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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2020 17(4): 4015-4020. **OPEN ACCESS**

De novo direct and indirect regeneration of *Rosmarinus officinalis*

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Regeneration of plants in vitro are crucial for both plant biotechnology and the fundamental study of plant biology. *Rosmarinus officinalis* is a well-known medicinal plant and its regeneration *in vitro* is of wide biotechnological applications. In this work, *R. officinalis* was regenerated directly from nodal segments of the mother plant and indirectly via de novo organogenesis using MS, BA and NAA. Primary callus was obtained using MS + 2.0 mg l^{-1} NAA + 1.0 mg l^{-1} BA within 5-6 weeks. One gram of the primary callus was transferred to MS media plus (0.1, 0.2 and 0.3 mg l^{-1}) NAA combined with (0.03 and 0.06 mg l^{-1} each) BA. For rooting, the obtainable shoots were transferred to 0.1, 0.2 and 0.3 mg l^{-1} NAA. MS plus 0.2 mg l^{-1} BA + 0.03 mg l^{-1} NAA was superior to the other formulations in inducing shoot morphogenesis both directly and indirectly. Using this growth regulators combination, there was no difference between both modes of regeneration with respect to the number of the shoots regenerated (2.33±0.33). The differences in the length of the shoots obtained were statistically insignificant, while the number of leaves increased from 21.67±0.88 in the regenerants directly to 28.67±1.45 in the shoots regenerated indirectly. The obtained shoots (direct and indirect) regeneration were successfully rooted using MS medium plus 0.02 mg l^{-1} NAA where the rooting percentage reached up 70%, the number of rootlets per regenerant reached up 16 and the average root length was 3.7 cm.

Keywords: Callus cultures; *in vitro* organogenesis; medicinal plants; tissue culture; *Rosemary*.

INTRODUCTION

Rosmarinus officinalis L. (Rosemary plant) is significant importance due to its medicinal and aromatic constituents. It is a member of the Labiatae family (Hussain et al. 2010) and widely distributed in Mediterranean countries.

It has to be mentioned that the genus *Rosmarinus* has been merged into the genus *Salvia* in a recent phylogenetic analysis. This means that the *Rosmarinus officinalis* is no longer the correct name of the species studied as declared by (Lucus et al. 2020). (*Rosmarinus officinalis* essential oil has many uses such as antioxidant, cytotoxic, antibacterial, antimutagenic and used in treatment of gastrointestinal ailments. It has a great effect in various spasmodic conditions such as renal and biliary colic spasm and potentially used to treat inflammatory bowel diseases (Minaiyan et al. 2011; Hussein et al. 2017).

Tissue culture used for a lot of biotechnological purposes like regeneration of plants *in vitro* or wide applications of callus and cell suspension cultures. Regeneration has been employed to enhance the mass production, quality, and health condition of the adopted or targeted plants (Ghorpade et al. 2012). Plants can be regenerated through somatic embryogenesis or de novo organogenesis as stated by many authors like Birnbaum and Sánchez Alvarado

2008; Duclercq et al. 2011; Sugimoto et al. 2011; Gaillochet and Lohmann 2015.

De-novo organogenesis it is mean *in vitro* genesis of organs (shoots or roots) from cultured explants. Indirect organogenesis (organogenesis with an intervening callus phase) is achieved by inducing callus formation from plant explants on a media for induced callus (CIM) followed by transfer to a shoot inducing medium (SIM). It can be understood from the work of (Skoog and Miller 1957; Duclercq et al. 2011; Cheng et al. 2013) that every step or stage has its own nutritional, hormonal, physical and cultural conditions. Many research reported that, de-novo shoot organogenesis is dependent many factors for example, (cell division progression, organogenetic competence on callus media CIM, the partition responses to auxin and cytokinin , cytokinin signal perception, and other (Che et al. 2002; Che et al. 2007; Gordon et al. 2007; Atta et al. 2009; Cheng et al. 2013; Kareem et al. 2015; Ikeuchi et al. 2016; Zhang et al. 2017).

Due to the shortage and low number of studies concerning regeneration of Rosemary *in vitro*, the main objective of the present study was set to regenerate *Rosmarinus officinalis* via indirect de novo shoot organogenesis.

MATERIALS AND METHODS

Plant material

The mother plant "*Rosmarinus officinalis* L.", from which explants were taken to initiate callus culture is a well identified growing medicinal herb of 8 years old at the botanical garden of Botany and Microbiology Department, Faculty of science, Al-Azhar University, Cairo, Egypt. The leaves and nodal segments of *Rosmarinus officinalis* were surface sterilized using Clorox at 10% (v/v) for 15 min.

Culture medium

The basal salts mixture of MS medium (Murashige and Skoog 1962) containing 25 g L^{-1} sucrose, solidified with 7.0 g L^{-1} agar and pH adjusted to 5.7 ± 1.

Treatments

For primary callus: It was obtained directly from leaf explants of new outgrowth of the mother plant according to (Hussein et al. 2017) by impregnating the MS culture media with NAA at 2.0 mg l^{-1} plus BAP at 1.0 mg l^{-1} after 5-6 weeks.

For both direct and indirect shoot organogenesis was studied by transferring nodal segments of the mother plant and equal pieces (one gram of the primary mother callus) to MS culture media containing the following plant growth regulators formulations:

a- 0.1 mg l^{-1} BA + 0.03 mg l^{-1} NAA.
b- 0.2 mg l^{-1} BA + 0.03 mg l^{-1} NAA.
c- 0.3 mg l^{-1} BA + 0.03 mg l^{-1} NAA.
d- 0.1 mg l^{-1} BA + 0.06 mg l^{-1} NAA.
e- 0.2 mg l^{-1} BA + 0.06 mg l^{-1} NAA.
f- 0.3 mg l^{-1} BA + 0.06 mg l^{-1} NAA.

Root morphogenesis

For root formation, the proliferated microshoots obtained *in vitro* were relocated to MS culture medium supplemented with NAA at 0.1, 0.2 and 0.3 mg l^{-1}. was investigated.

Cultural conditions:

Cultures were generally incubated in growth room under controlled conditions, where temperature was maintained at 25 ± 1 °C day/night schedules at light intensity of 1500 lx using white cool fluorescent lamp 120 cm long 40 watts.

Statistical analysis:

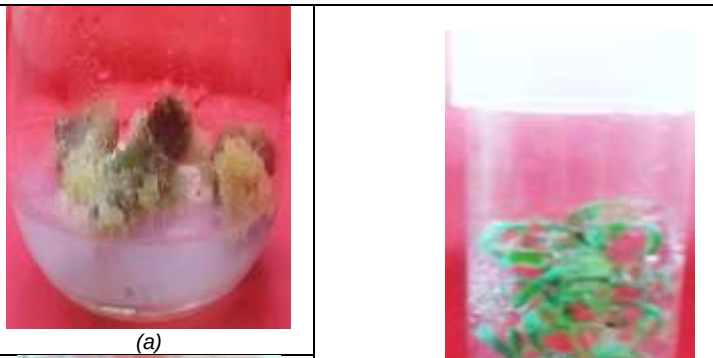
The obtained results are exhibited as the mean of three replicates. Analysis of data was conducted by variance (ANOVA) through a statistical package SigmaPlot v12.5. However, the chief difference between the treatments was analyzed by Tukey HSD test at $P < 0.05$.

RESULTS AND DISCUSSION

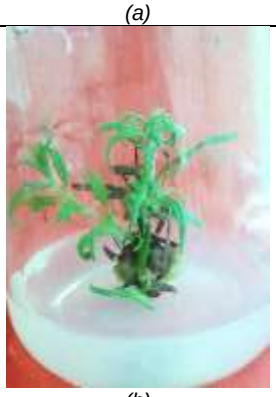
Regeneration of shoots *in vitro*

Several of trials were done to regenerate shootlets of *R. officinalis* both directly (from stem nodal explants without an intervening callus phase) and indirectly (from a callus initiated from stem segments of the mother plant) using MS culture media supplemented with various BA and NAA formulations.

The results illustrated in table (1) show the effect of different BA/NAA concentrations on the regeneration process from rosemary nodal segments. The number of shoots obtained per single nodal segment ranged between 2.33±0.33 to 1.0±0.0 and the average length of the shoots obtained per segment ranged between 7.7±0.5 cm and 1.17±0.03 cm while the number of leaves ranged between 21.67 ± 0.88 and 4.0 ± 0.0.



(a)



(b)

(c)

(d)

Figure 1: Regeneration of R. officinalis in vitro. a: Primary callus obtained on MS + 2.0 mg/L NAA + 1.0 mg/L BAP after 5 weeks of growth, b: Indirect shoot morphogenesis from callus obtained from primary callus after being transferred to MS + 0.2 mg/L BA + 0.03 mg/L NAA, c: Direct shoot morphogenesis from sterile stem nodal explants of the mother plant using MS + 0.2 mg/L BA + 0.03 mg/L NAA and d: Rooted regenerants on MS + 0.2 mg/l NAA.

Table 1: Regeneration of shoots *in vitro* from stem nodal segments of *R. officinalis*.

No.	Treatment	Shoot length (cm)	Leaves no.	Shoot no.
		Mean±SE	**Mean±SE**	**Mean±SE**
1	0.1 mg l^{-1} BA + 0.03 mg l^{-1} NAA	4.4±0.153 b	16.33±0.33 b	1.33±0.33 b
2	0.2 mg l^{-1} BA + 0.03 mg l^{-1} NAA	7.7±0.5 a	21.67±0.88 a	2.33±0.33 a
3	0.3 mg l^{-1} BA + 0.03 mg l^{-1} NAA	3.53±0.291 bc	10±1 c	1±0 b
4	0.1 mg l^{-1} BA + 0.06 mg l^{-1} NAA	2.97±0.033 c	8.67±0.67 c	1±0 b
5	0.2 mg l^{-1} BA + 0.06 mg l^{-1} NAA	3.33±0.88 bc	15.33±1.86 c	1±0 b
6	0.3 mg l^{-1} BA + 0.06 mg l^{-1} NAA	1.17±0.03 d	4±0 d	1±0 b
	F ratio	24.76	41.49	7.7
	P value	***	***	**

Each value is a mean of three determinations± standard error. Columns with similar letters are non-significant difference according to Fisher LSD. ** = significant at P < 0. 01, *** = significant at P < 0. 001, (Note: each Colum of means compared according to different treatments showed in first Colum).

Table 2: Regeneration from callus cultures of *R. officinalis*.

No.	Treatment	Shoot length (cm)	Leaves no.	Shoot no.
	0.1 mg l^{-1} BA + 0.03 mg l^{-1} NAA	Mean±SE	Mean±SE	Mean±SE
1		4.87±0.18 b	20±1.16 b	1.33±0.33 b
2	0.2 mg l^{-1} BA + 0.03 mg l^{-1} NAA	8.4±0.61 a	28.67±1.45 a	2.33±0.33 a
3	0.3 mg l^{-1} BA + 0.03 mg l^{-1} NAA	4.1±0.23 b	13.33±0.88 c	1±0 b
4	0.1 mg l^{-1} BA + 0.06 mg l^{-1} NAA	3.43±0.19 bc	12±0.58 c	1±0 b
5	0.2 mg l^{-1} BA + 0.06 mg l^{-1} NAA	5.17±1.55 b	17±4.04 bc	1±0 b
6	0.3 mg l^{-1} BA + 0.06 mg l^{-1} NAA	1.8±0.17 c	5.33±1.16 d	1.33±0.33 b
	F ratio	9.98	17.96	7.7
	P value	***	***	**

Each value is a mean of three determinations± standard error. Columns with similar letters are non-significant difference according to Fisher LSD. ** = significant at P < 0. 01, *** = significant at P < 0. 001, (Note: each Colum of means compared according to different treatments showed in first Colum).

Table 3: Comparison of regeneration from nodal segments and callus using 0.2 mg l-1 BA + 0.03 mg l-1 NAA

Regeneration from	Shoot length (cm)	Leaves no.	Shoot no.
	Mean±SE	Mean±SE	Mean±SE
Nodal segments	7.7±0.5 a	21.67±0.88 a	2.33±0.33 a
Callus culture	8.4±0.61 a	28.67±1.45 b	2.33±0.33 a

Each value is a mean of three determinations± standard error. Columns with similar letters are non-significant difference according to Fisher LSD.

Statistical analysis of the obtained data may show that the treatment with 0.2 mg l^{-1} BA + 0.03 mg l^{-1} NAA is probably the most reproducible combination because the number of shoots, the sum of the shoot lengths and the number of leaves obtained per segment increased significantly over all the other treatments.

The results illustrated in table (2) may reveal that the number of shoots obtained per a gram callus ranged between 2.33± 0.33 to 1.0±0.0 and the average of the lengths of the shoots obtained ranged between 8.4±0.61 and 1.8±0.17 while the number of leaves ranged between 28.67±1.45 and 5.33±1.16. Statistical analysis of the obtained data may show that the treatment with 0.2 mg l^{-1} BA + 0.03 mg l^{-1} NAA is probably the most reproducible combination because the number of shoots obtained, the shoot lengths and the number of leaves obtained were significant over all the other treatments. Otherwise, the lowest number of shootlets (1) was obtained when callai were cultured in MS + 0.3 mg l^{-1} BA + 0.03 mg l^{-1} NAA and MS supplemented with (0.1 or 0.2) mg l^{-1} BA + 0.06 mg l^{-1} NAA and the lowest significant shoot length (1.8 cm) and leaves number (5.33) resulted from the treatment of primary callus with 0.3 mg l^{-1} BA + 0.06 mg l^{-1} NAA**.** The whole regeneration process is illustrated in figure (1).

The results illustrated in table (3) may show some similarities and differences between the regeneration of shoot of *R. officinalis in vitro*. There was no difference between both modes of regeneration with respect to the number of the shoots regenerated (2.33±0.33). The differences in the length of the shoots obtained is statistically insignificant, while the number of leaves increased from 21.67±0.88 in the regenerants obtained from nodal segments directly to 28.67±1.45 in the shoots regenerated via callus culture indirectly.

In vitro rooting of *the* obtained shoots

Through this study, it was observed that rooting of the shootlets obtained *in vitro* was not easy. Also, one concentration of NAA (0.2 mg l^{-1}) had effect on the rhizogensis behaviors of plantlet *in vitro* (i.e. rooting percentage, number of rootlets and root length) of *Rosmarinus officinalis*. When plantlets were grown on full strength MS medium + 0.2 mg l^{-1} NAA showed that the rooting percentage (70%), number of rootlets per regenerant reached up 16 and the average root length reached up (3.7 cm) and was observed that other treatment of NAA had no effect on the rhizogensis behaviors of plantlet *in vitro* Fig. 1 (D).

With respect to the indirect regeneration of shoots, primary callus was first initiated from sterile explants which take from mother plant of *R. officinales* after 5-6 weeks according to (Hussein et al*.* 2017) using MS culture media supplemented with 2.0 mg l^{-1} NAA plus 1.0 mg L^{-1} BA. This may be more or less similar to the results obtained by the others like (Coskun et al. 2019) who used MS

medium plus 1.0 mg l^{-1} BAP and 2.0 mg l^{-1} NAA was used for callus production for determining the changes in secondary metabolites, (Perez-Mendoza et al. 2019) who used MS medium supplemented with growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) (2.6 µM) and 6-benzylaminopurine (8.8 µM) for callus initiation from rosemary leaf segments and (Dong et al. 2012) who reported that BAP at 0.5 mg l^{-1} plus NAA at 0.5 mg l-1 and 50 g L^{-1} sucrose could be used successfully to induce callus cultures from Rosmarinus officinalis *in vitro*. (Leelavathi and Kupp 2013) obtained satisfactory callus growth when rosemary explants were planted in MS + B AP (8.88 µM) + I AA (5.70 µM).

(Dong et al. 2012), on Rosmarinus officinalis who disclosed that MS medium plus BA at 1.5 mg l-1, KT at 0.5 mg l-1 and NAA at 0.5 mg l-1 showed relatively good result with 50% regeneration rate. But when grown in 0.8 mg l-1 BA plus 0.5 mg l-1 NAA, in this case the growth rate increased more than 300%. On other hand, (Misra and Chaturvedi 1984) on *Rosmarinus officinalis* reported that BA was more effective than others in shoot induction and the best result of shoot buds (14) per explant was formed when used BA at 0.2 mg l^{-1}.

(Dong et al. 2012), on *Rosmarinus officinalis* reported that the best result in rooting stage with rooting rate reaching 65% when plantlets were planted on MS containing 0.1 mg l^{-1} NAA. But (Misra and Chaturvedi 1984) on *Rosmarinus officinalis* showed that rooting rate reaching 80% when plantlets were transferred to MS plus 0.25 mg l^{-1} IPA. (Lan et al*.* 2018) reported that the best regeneration medium treatment to induce multiple bud regeneration (3.14 shoots/explant) from rosmary stem segments was achieved by using MS augmented with BAP at 1.5 mg l^{-1}. The rate of rooted shoots was 82.22% with an average root number of 2.83 roots/shoot after Incubation culture for 4 weeks of on MS medium plus NAA at 0.3 mg l^{-1}.

CONCLUSION

Rosmarinus officinalis is a medicinal plant and its regeneration *in vitro* is of wide biotechnological applications. Callus cultures of *Rosmarinus officinalis* were obtained from leaf explants using MS + 2.0 mg/L NAA + 1.0 mg/L BA within 5-6 weeks. MS medium + 0.2 mg/L BA + 0.03 mg/L NAA was the best formulations in inducing shoot morphogenesis and complete plantlets regeneration. The obtained shoots (direct and indirect) were successfully rooted using MS medium plus 0.02 mg/L NAA. The results obtained in this study may not only help in micropropagation of *R. officinalis* but also facilitate the more perspective and advanced biotechnological studies on this species including recombinant and/or non- recombinant gene technology.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

All authors wish to acknowledge lab. of plant tissue culture, botany and microbiology department and Faculty of science and Al-Azhar university for providing the research facilities.

AUTHOR CONTRIBUTIONS

MSA, regeneration experiments supervision, analyzed the results and wrote the research. MRM data collection and performed the callus experiments. EAH edited the manuscript and data interpretation.

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