were significantly improved under light (especially red light) compared to the dark treatment, while IBA in the medium enhanced root production under all light treatments.

In acclimatization of blackberry plantlets ex vitro, the plantlets previously rooted in vitro on medium supplemented with both types of auxins, especially NAA, had more SDW, RDW, RFW and RN than control, indicating the importance of in vitro PGRs treatments on subsequent ex vitro growth and rooting for better adaptation to field growing conditions. After being fully acclimatized, the shoot/root ratios were not different among auxin treatments. Almost similar results were found by Lebedev et al. (2018). In vitro auxin treatment had also increased blackberry SDW which could have impacted their survival and growth ex vitro. In line with this, the study of Laforge et al. (1991) on strawberry and raspberry suggested that the in vitro leaves could be a source of nutrients reserved for leaves initiated ex vitro, resulting in enhanced plat performance in acclimatization.

Conclusion

A protocol for micropropagation of thorny blackberry was established under the influence of PGRs and dark pretreatment. Shoot multiplication was significantly increased on MS medium + 1.5 mg l^{-1} BA, alone or combined with 0.5 mg l^{-1} GA_3. Shoot proliferation and growth were also enhanced by dark pretreatment and growth on MS + IBA for two weeks. The best rooting and growth potentials were achieved on MS + 1.0 mg l^{-1} NAA under normal light, compared to dark conditions. The in vitro auxin treatment during the rooting stage had positively influenced the ex vitro growth and rooting of plantlets in acclimatization.

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Conflict of interests

The authors declare no conflict of interest in the publication of this work.

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تأثير منظمات النمو النباتية ومعاملة الإظلام على التضاعف والتجذير داخل المعمل والنمو الخارجي خلال مرحلة الأقلمة لنباتات التوت الاسود الشوكي

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أجريت هذه الدراسة لايجاد بروتوكول للإكثار المعملي لنباتات التوت الاسود الشوكي. زرعت النباتات الناتجة من القمة النامية علي بيئة MS المحتوية علي البنزيل أدينين بتركيزات ٠,٥ و ١,٥ ملجم/لتر وكذلك حمض الجبريليك بتركيزات صفر و ٠,٥ ملجم/لتر حيث تم الحصول علي أكبر عدد من النباتات المتضاعفة وكذلك الطول والوزن الطازج للكتلة المتبرعمة علي بيئة MS + ١,٥ ملجم/لتر BA+ ٠,٥ ملجم/لتر GA_3. في التجربة الثانية تم اختبار التضاعف والنمو للنباتات المعاملة مبدأياً بالتحضين لمدة أسبوعين في الظلام والزراعة علي بيئة التضاعف (MS+1.5 BA) المحتوية علي IBA و NAA كل منهما بتركيز ٠,٥ و ١,٠ ملجم/لتر وتم إستكمال النمو لمدة ٤ أسابيع تحت ظروف الاضاءة حيث أوضحت النتائج حدوث زيادة معنوية في الوزن الطازج وارتفاع النبتات المعاملة بالاظلام في البيئة المحتوية علي ٠,٥ ملجم/لتر IBA مقارنة بباقي المعاملات وكذلك أعلي زيادة معنوية في عدد النبتيات المتضاعفة (متوسط ٩,٧) ولكن تحت ظروف الاضائة العادية أعطت الكتلة المتضاعفة أعلى عدد من الجذور. تم في التجربة الثالثة إختبار النمو و التجذير تحت ظروف المعاملة بالاظلام أو الضوء في بيئة تحتوي علي IBA و NAA. أوضحت النتائج الحصول علي أعلي طول للجذور في البيئة المحتوية علي IBA أو NAA بتركيز ٠,٥ ملجم/لتر وأعلي عدد للجذور في البيئة المحتوية علي ١,٠ ملجم/لتر NAA تحت ظروف الضوء بينما أعطت النبتات النامية في بيئة MS بدون أكسينات وظروف الإظلام أعلي نسبة للمجموع الخضري/الجذري. التجربة الرابعة تم خلالها إختبار تأثير المعاملات السابقة خلال مرحلة التجذير المعملي علي النمو خارج المعمل خلال مرحلة الأقلمة حيث دلت النتائج علي أن النبتات السابق نموها في بيئة MS المحتوية علي IBA أو NAA بتركيز ١,٠ ملجم/لتر قد أعطت أعلي عدد ووزن طازج وجاف للجذور.