Effect of Growth Regulators on In Vitro Callus Formation of Rosemary Plant (*Rosmarinus officinalis* L.)

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ABSTRACT

The study included the use of tissue culture technique in rosemary (*Rosmarinus officinalis* L.) plant propagation starting from leaf explants and the production of callus. The first experiment consisted in the leaf explants sterilization using ten randomized replications of ethanol (C₂H₅OH), mercuric chloride (HgCl₂) and sodium hypochlorite (NaOCl) respectively, on six concentrations and five treatment periods, each. The results showed that the use of sodium hypochlorite in a concentration of 0.75% for a period of 15 minutes was the most effective by giving sterile explants after two weeks of culture. After the explants sterilization, the effect of six concentrations of benzyl adenine (BA) and six concentrations of naphthalene acetic acid (NAA) and the overlap between them on the callus formation were studied. Highest callus size (mm³), callus fresh and dry weight (g) occurred at 0.2 mg/l BA and 1.5 mg/l of NAA.

Keywords: *hormones, leaf explant, sterilization.*

INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.) is a very important medicinal plant which belongs to the Lamiaceae Family and has been cultivated for a long time (Eva *et al.*, 2003). The Lamiaceae family includes an extremely wide variety of aromatic plants, among this rich array of plants yielding essential oils (Chalchate *et al.*, 1993). Medicinal plants are used in crude or purified form in the preparation of drugs in different systems (Robins *et al.*, 1999).

Rosemary is widely used as a culinary spice and is also used for its fragrance in soaps and cosmetics.

Traditionally, rosemary has been used for medicinal cures to improve memory, relieve muscle pain and spasm, stimulate hair growth, support the circulatory and nervous systems (Foster and Tyler, 1999).

The leaves of rosemary contain 1.0-2.5% essential oils and such composition may vary according to the chemo type and the development stage at which the plant has been harvested. The essential oil is almost colorless to pale yellow liquid with a characteristic refreshing and pleasant odor (Bauer *et al.*, 1997).

Plant cells and callus cultures have been extensively used to explore the possibility of producing useful secondary metabolites through biotechnology methods.

With the stimulus of endogenous growth substances or by addition of exogenous growth regulators to the nutrient medium, cell division, cell growth and tissue differentiation are induced on *in vitro* cultivated medicinal plants (Leena and Jaindra, 2003).

MATERIALS AND METHODS

The study was conducted at the micropropagation laboratory of the Faculty of Horticulture, University of Agronomic Sciences and Veterinary Medicine of Bucharest during the period October 2013 - June 2014 on rosemary plant (*Rosmarinus officinalis* L.).

Explants preparation and sterilization.

Leaves of random length (0.5 - 1.5 cm) were taken from container grown rosemary plants. For leaves sterilization before the *in vitro* stabilization three types of chemical sterilizers were used: ethanol (C_2H_5OH), mercuric chloride ($HgCl_2$) and sodium hypochlorite ($NaOCl$).

The ethanol was used in ten full random replicates of six concentrations (20, 30, 40, 50, 60 and 70%) and five periods (0.5, 1, 2, 3 and 5 minutes).

The mercuric chloride ($HgCl_2$) was applied in ten randomized replicates of six concentrations (0.03, 0.06, 0.10, 0.15, 0.20 and 0.30 mg/l) and five treatment durations of 4, 6, 8, 10 and 12 minutes.

The leaves sterilization with sodium hypochlorite ($NaOCl$) was applied following the same experimental design with six concentrations (0.50, 0.25, 1.5, 1.25, 1.0, 0.75%) and five treatment periods (5.10, 15, 20 and 25 minutes respectively). The solution was obtained from the commercial sodium hypochlorite (Clorox) with a content of 5.25% chlorine.

The explants sterilization was done by solutions continuously stirring. After the end of the sterilization process, leaves were washed with distilled sterilized water for three times.

Preparation of nutrient medium. Murashige & Skoog medium (MS, 1972) supplemented with sucrose as a source of energy as well as vitamins (Walkey Vitamins) and plant growth regulators was used for explants inoculation.

As growth regulators, 6 x 6 combinations of benzyl adenine (BA) and naphthalene acetic acid (NAA) in concentration of 0.0, 0.5, 1.0, 1.5, 2.0 and respectively, 3.0 mg/l, were added at the culture medium to stimulate callus formation.

Sterilized explants were inoculated on culture media and then placed in an incubation room at $25 \pm 1^\circ C$, 1,000 Lux light intensity and 16 hours daily lighting.

Measurement of the callus size (volume).

For measuring the size, the mass callus was extracted by sterile forceps and washed with

distilled sterile water several times to remove the remaining culture medium. The block callus was then put inside a graduated cylinder containing sterile distilled water at a given volume and the supplementary volume was recorded and expressed in mm^3 .

Measurement of the callus fresh weight.

The mass callus growth was calculated by the difference between the weight of the culture jar at the end of and at the beginning of the experiment, determined with a sensitive balance.

Estimate the dry weight of callus. After calculating the fresh weight of the soft callus it was placed on filter paper in the oven at $70^\circ C$ for 24 hours. The dry weight was then recorded.

RESULTS AND DISCUSSION

The effect of ethylic alcohol (C_2H_5OH) on rosemary leaves explants sterilization

The results indicated in the Table 1 shows the lack of significant differences between the concentrations of 20%, 60% and 70% of ethylic alcohol, where the average number of healthy leaves was (1.00, 1.04 and respectively 1.00).

The concentration of 50% alcohol, gave the highest number of healthy leaves (2.62), while for the sterilization time, the highest average value (1.95 leaves) was obtain at a duration of 3 minutes.

The results also showed that there were no significant differences between the average number of healthy leaves (1.47 and 1.40) when the duration of the sterilization was 1 and respectively, 5 minutes.

The results also showed that the overlap between the alcohol concentration and the treatment duration affected significantly the average number of healthy leaves. The top number of 3.90 healthy leaves was reached when used alcohol concentration of 50% for 3 minutes.

The increase of alcohol concentration to more than 50% led to the increase in the number of dead leaves in all the variants, confirming the data published by Kosaka and Yokoi (2003).

The effect of mercuric chloride ($HgCl_2$) on rosemary leaves explants sterilization

The results in Table 2 showed that mercuric chloride ($HgCl_2$) had a significant effect on leaves sterilization with the best effect at a concentration of 0.06 mg/l that gave the highest number of healthy leaves (3.52). It was further noted that the period of sterilization affected the number

of healthy leaves. For 6-8 minutes sterilization time was reached the highest average number of sterile and healthy leaves (2.30) for all used concentrations.

The highest number of healthy sterile leaves (5.10) was obtained at the concentration of 0.06 mg/l and 6 minutes sterilization time. By increasing the concentration of HgCl_2 , the number of burned or dead leaves, increased.

It is therefore concluded that, the low concentrations of mercuric chloride led to the contamination of leaves at high rates, either high concentrations determined leaves death and burning due to the toxic effect.

The effect of sodium hypochlorite (NaOCl) on rosemary leaves explants sterilization

The results in the Table 3 indicate significant differences between different concentrations of sodium hypochlorite (NaOCl) and the number of survival leaves after 1 mg/l.

The number of healthy leaves began to decline the greater the duration of sterilization after more than 15 minutes, and reached the highest number of 3.10 leaves when using the concentration of 0.75 mg/l sodium hypochlorite.

The highest average number of healthy leaves (2.72) was obtained when the duration of sterilization was 15 minutes. The values decline to 1.87 healthy leaves at the duration of 20 minutes

Tab. 1. Effect of the concentration of ethanol ($\text{C}_2\text{H}_5\text{OH}$) solution (%) and the duration of sterilization (minutes) on the average number of healthy leaves of rosemary.

Time $\text{C}_2\text{H}_5\text{OH}$	0.5	1	2	3	5	Mean
0	0.00	0.00	0.00	0.00	0.00	0.00
20	1.00	1.00	1.00	1.00	1.00	1.00
30	1.10	1.30	1.40	1.60	1.80	1.44
40	1.50	1.70	3.00	3.20	1.60	2.20
50	1.50	2.80	3.10	3.90	1.80	2.62
60	1.00	1.00	1.00	1.00	1.20	1.04
70	1.00	1.00	1.00	1.00	1.00	1.00
Mean	1.18	1.47	1.75	1.95	1.40	
LSD $\leq$ 0.05	$\text{C}_2\text{H}_5\text{OH} = 0.1814$		Time = 0.1534		$\text{C}_2\text{H}_5\text{OH} \times \text{Time} = 0.4057$	

Tab. 2. Effect of the concentration of mercuric chloride (mg/l) and the duration of sterilization (minutes) on the average number of healthy leaves of rosemary.

Time HgCl_2	4	6	8	10	12	Mean
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.03	1.90	1.80	1.60	1.10	1.00	1.48
0.06	2.90	5.10	4.00	3.40	2.20	3.52
0.10	2.70	3.50	3.20	1.60	1.10	2.42
0.15	1.60	1.20	1.10	1.00	1.00	1.18
0.20	1.40	1.20	1.00	1.00	1.00	1.12
0.30	1.00	1.00	1.00	1.00	1.00	1.00
Mean	1.92	2.30	2.30	1.52	1.22	
LSD $\leq$ 0.05	$\text{HgCl}_2 = 0.1401$		Time: 0.1148		$\text{HgCl}_2 \times \text{Time} = 0.3332$	

while the lowest number of 1.33 leaves was reached at 25 minutes sterilization time (Caruso *et al.*, 2000).

The results of statistical analysis showed that the overlap between the variants has affected the average number of healthy leaves, best result being of 5.00 leaves when using a solution of 0.75 mg/l sodium hypochlorite for a period of 15 minutes.

Used by many researchers, the solution of sodium hypochlorite for superficial sterilization of plant parts was efficient and didn't damage the explants at appropriate concentrations (Gertlowski *et al.*, 1993). These findings are similar with those of Hippolyte. (2000), which pointed out that the high concentrations of sodium hypochlorite can be effective in sterilizing the superficial plant parts cultivated *in vitro*, but it is accompanied by the death of plant parts. The

reason may be attributed to the different type of plant and to the degree of persistent injury.

The effect of BA and NAA and the overlap between them on the rosemary callus volume (mm³)

Data shown in Table 4 indicate that the levels of BA and NAA and overlap of two, significantly affected the volume of formed callus. The concentration of 1.5 mg/l BA gave the highest volume of callus (8.4 mm³), which was superior to the average volume of callus produced by different concentrations of NAA (1.68 mm³).

It is noted in the same table that, the highest volume of callus was 6.1 mm³ at concentrations of 1.5 and 2.0 mg/l of NAA and the lowest volume (1.1 mm³) at 0.0 mg/l NAA.

By increasing the concentrations of BA and NAA to 2.0 mg/l or more, the callus size reduced and this shown that high concentrations led to

Tab. 3. The effect of the concentration of sodium hypochlorite NaOCl (mg/l) and duration of sterilization (minutes) and overlap on the average number of healthy leaves of rosemary.

Time NaOCl	5	10	15	20	25	Mean
0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.25	1.00	1.00	1.80	1.60	1.00	1.28
0.50	1.60	2.00	3.40	2.10	1.60	2.14
0.75	2.00	3.00	5.00	3.50	2.00	3.10
1.00	1.60	2.40	3.60	2.00	1.40	2.20
1.25	1.40	1.40	1.50	1.00	1.00	1.26
1.50	1.00	1.00	1.00	1.00	1.00	1.00
Mean	1.43	1.80	2.72	1.87	1.33	
LSD ≤ 0.05	NaOCl = 0.1004		Time = 0.1188		NaOCl x Time = 0.2713	

Tab. 4. Effect of NAA and BA and overlap on callus volume (mm³).

NAA (mg/l) BA (mg/l)	0.0	0.5	1.0	1.5	2.0	3.0	Average BA
0.0	0.0	2.0	2.2	2.4	1.9	1.6	1.68
0.5	0.9	2.6	2.9	3.5	4.4	2.3	2.76
1.0	1.2	3.1	3.7	4.3	6.1	2.9	3.55
1.5	1.6	6.2	7.4	14.4	11.5	9.3	8.40
2.0	1.3	3.3	6.9	6.2	7.5	8.2	5.56
3.0	1.1	2.6	5.4	3.5	5.4	7.5	4.25
Average NAA	1.1	3.3	4.8	6.1	6.1	5.3	
LSD ≤ 0.05	BA = 0.1532		NAA = 0.1532		BA x NAA = 0.3754		

inhibit the callus growth. The variants free of NAA determined the severe inhibition of cells, leading to weak growth. The overlap between BA and NAA

in concentration of 1.5 mg/l each, produced the highest callus volume of 14.4 mm³.

Effect of BA and NAA and the overlap between them on the rosemary callus fresh weight (g).

The Table 5 shows that, the treatment with BA and NAA have led to a significant increase in the fresh weight of the soft callus (g). The concentration of 2.0 mg/l BA determined an increase in callus fresh weight of 0.68 g compared with only 0.32 g in the control treatment. The levels of NAA has significantly affected the fresh weight of callus reaching a top value of 0.75 g at a concentration of 1.5 mg/l and only 0.11g for control.

The highest impact of the overlap between BA and NAA on the callus fresh weight was obtained at 2.0 mg/l BA and 1.5 mg/l NAA with a growth of 1.53 g.

Fig. 1. Rosemary callus formation after the sterilization process.

Tab. 5. Effect of BA and NAA and overlap on callus fresh weight (g).

NAA (mg/l) \ BA (mg/l)	0.0	0.5	1.0	1.5	2.0	3.0	Average BA
0.0	0.00	0.29	0.28	0.51	0.64	0.20	0.32
0.5	0.09	0.62	0.41	0.59	0.83	0.31	0.47
1.0	0.13	0.34	0.72	0.73	0.53	0.32	0.46
1.5	0.14	0.39	0.81	0.74	0.23	1.05	0.49
2.0	0.16	0.44	0.42	1.53	0.63	0.93	0.68
3.0	0.18	0.24	0.62	0.42	1.32	0.82	0.60
Average NAA	0.11	0.39	0.52	0.75	0.69	0.60	
LSD ≤ 0.05	BA = 0.0137		NAA = 0.0137		BA x NAA = 0.0337		

Tab. 6. Effect of BA and NAA and overlap on the rosemary callus dry weight (g).

NAA(mg/l) \ BA (mg/l)	0.0	0.5	1.0	1.5	2.0	3.0	Average BA
0.0	0.00	0.10	0.12	0.22	0.29	0.09	0.13
0.5	0.04	0.23	0.19	0.24	0.37	0.10	0.19
1.0	0.06	0.21	0.39	0.38	0.22	0.11	0.23
1.5	0.07	0.17	0.20	0.32	0.10	0.37	0.27
2.0	0.08	0.13	0.31	0.13	0.63	0.44	0.28
3.0	0.08	0.09	0.29	0.21	0.29	0.48	0.24
Average NAA	0.055	0.160	0.250	0.3208	0.3227	0.260	
LSD ≤ 0.05	BA = 0.0263		NAA = 0.0263		BA x NAA = 0.0263

Fig. 2. Rosemary callus produced under the effect of BA and NAA treatment.

Effect of BA and NAA and the overlap between them on rosemary callus dry weight (g).

The results shown in Table 6 underline that the treatment of BA and NAA have led to a significant increase in callus dry weight. The concentration of 2.0 mg/l BA had a significant effect on callus dry weight growth (0.28 g), while the lowest rate (0.13 g) was registered in the control treatment.

Similar results, with a value of callus dry weight which stood above 0.32 g, were registered at a concentration of 1.5 mg/l NAA.

The overlap between BA and NAA had significant effect on the callus dry weight (0.63g), at 1.5 mg/l of BA and 2.0 mg/l of NAA. No increase in callus dry weight was registered at control treatment.

CONCLUSIONS

The recommended concentration of ethylic alcohol for rosemary leaves explant sterilization is 50% for a duration of 3 minutes.

The mercuric chloride (HgCl_2) gave the highest number of healthy sterile rosemary leaves (5.10) at the concentration of 0.06 mg/l and 6 minutes sterilization time. By increasing the concentration of HgCl_2 , the number of burned or dead leaves increased.

The highest average number of healthy rosemary leaves (2.72) was obtained when the sodium hypochlorite (NaOCl) was used at

a concentration of 0.75 mg/l for a sterilization duration of 15 minutes.

Callus growth expressed by callus volume (mm^3), callus fresh and dry weight (g) was significantly influenced by the combination of BA and NAA. Best results were obtained at concentrations of 2.0 mg/l BA and 1.5mg/l NAA.

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