Asian Journal of Agricultural and Horticultural Research

5(1): 37-48, 2020; Article no.AJAHR.54489
ISSN: 2581-4478

Trials on *In vitro* Propagation and Using Natural Additives for *Myrtus communis L.* Plant

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

Article Information

DOI: 10.9734/AJAHR/2020/v5i130042
Editor(s):
(1) Dr. Ahmed Medhat Mohamed Al-Naggar, Professor of Plant Breeding, Department of Agronomy, Faculty of Agriculture, Cairo University, Egypt.
Reviewers:
(1) Ufuoma Bigila Shemishere, Federal University Birnin Kebbi, Nigeria.
(2) Junir Antônio Lutinski, Brazil.
(3) John Kimondo Kariuki, Jomo Kenyatta University of Agriculture and Technology, Kenya.
Complete Peer review History: http://www.sdiarticle4.com/review-history/54489

Original Research Article

Received 06 December 2019
Accepted 12 February 2020
Published 19 February 2020

ABSTRACT

Explant types, anti-oxidant pre-treatments, organic additives, natural additives, vitamins mix strengths, cytokinin types and concentrations, different medium strengths, GA3 concentrations, auxin types and concentrations were studied during the period from 2017 to 2018 to establish a protocol for *in vitro* propagation of Myrtle. It was found that culturing of pre-treated shoot tips with anti-oxidant solution (A.O.S) on modified Murashige and Skoog (MS), or Murashige and Skoog medium supplemented with PVP as anti-oxidants induced the best results in reducing free phenolic compounds and enhancing explant development parameters. Also, adding combination of tryptophan, adenine sulphate and coconut water as organic additives maximized survival percentage and improved explant development. In the same time, adding combination of coconut water at 5% plus Banana pulp plus Papaya extract at 50 g/L of were helpful in maximizing number of shoots/plant, shoot length and greening parameters. Also duplicating the dose of vitamin mix of Gamborg medium improved explants development and survival (%) of explant. Meanwhile, using of 1.0 mg/ L BAP increased proliferation. Meanwhile, addition of 2.0 mg/ L GA3 to half strength medium maximized shoot length. Moreover, the addition of 2.0 mg/L IBA to the culture medium induced the highest number of roots/plant.

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Keywords: Micropropagatin; In vitro; natural additives; myrtle and ornamental shrubs.

1. INTRODUCTION

Myrtus communis L. (Myrtle) plant is an evergreen shrub belongs to family *Myrtaceae*. It occupies an important position in folk medicine through alleviating some ailments and with multipurpose as the scent and the essential oils [1]. It is used as hedges, in the ornamental trade in the Italian and European markets in which green cut branches with flowers or colored fruits (berries) were fruitfully commercialized for the pot plant production and for gardening. Myrtle is also important for the reforestation of Coastal zones injured by fires. Meanwhile, the extracted active ingredient have many uses in chemical and medicinal industries. The fruits and leaves of Myrtle are used for anti-genotoxic, anti-mutagenic, antiseptic, anti-inflammatory and in the treatment of internal and topical infections [2,3]. Moreover, its essential oils can be used for the production of natural medicines and their leaves can be consumed as a drink like tea [4] and [5]. The fruits and leaves of wild myrtles are picked and evaluated by the growers who grow the promising genotypes with superior fruit quality, and then sold at local bazaars in Turkey. Furthermore, it is used in industries related to production of liqueurs from mature fruits, as food additives and handy crafts. Myrtus plants are propagated mainly by seeds or woody cuttings which can not cover an increasing demand every year. Thus, *in vitro* technique is considered the best alternative method that produce a huge numbers of plants for commercial planting and further studies. Also, it is a sophisticated technique that involves different stages which have to be performed carefully for successfully production of the planting material. Thus, application of tissue culture is greatly recommended for enhancing the scope and potentiality of mass propagation by exploiting regeneration behavior in a wide rang of selected horticultural plants [6-8]. They stated that Murashige and Skoog medium was suitable for micro-propagating of Myrtus plants. However, [9] reported that woody plant medium was superior in micro-propagating of Myrtle plants. Chemicals for tissue culture technique are very expensive. Thus, decreasing the costs of tissue culture industry can be achieved through use of alternatives or replacement of highly expensive chemicals component of the tissue culture media with a suitable cheap natural ones. Different trials were taken for testing some of these natural additives (natural fruit juices extracts) as alternatives to some chemicals (medium components or additives) specially, growth regulators are required for reducing expenses and improving growth and development of *in vitro* plants. Natural organic additives including coconut water, banana pulp, papaya extract, and others are used as additives for i*n vitro* culture [10,11]. Organic additives help in producing more protocorm like bodies, shoots and leaves of *Dendrobium orchid* [12]. It also**,** increases the size of somatic embryos [13]. In addition, they promote growth and development of a symbiotic seeds and regeneration of plantlets [14]. Natural additives consider as a natural source of carbon and contain natural vitamins, phenols, fiber, hormones, and proteins [15]. Moreover [13] mentioned that organic additives contained in addition to sugar other nutrients as proteins, lipids and minerals.

The ultimate goals of this study were to investigate the possibilities of establishing tissue culture protocol for raising huge quantities of healthy plants in shorter time with fewer expensive. Also, as a trial for exchanging of highly expensive components of some chemicals of tissue cultured medium by very cheap natural components (fruit juice extract) which are greatly valuable in reducing the costs of tissue culture industry.

2. MATERIALS AND METHODS

This study was carried out in Commercial Laboratory at El-Haram Street, Giza Governorate during the period from 2017 to 2018. New growing branches from good growing, healthy trees of *Myrtus communis* were taken, subjected to running water for five minutes and divided into small parts. Then sterilized using 10% Clorox with two drops of Tween-20 for fifteen minutes and immersed in sterilized distilled water three times for five minutes each under aseptic conditions. Then shoot tips were excised from terminal parts with 0.5-1.0 mm length. The remaining parts were divided into one nodal cutting as explants. The prepared explants were cultured on different nutrient media supplemented with 30 gm/ L sucrose, 1.0 mg /L 6-benzylaminopurin (BAP), 0.1 mg/ L indole-3-butyric acid (IBA) and 7.0 gm/ L Difco bacto agar. The PH was adjusted to 5.7 and autoclaved at 121ºC for 15 minutes. The cultured explants were incubated under 16 hours of artificial light (Fluorescent light) and 8 hours of darkness at average temperature of 27-28ºC.

2.1 The Following Experiments were Carried Out

2.1.1 Establishment stage

2.1.1.1 Effect of the culture medium and explant type

Different explants i.e. shoot tips and one nodal cuttings were cultured on different nutrient media i.e. Murashige and Skoog (M.S.); modified Murashige and Skoog; and Woody Plant Medium (W.P.M) to determine the best suitable medium and explant type.

2.1.1.2 Effect of anti-oxidant treatments

Variable anti-oxidant compounds were tested either alone or in combination to select the best anti-oxidant treatment minimized accumulation of the free phenolic compounds which is toxic and causing necrosis for the explants.

The anti-oxidant treatments were used as follow:

1. **Control:** the explants were immersed in sterilized distilled water as pre-treatment for 2 hours.
2. Polyvinylpyrrolidone (PVP) was added to the culture medium at 100 mg/ L as recommended by Siqueira, et al. [16].
3. **Anti-oxidant solution (A.O.S.):** The explants were immersed in A.O.S mixture which consists of a mixture of 100 mg/L ascorbic acid and 150 mg/ L citric acid) for 2 hours as recommended by [17].
4. Activated charcoal (A.C.) was added to the culture medium at 3000 mg/ L.
5. Combination of (PVP) and (A.C).
6. Combination of (PVP) and (A.O.S.)
7. Combination of (A.O.S.) and (A.C.) and (P.V.P).

2.1.1.3 Effect of organic additives

Different organic additives were added to the culture medium to select the best organic additive encouraged the best growth and development of the explants.

The following additives were tested:

1. Control (no additives were used in the culture medium)
2. Tryptophane was added at 100 mg/ L to the culture medium.
3. Coconut water: 10% of coconut water was supplemented to the culture medium.
4. Adenine sulphate: at 80 mg /L was added to the culture medium.
5. Tryptophan+ Coconut water.
6. Tryptophan+ Adenine sulphate.
7. Coconut water+ Adenine sulphate.
8. Tryptophan+ Coconut water+ Adenine sulphate.

2.1.1.4 Effect of natural fruit extracts additives

Ripened fruit of banana and papaya were taken, peeled and the pulps of the these fruit were cut into 1cm squares and 50,100,and 200 g/ L of each tested fruit type were taken and homogenized in presence of distilled water for 2 or 3 minutes in kitchen blender and added to MS medium [18]. However, coconut water was extracted from tender fruit then filtered with filter paper to get rid of unwanted debris to testify their beneficial effect on different parameters of *in vitro* plants as a substituent of some growth regulators. Also, to select the most suitable additives and the best concentration that maximizes growth, greening, and survival (%) parameters. The experiment was conducted as follow:

1. **Control:** The culture medium was supplemented with 1.0 mg/L 6-benzylaminopurin (BAP) and 0.1 mg/L Indole-3-butyric-acid (IBA).
2. **Coconut water (CW):** The coconut water was added at levels 5,10 and 20%
3. **Banana pulp (B p):** The homogenized pulp was added at rats of 50, 100 and 200 g/ L
4. **Papaya juice (PJ):** The juice was supplemented at levels 50, 100 and 200 g /L

2.1.1.5 Effect of natural additives combinations

This experiment was carried to find out the effect of different combinations of coconut water, banana pulp ,and Papaya juice in relation to control(the best treatment in the previous experiment) to determine the most effective combination that enhanced the highest No. of shoots/plant, Shoot length, and Greening parameters as follow:

1. **Control:** The culture medium was supplemented with coconut water at 10% (as this treatment gave the best results in the previous experiment)

2. Combination of Coconut water at 5% and Banana pulp at 50 g/ L
3. Combination of Coconut water at 5% and papaya juice at 50 g/ L.
4. Combination of Banana pulp at 5% and papaya juice at 50 g /L.
5. Combination of coconut water at 5%, banana pulp at 50 g/L and papaya juice at 50 g/L.

2.1.1.6 Effect of vitamin mix strengths

The vitamins mix of Murashige and Skoog [18] was used as control. However, vitamins mix of Gamborg medium as recommended by [19] which include Ca-pantothenate, Nicotinic acid, Pyrodoxine hcl, Thiamin hcl, Riboflavin at concentration 1.0 mg/L, Folic acid at 2.0 mg/L and 0.5 mg/L Glycine were prepared. Different strengths of vitamins mixes of Gamborg medium were evaluated (double, full or half strength) to study the effect of these strengths of vitamins and select the best level maximized Survival % and Explant development parameters of the explants.

2.1.2 Proliferation stage

2.1.2.1 Effect of different cytokinin types and concentrations

Different cytokinin types i.e. kinetin (Kin.), 6-benzylaminopurin (BAP) and 2-isobentenyl-adenine (2-ip) with different concentrations i.e. 0.0, 0.5, 1.00 and 2.00 mg /L were studied to determine the best cytokinin type with suitable concentration that enhance the best growth and proliferation parameters.

2.1.3 Rooting

2.1.3.1 Shoot elongation

Effect of medium strength: Different medium strengths i.e. full, one half, one-fourth, and one eighth were tested to find out the best medium strength induced the longest Shoot length and root primordial.

Effect of gibberellic acid (GA3) concentrations: GA3 at different concentrations i.e- 0.0, 1.0, 2.0 and 3.0 mg/L were evaluated to select the recommended concentration of GA3 that enhance the shoot length and number of roots.

2.1.3.2 Root formation

Effect of auxin type: Different auxin types i.e. indole acetic acid (IAA), indole-3-butyric acid (IBA) and Naphthalene acetic acid (NAA) at 1.0 mg/L were evaluated to find out the best auxin type maximized Shoot and Root length.

Effect of indole-3-butyric acid (IBA) concentrations: Different IBA concentrations i.e. 0.0, 0.5, 1.0 and 2.0 mg/ L were studied to detect the best IBA concentration that induced the best Shoot length and number of roots.

2.1.4 Data and calculations

Scores were given for Growth, Greening, and Explant development parameters. These scores were estimated as follow: negative results = 1, below average = 2, average = 3, above average = 4 and excellent results = 5. .However, the reverse is true for browning, and necrosis according to [20]. On the other hand, proliferation and number of roots parameters are estimated by counting their numbers. Shoot length determined by measuring the shoot length (cm). Meanwhile, Survival percentage was calculated as follow: Survival % = number of survived plants ×100 / total number of starting plants.

2.1.5 Statistical analysis

All tested treatments in this study were arranged in a complete randomized block design and replicated four times with three jars for each replicate. The obtained date were subjected to analysis of variance and statistically analyzed according to Duncan's multiple range test [21] at 1% level.

3. RESULTS AND DISCUSSION

3.1 Establishment Stage

3.1.1 Effect of culture medium and explants type

Table 1 reveals the effect of different medium and explant types on Necrosis, Explant development, Survival percentage, Greening and Browning scores of *Myrtus communis*. Referring to the effect of medium type, it is clear from Table (1a) that both Murashige and Skoog medium and Modified Murashige and Skoog medium significantly increased Survival percentage, explant development and greening

scores as compared with Woody Plant Medium (W.P.M). In the same time, they reduced both necrosis and browning scores in comparison with Woody Plant Plant Medium. Concerning the effect of explant types, it is obvious from Table (1b) that shoot tips were significantly superior in explant than one node cuttings in maximizing survival (%), explant development and greening parameters. However, the reverse was true when necrosis and browning parameters were considered. Referring to the interaction between medium types and explants, its appear from Table (1c) that culturing of shoot tip as explant on either MS. or modified MS. medium was significantly surpassed other tested combinations in improving survival (%), explants development, and greening parameters. Otherwise, necrosis and browning parameters behaved differently. The aforementioned results conclude that using of either Murashige and Skoog or modified Murashige and Skoog medium in combination with shoot tip as explant were significantly induced the highest survival (%), explants development and greening parameters. These results are somewhat in line partially with the findings of [8] who recommended using of Murashige and Skoog medium for micro-propagation of Myrtus plants. Also, with the findings of [22], they stated that shoot tips were preferred in improving explants development parameters of myrtle. However, these results disagrees with the findings of [9]. They found that Woody Plant Medium surpassed other media in micro-propagating of Myrtle plants.

3.1.2 Effect of anti-oxidant treatments

Table 2 clarifies the effect of different anti-oxidant treatments on explants development and greening parameters. It is clear that treating of the explants with the combination of anti-oxidant solution as pre-treatment and addition of Polyvinylpyrrolidone to the cultured medium induced significant enhancement of both explant development and greening parameters in relation to other anti-oxidant treatments. However, the opposite was true in case of necrosis and Browning parameters. Similarly, combination of anti-oxidant solution, Polyvinylpyrrolidone, and Activated charcool took the second rank in reducing free phenolic compounds and induced suitable conditions for improving micro-propagation of Myrtus plant. The aforementioned results summarized that combination of anti-oxidant solution as pre-treatment and addition of Polyvinylpyrrolidone to cultured medium encouraged the best improvement of both explants development and greening while, reduced both necrosis and browning parameters. This combination was valuable in changing free phenols to conjugated ones which are less harmful to the explants and in turn maximizing explants development and greening parameters. These results are in general agreement with the findings of Abd [23] who found that combination of anti-oxidant solution and Polyvinylpyrrolidone solution, enhanced a significant increase of explants development and greening parameters while the reverse was true for both necrosis and browning parameters as compared with the others.

3.1.3 Effect of additives

Data in Table 3, showed the effect of different additives on necrosis, survival percentage, and explants development parameters. It is appear that supplementation of culture medium with combined treatment of tryptophan plus coconut water plus adenine sulphate induced a significant increase in Survival percentage, and explants development parameters, while reduced necrosis parameter as compared with the other treatments. Meanwhile, all other tested combined treatments came next in enhancing a significant increase of survival (%) and explants development parameters and reduced necrosis in relation to other treatments. Furthermore, addition of coconut water to the culture medium promoted significant enhancement in all parameters under study in comparison with tryptophan and adenine sulphate treatments. In General conclusion, supplementation of the culture medium with Tryptophan plus coconut water plus adenine sulphate enhanced all studied parameters since they contains some traces of cytokinins which valuable in inducing a promotive effect as recommended by [24].

3.1.4 Effect of natural (fruit extracts) additives

Table 4 verifies the effect of different natural additives i.e (Coconut water, banana pulp, papaya extract) on Necrosis, Growth, Greening, and Survival (%). It is quit evident that using of natural fruit extract either coconut water, banana pulp, or papaya juice were significantly improved all parameters under study as compared with control. Also, coconut water at 10 % statistically improved most studied parameters as it increased growth, greening, and survival (%) parameters while, decreased necrosis in comparison with the other additives. Moreover, papaya juice at different concentrations

statistically surpassed banana pulp in improving growth, greening and survival % parameters. Meanwhile, successive increase from 100 to 200 g/L of natural additives showed more or less promising effect on most parameters under study. Using of natural additives were recommended in improving of most parameters under study. The above results conclude that addition of natural additives (fruit extracts) to the culture medium were significantly surpassed control in improving most of parameters under study. Also, coconut water at level of 10% was recommended as it improved the most studied parameters i.e growth, greening and survival (%).

Table 1a. Effect of medium type on survival (%) and explant development parameters of *Myrtus communis* plant

Parameters / Medium type	Necrosis (Scores)	Survival (%)	Explant development (Scores)	Greening (Scores)	Browning (Scores)
Murashige and Skoog	2.28b	21.33a	2.11a	2.42a	2.12b
Woody Plant Medium	3.02a	7.15b	1.17b	1.30b	3.26a
Modified Murashige and Skoog	2.85a	17.00a	1.97a	2.27a	2.30b

Means of medium type followed with the same letter (s) within each column are not significantly different at 1% level

Table 1b. Effect of explant type on survival (%) and explant development parameters of *Myrtus commmunis* plant

Parameters / Explant type	Necrosis (Scores)	Survival (%)	Explant development (Scores)	Greening (Scores)	Browning (Scores)
Shoot tips	2.18b	21.22a	2.04a	2.36a	2.86a
One node cuttings	3.25a	9.10b	1.45b	1.63b	2.25b

Means of explants type followed with the same letter (s) within each column are not significantly different at 1% level

Table 1c. Effect of combinations of medium and explant types on survival (%) and explants development parameters of *Myrtus communis* plant

Explant type	Medium type	Necrosis (Scores)	Survival %	Explant development (Scores)	Greening (Scores)	Browning (Scores)
Shoot tip	MS.	2.04c	29.33a	2.63a	2.96a	2.33cd
	W.P.M	2.42b	11.00b	1.00b	1.37bc	3.76a
	Modified MS.	2.08c	23.33a	2.49a	2.75a	2.50bc
One node cuttings	MS.	2.52b	13.33b	1.59a	1.88b	1.90e
	W.P.M	3.62a	3.29c	1.33b	1.23c	2.75b
	Modified MS.	3.62a	10.67b	1.44b	1.79bc	2.10de

Means of combinations of medium and explants types followed with the same letter (s) within each column are not significantly different at 1% level

Table 2. Effect of different antioxidant treatments on Explant development parameters of *Myrtus communis plant*

Parameters / Antioxidant treatments	Necrosis (Scores)	Browning (Scores)	Greening (Scores)	Explant development (Scores)
Control (sterilized distilled water)	4.00a	4.00a	1.32e	1.27f
Poly vinyl pyrrolidone (P.V.P)	3.23b	3.64a	2.82b	1.82df
Antioxidant solution (A.O.S)	2.92bc	2.70b	2.40c	2.30c
Activated charcoal (A.C)	3.74a	3.82a	1.84d	1.62e
P.V.P + A.C	2.60c	2.23c	2.39c	2.05cd
A.O.S + P.V.P	2.03d	2.10c	3.85a	3.90a
A.O.S+ P.V.P+ A.C	2.68c	2.88b	2.86b	3.11b

Means of antioxidant treatments followed with the same letter (s) within each column are not significantly different at 1% level

Table 3. Effect of different additive treatments on explant development parameters of *Myrtus communis* plant

Parameters / Treatments	Necrosis (Scores)	Survival (%)	Explant development (Scores)
Control	3.70a	5.00h	1.60h
Tryptophane	3.20b	30.00g	2.20g
Coconut water	3.00cd	50.00e	3.00e
Adenine sulphate	3.10bc	40.55f	2.60f
Tryptophan+ Coconut water	2.50e	63.30c	3.70c
Tryptophan+ Adenine sulphate	2.90d	50.20d	3.20d
Coconut water+ Adenine sulphate	2.40e	72.00b	3.90b
Tryptophan+ Coconut water+ Adenine sulphate	1.90f	79.00a	4.10a

Means of different additives treatments followed with the same letter (s) within each column are not significantly different at 1% level

Table 4. Effect of different natural additives types with different concentrations on growth, greening and survival (%) parameters of *Myrtus communis* plant

Natural additives types	Concentrations (%)	Necrosis (Scores)	Growth (Scores)	Greening (Scores)	Survival (%)
Control	0.0	2.20cd	1.70h	1.50f	5.50h
Coconut water	5%	1.80e	2.50c	2.60b	40.00c
(CW)	10%	1.90e	3.00a	2.80a	49.85a
	20%	2.20cd	2.30d	2.88a	45.00b
Banana pulp	50g/L	2.30c	1.50i	1.60f	16.00f
(BP)	100 g/L	2.60b	2.00fg	1.80e	15.00f
	200 g /L	3.00a	2.10ef	1.90de	12.85g
papaya extract	50 g/L	2.10d	1.90g	2.00d	16.00f
(PE)	100 g/L	2.30c	2.20de	2.40c	18.00e
	200 g /L	2.70b	2.80b	2.00d	20.00d

Means of different natural additives types with different concentrations%. Followed with the same letter (s) within each column are not significantly different at 1% level

This indicates that natural additives are valuable in supplying the explants with suitable contents beneficial for growth and development. These results confirmed the findings of [15]. They stated that natural additives consider as a natural source of carbon and contain natural vitamins, phenols, fiber, hormones, and proteins.

3.1.5 Effect of different combinations of natural additives

Referring to the effect of using of combined of natural additives, Table 5 shows the effect of different combinations on necrosis, shoot numbers, shoot length, and greening parameters. It is quit evident that addition of combination of coconut water plus banana pulp plus papaya extract were encouraged a significant increase in number of shoots /plant, shoot length and greening, parameters in comparison with the other tested treatments. Otherwise, this combination statistically induced the lowest adverse effect of necrosis in relation the others. Moreover, all tested additives combinations supplemented to the culture medium showed more or less significant improvement in studied parameters under study as compared with control.

In general, the aforementioned results conclude that the natural additives are recommended to substitute the highly expensive chemicals specially growth regulators as compared with control. This may be due to presence of traces of promising substances which encourage growth and other measurements. Also, the natural additives contain others like organic carbohydrates, vitamins, enzymes, and many traces. These results are somewhat agree with the findings of [13] who reported that natural additives are recommended as it contained in addition to sugar other nutrient as proteins, lipids and minerals.

3.1.6 Effect of vitamins combination strengths

Table 6 explains the effect of different vitamins mix strengths on necrosis, explant development and survival (%) of *Myrtus communis*. It is clear that duplicating vitamins mix strength in the culture medium induced a significant increase in Survival (%) as compared with control or half vitamins mix strength. However, a statistical increase in explant development parameter was showed as half, full or duplicate vitamins mix strengths were added to the culture medium in relation to control. Also, the opposite result was true when necrosis parameter was considered.

3.2 Proliferation

3.2.1 Effect of different cytokinin types and concentrations

Table 7 shows that supplementing the culture medium with 0.5 mg /L kinetin induced a significant enhancement of growth parameters while produced the lowest significant Necrosis parameter in comparison with the other treatments. However, proliferation parameter was significantly maximized by using 1.0 mg/ L from 6-benzylaminopurin (BAP) as compared with the other cytokinin types and concentrations. On the other hand, using higher concentrations of all tested cytokinins induced an adverse effect on either growth or necrosis parameters. However, the lower cytokinin concentrations induced the best effect on growth and necrosis parameters. Moreover, BAP is more effective than both 2-ip and kinetin in increasing proliferation parameter. The aforementioned results summarized that lower concentration (0.5 mg L) is recommended for Growth and Necrosis while 1.0 mg L from BAP induced the highest proliferation parameter. These results are in general agreement with the findings of [25]. They declared that adding 1.0 mg / L of BAP to MS medium enhanced the best shoot proliferation of carnation.

3.3 Rooting

3.3.1 Shoot elongation

3.3.1.1 Effect of medium strength

Referring the effect of different medium strengths, Table 8 shows that using half medium strength induced a significant increase in shoot length as compared with the other medium strengths. However, number of roots / plant were statistically increased due to either one half or one fourth medium strength in relation to one-eighth medium strength. The abovementioned results conclude that one-half medium strength enhanced the highest Shoot length and number of roots. This occurred due to increase the content of free water which decreased osmotic pressure of the cultured medium and in turn increased absorption ability and finally improved most of the studied parameters. These results are in harmony with the results of [26] who mentioned that half strength M.S. medium maximized the best shoot length of *Paulownia tomentosa* plants.

3.3.1.2 Effect of gibberellic acid (GA3) concentrations

Table 9 reveals that supplementing the culture medium with 2.0 mg/L GA3 was effective in increasing Shoot length as compared with the other tested concentrations. Meanwhile, control treatment was valuable in significantly increasing the no. of roots/plant parameter. In general the aforementioned results verify that using of 2.0 mg L GA3 maximized Shoot length parameter. These results are somewhat in line with the findings of [23]. Who found that dwarf shoots of Cupressus sempervirens were elongated on MS medium supplemented with 2.0 mg/ L of GA3.

3.3.2 Root formation

3.3.2.1 Effect of auxin type

Table 10 explains the effect of different auxin types on shoot length and number of roots/plant parameters. It was found that using of naphthalene acetic acid (NAA) was significantly increased shoot length parameter as compared with the other used auxins (indole-3-butyric acid and indole acetic acid). However, number of roots/plant parameter was statistically maximized as indole-3-butyric acid (IBA) was used in relation to other auxin types. However, Necrosis parameter was significantly decreased when either NAA or IAA auxin types was used in comparison with IBA auxin type. The aforementioned results indicated that IBA is recommended for increasing Number of roots while NAA improved Shoot length parameter. These results are in harmony with the findings of [27]. They found that IBA was more active than IAA or NAA in promoting root development of *Salvia fruticosa*, Mill.

3.3.2.2 Effect of indole-3-butyric acid (IBA) concentrations

Table 11 verified that control treatment followed with lower IBA concentrations (0.5 and 1.0 mg L) were valuable in inducing the highest Shoot length as compared with the highest concentrations. On the other hand, the Number of roots parameter was significantly increased due to adding 2.0 mg/ L of IBA to the culture medium in relation to the other tested concentrations. The above study conclude that adding IBA at 2.0 mg/ L was effective in maximizing the Number of roots / plant. These results are in harmony with findings of [28]. They mentioned that the best

Table 5. Effect of different combinations of natural additives on number of shoots/plant, shoot length and Greening parameters of *Myrtus communis* plant

Parameters / Natural additive treatments	Necrosis (scores)	No. of shoots/ plant (scores)	Shoot length (cm)	Greening (scores)
Control	2.20a	2.40e	0.75d	2.10d
Coconut water+ Banana pulp	1.99b	2.48d	0.83d	2.70b
Coconut water+ papaya extract	1.80c	2.65d	0.92c	2.20c
Banana pulp+ papaya extract	1.60d	2.50c	1.05b	2.00e
Coconut water + Banana pulp+ papaya extract	2.03b	2.89a	1.15a	3.50a

Means of different natural additives treatments with different combinations followed with the same letter (s) within each column are not significantly different at 1% level

Table 6. Effect of vitamins combination strength on necrosis, explant development and survival (%) of *Myrtus communis* plant

Parameters / Vitamins treatments	Necrosis (scores)	Survival (%)	Explant development (scores)
Control (MS vitamins)	3.67a	7.67c	1.63b
Vitamine mix(half)	1.98b	62.33b	3.47a
Vitamin mix(full)	1.33b	71.00ab	4.00a
Vitamin mix (duplicate)	1.17b	79.00a	4.23a

Means of pretreatments of vitamins followed with the same letter (s) within each column are not significantly different at 1% level

Table 7. Effect of different cytokinin types with different concentrations on proliferation and growth parameters of *Myrtus communis*

Cytokinin type	Concentrations (mg/L.)	Necrosis (scores)	Growth (scores)	Proliferation (scores)
Control	0.0	2.17d	2.16e	1.95e
Ki.	0.5	1.17e	3.50a	1.97e
	1.0	2.09d	3.00bc	2.90cd
	2.0	3.17b	2.13e	3.37b
BAP	0.5	2.73c	2.60d	2.68d
	1.0	2.93bc	1.93e	4.70a
	2.0	3.87a	1.50f	2.86cd
2-IP	0.5	1.97d	3.10b	2.10e
	1.0	2.03d	2.75cd	3.07c
	2.0	3.17b	1.25f	3.40b

Means of Cytokinin type with concentrations mg/L. followed with the same letter (s) within each column are not significantly different at 1% level

Table 8. Effect of different medium strengths on shoot length and number of roots/plant parameters of *Myrtus communis* plant

Parameter / Medium strength	Necrosis (scores)	Shoot length (cm)	No. of roots/plant (scores)
Full strength	2.06a	3.26b	1.77b
One half strength	1.70b	4.00a	2.17a
One fourth strength	1.41c	2.17c	1.89ab
One eight strength	1.25c	1.63d	1.03c

Means of medium strength followed with the same letter (s) within each column are not significantly different at 1% level

Table 9. Effect of GA3 concentration on shoot length and number of roots/plant of *Myrtus communis* plant

Parameters / Treatments	Necrosis (scores)	Shoot length (cm)	No. of Roots/plant (scores)
Control	1.96c	1.93d	2.63a
1.0 mg/L GA3	1.50d	3.21b	1.62b
2.0 mg/L GA3	2.93b	4.51a	1.83b
3.0 mg/L GA3	3.44a	2.77c	1.24c

Means of GA3 concentrations/L. followed with the same letter (s) within each column are not significantly different at 1% level

Table 10. Effect of different auxin type on shoot length and number of roots parameters of *Myrtus communis* plant

Parameters / Treatments	Necrosis (scores)	Shoot length (cm)	No. of roots/plant (scores)
IBA	2.17a	5.72b	3.50a
NAA	1.17b	7.00a	2.34b
IAA	0.99b	5.71b	1.82c

Means of different auxin type followed with the same letter (s) within each column are not significantly different at 1% level

Table 11. Effect of different IBA concentrations on shoot length and number of roots parameters of *Myrtus communis* plant

Parameters / Treatments	Necrosis (scores)	Shoot length (cm)	No. of Roots (scores)
Control	1.50d	6.21a	1.00c
0.5 mg/L IBA	1.86c	5.14b	2.04b
1.0 mg/L IBA	2.69b	4.64b	2.19b
2.0 mg/L IBA	3.73a	3.63c	3.15a

Means of different IBA concentrations followed with the same letter (s) within each column are not significantly different at 1% level

rooting of Rosa hybrida L. was obtained when using 2.0 mg/L of IBA was used. Also, [29] who stated that rooting Rosa damascena plant occurred with the highest frequency of cultured on a medium containing 2.0 mg/ L of IBA.

The following photos represent different stages of *in vitro Myrtus communis* plants from establishing to rooting stages. Photo (A) explain the development of the explant at the final of establishment stage. Also, photo (B) showed increasing the numbers of plantlets at the end of proliferation stage Moreover, during rooting stage, two phases appear the photo (C) represent the Shoot elongation phase and finally root formation phase appeared in both (D and E) photos.

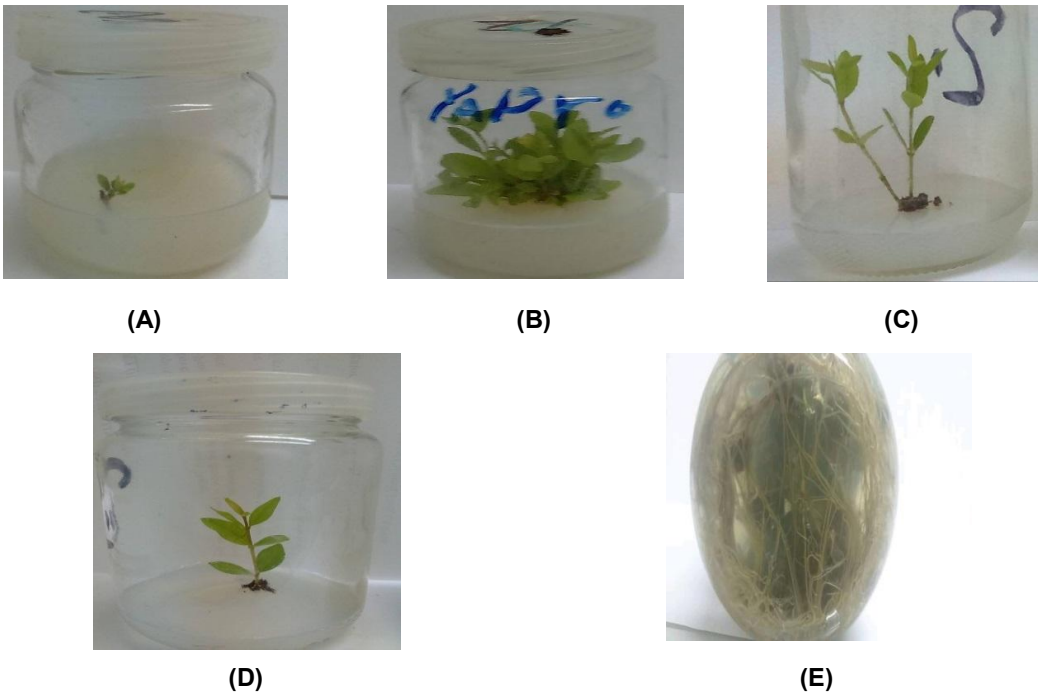

Fig. 1. From (A to E). Developmental stages *In vitro* propagation of *Myrtus communis* plant

4. CONCLUSION

Consequently, it ispreferable to obtain an integrated protocol for in vitro propagation of *Myrtus communis* plant, Treating the shoot tip with anti-oxidant solution and culturing on Murashige and Skoog or modified Murashige and Skoog medium supplemented with P.V.P and A.O.S as anti-oxidant treatment, as well as adding combination of tryptophan, adenine sulphate and coconut water as additive, duplication of vitamin mix strength of Gamborg medium during establishment stage. Moreover, addition of 1.0 mg / L of BAP to induce the highest proliferation. Meanwhile, addition of 2.0 mg/L of GA3 to half strength medium for enhancing shoot length. Also, addition of 2.0 mg / L IBA to encourage the highest number of roots The coefficient of the combination of coconut water at 5%, banana pulp and papaya pulp at 50 g/L in this study achieved clear superiority in the all studied parameters.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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