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Effect of growth regulators for the induction of Callus from the apical bud on In Vitro of Rosemary Plant (Rosmarinus officinalis L.)

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Abstract - The study included the use of tissue culture technique in rosemary (Rosmarinus officinalisL.) plant propagation starting from Apical buds explants and the production of callus Apical buds of Rosmarinus officinalis L. – one of the most important sources for the extraction of phenolic compounds with strong antioxidant activity, were used to evaluate the effect of growth regulators on in vitro callus formation. The first experiment consisted inthe aplical buds explants sterilization using ethanol 70% for 30 seconds (C2H5OH) and sodium hypochlorite (NaOCl). After sterilization with sodium hypochlorite, the highest average number of healthy buds (3.24) was obtained when the duration of sterilization was 20 minutes. The values drop to 2.70 healthy buds at the duration of 15 minutes while the lowest number of 1.18 buds was obtained at 5 minutes sterilization time. The study showed that he highest callus volume (10.2 mm3) was produced by the overlap between BA and NAA in concentration of 2.0 and 1.5 mg/l. 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 2.0 mg/l NAA.

Key words: rosemary, grown regulators, hormons, apical bud explant, sterilization.

1. INTRODUCTION

Rosmarinus officinalis L. belongs to the class Dicotyledon order Tubiflorae family Lamiaceae. Rosemary is a native of Mediterranean regions of Europe, Asia Minor and North Africa. Rosemary is grown in Spain, Italy, France, Algeria, Morocco and Portugal for its essential oil. The Lamiaceae family seems to be a rich source of plant species containing large amounts of phenolic acids, so it is considered to be a promising source of natural antioxidans[1]. Rosemary is an aromatic plant and thus a flavoring agent, widely used in foods. Its extracts have been introduced as preservatives in the food industry[2].
Rosemary is considered one of the most important sources for the extraction of phenolic compounds with strong antioxidant activity. This specie grows worldwide and has been cultivated since long ago, in ancient Egypt, Mesopotamia, China and India[3].
Rosemary extracts, enriched in phenolic compounds are effective antioxidants due to their phenolic hydroxyl groups but they also possess plenty of other beneficial effects like antimicrobial, antiviral, anti-inflammatory, anticarcinogenic activities and is also known to be an effective chemopreventive agent[4]. Rosemary extract formulations are the only ones commercially available for use as antioxidants in the European Union and the United States, and they are marketed in an oil-soluble form, as a dry powder, and in water-dispersible or water-miscible formulations[5]. Also, rosemary is widely used as a culinary spice and is also used for its fragrance in soaps and cosmetics. 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[6].
Apical meristems are located on top of the strain ramifications, ensuring their growth in length. These meristems are formed when the embryo is born and remain active for a long time. Meristems are protected by structures such as buds. It is known that the correlative inhibition process that apical meristems develop in relation to axillary meristems, fact explained through high content of auxine of apical meristems[7].

2. 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 the rosemary plant (*Rosmarinus officinalis* L).

2.1 Explants preparation and sterilization

Apical buds of different lengths (0.5 - 1.5 cm) were taken from rosemary plants that were container grown. For buds sterilization, before the *in vitro* stabilization,

two types of chemical sterilizers were used: ethanol (C_2H_5OH) and sodium hypochlorite (NaOCl). The ethanol was used in concentration 70% for 30 seconds. The buds sterilization with sodium hypochlorite (NaOCl) was applied following the same experimental design with six concentrations (1, 2, 3, 3.50, 4, 4.50%) and five treatment periods (5, 10, 15, 20 and 25 minutes respectively). After the end of the sterilization process, the buds were washed with distilled sterilized water for three times.

2.2 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) in concentration of 0.0, 0.5, 1.0, 1.5, 2.0 and respectively, 3.0 mg/l and naphthalene acetic acid (NAA) in concentration of 0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l, were added to the culture medium to stimulate callus formation. The autoclave was used for 20 minutes at 120°C and pressure of 104 bar for sterilization. Then, the sterilized apical bud explants were inoculated on culture media and then placed in an incubation room at 25±1°C, 1,000 Lux light intensity and 16 hours daily lighting.

2.3 Measurement of the callus size (volume)

In order to measure the size, the mass callus was extracted using a sterile forceps and was then 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.

2.4 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.

2.5. 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°C for 24 hours. After this process the dry weight of the callus was then recorded.

3. RESULTS AND DISCUSSION

3.1 The effect of sodium hypochlorite (NaOCl) on rosemary axillary bud explant sterilization

The results in the Table 1 indicate major differences between different concentrations of sodium hypochlorite (NaOCl) and the number of survival buds after 1 mg/l. The number of healthy buds began to decline the greater the duration of sterilization after more than 15 minutes, and reached the highest number of 3.82 leaves when using the concentration of 3.00 mg/l sodium hypochlorite. The highest average number of healthy buds (3.24) was obtained when the duration of sterilization was 20 minutes. The values decline to 2.70 healthy buds at the duration of 15 minutes while the lowest number of 1.18 buds was reached at 5 minutes sterilization time.

Table -1. 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
1.00	1.10	1.30	1.60	1.80	1.00	1.36
2.00	1.40	1.80	2.50	4.90	2.20	2.56
3.00	1.80	3.20	6.30	5.30	2.50	3.82
3.50	1.70	2.70	4.70	4.80	2.10	3.20
4.00	1.30	2.10	2.50	3.20	1.40	2.10
4.50	1.00	1.40	1.60	1.70	1.00	1.34
Mean	1.18	1.78	2.70	3.24	1.45	
LSD≤ 0.01	NaOCl = 0.62 Time =0.5 NaOCl x Time = 1.2					

The results of statistical analysis determined that the overlap between the variants has affected the average number of healthy buds, best result being of 6.30 buds when using a solution of 3.00 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[8]. These findings are similar with those of Hippolyte[9], 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.

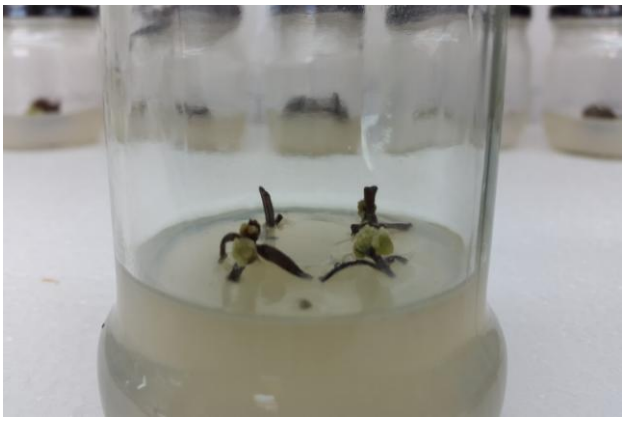

Fig -1. Rosemary callus formation after the sterilization process

3.2 The effect of BA and NAA and the overlap between them on the rosemary callus

Data shown in Table 2 indicate that the levels of BA and NAA and overlap of two, significantly affected the volume of formed callus. The concentration of 2.0 mg/l BA gave the highest volume of callus (7.8 mm^3), which was superior to the average volume of callus produced by different concentrations of NAA (2.95 mm^3). It is noted in the same table that, the highest volume of callus was 7.2 mm^3 at concentrations of 1.5 mg/l of NAA and the lowest volume (1.7 mm^3) 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 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 2.0 and 1.5 mg/l, produced the highest callus volume of 10.2 mm^3.

Table -2. Effect of NAA and BA and overlap on callus formation from apical bud of Rosemary explant

NAA (mg/l) / BA (mg/l)	0.00	0.50	1.00	1.50	2.00	2.50	Average BA
0.0	0.0	1.8	4.4	3.2	4.8	3.5	2.95
0.5	1.3	2.3	4.5	4.4	5.0	4.8	3.71
1.0	1.6	3.3	5.3	7.4	6.5	5.7	4.96
1.5	2.0	3.4	7.2	8.8	8.6	6.3	6.05
2.0	3.4	5.2	8.9	10.2	9.4	9.7	7.80
3.0	1.9	4.5	6.6	9.0	7.4	7.8	6.2
Average NAA	1.7	3.4	6.2	7.2	7.0	6.3	
LSD≤ 0.05		BA = 0. 185 BA x NAA = 0.3869			NAA =0. 158		

3.3 The effect of BA and NAA and the overlap between them on the rosemary callus fresh weight (g)

Table 3 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 a rise in callus fresh weight of 0.96 g compared with only 0.33 g in the control treatment. The levels of NAA has significantly affected the fresh weight of callus reaching a top value of 0.87 g at a concentration of 1.5 mg/l and only 0.20 g 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 2.0 mg/l NAA with a growth of 1.55 g.

Table -3. 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	0.20	0.39	0.41	0.58	0.42	0.33
0.5	0.16	0.28	0.60	0.54	0.60	0.54	0.45
1.0	0.23	0.38	0.63	0.84	0.77	0.62	0.58
1.5	0.24	0.39	0.79	1.00	0.87	0.66	0.66
2.0	0.39	0.58	0.99	1.22	1.55	1.01	0.96
3.0	0.22	0.52	0.70	0.99	0.99	0.90	0.72
Average NAA	0.20	0.39	0.68	0.83	0.87	0.69	
LSD≤ 0.05	BA = 0.0421 x NAA = 0.1032			NAA = 0.0421			BA

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

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

The results shown in Table 4 show that the treatment of BA and NAA have determined a significant increase in callus dry weight. The concentration of 2.0 mg/l BA had a significant effect on callus dry weight growth (0.45 g), while the lowest rate (0.14 g) was registered in the control treatment.
Similar results, with a value of callus dry weight which stood above 0.43 g, were registered at a concentration of 1.5 mg/l NAA.

Table -4. 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	0.14	0.19	0.23	0.20	0.14
0.5	0.06	0.12	0.27	0.39	0.29	0.24	0.23
1.0	0.10	0.14	0.29	0.49	0.33	0.29	0.28
1.5	0.14	0.14	0.32	0.50	0.43	0.31	0.31
2.0	0.14	0.23	0.36	0.59	0.78	0.60	0.45
3.0	0.10	0.20	0.22	0.40	0.32	0.34	0.26
Average NAA	0.09	0.15	0.27	0.43	0.40	0.33	
LSD≤ 0.05	BA = 0.0139 x NAA = 0.0341			NAA = 0.0139			BA

The overlap between BA and NAA had significant effect on the callus dry weight (0.78g), at 2.0 mg/l of BA and 2.0 mg/l of NAA. No increase in callus dry weight was registered at control treatment.

4. CONCLUSIONS

After sterilization with sodium hypochlorite, the highest average number of healthy buds (3.24) was obtained when the duration of sterilization was 20 minutes. The values drop to 2.70 healthy buds at the duration of 15 minutes while the lowest number of 1.18 buds was obtained at 5 minutes sterilization time.
The highest callus volume (10.2 mm^3) was produced by the overlap between BA and NAA in concentration of 2.0 and 1.5 mg/l.
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 2.0 mg/l NAA.

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