



Callus Induction and Plant Regeneration from Rosemary Leaves

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Abstract *Rosmarinus officinalis* L. leaves were used as explants in the study to investigate factors affecting callus induction and plant regeneration. It was found that the culture medium with higher concentration of sucrose promoted callus induction. MS medium supplemented with 6-BA 0.5 mg/L, NAA 0.5 mg/L, and sucrose 50 g/L showed the best result with an induction rate of 88.8%. When regenerated callus develops shoots, it was found that MS medium containing 6-BA 1.5 mg/L, KT 0.5 mg/L and NAA 0.5 mg/L showed relative good result with 50% regeneration rate. When grown in 6-BA 0.8 mg/L and NAA 0.5 mg/L, the amount of growth was increased by more than 300%. MS medium containing NAA 0.1 mg/L showed better result in inducing roots with rooting rate reaching 65%. Additionally, the study also showed that even though the explants were sterile, solution containing 75% ethanol with other sterilizing reagents would result in mass explant deaths.

Keywords *Rosmarinus officinalis* L.; Leaf explant; Callus; Differentiation; Regeneration

Introduction

Rosmarinus officinalis L. (Family Lamiaceae), which is known as Rosemary, is a kind of perennial evergreen shrub. It originates in Mediterranean region, and has been cultivated as a common household plant around the world for long time (Zhang et al., 2006; Minaiyan et al., 2011). *R. officinalis* L. usually has three basic function as raw materials. Firstly, it is used as spice products in food and beverage, secondly, the essential oil can be added into cosmetic production, lastly, in terms of numerous antioxidants, the plant or the essential oil is the materials of gastrointestinal ailments, which has great effect in various spasmodic conditions such as renal and biliary colic. So far, numerous pharmacological studies have suggested *Rosmarinus officinalis* L. may have a high therapeutic potential in inflammatory bowel diseases (Minaiyan et al., 2011).

Nowadays, the nature extracts efficacy is a hot spot research. For example, Rosa et al (2011) have utilized ethanol extract from *R. officinalis* L to investigate that the spasmolytic activity has relationship with

calcium channels switch in guinea pig ileum. Omri et al (2011) have tested the expression of nucleoside diphosphate kinase (NDPK) and heat shock protein (HSP) was controlled by luteolin, carnosic acid, and rosmarinic acid. Interestingly, essential oils from *R. officinalis* L. are likely to kill mites (Martinez-Velazquez et al., 2011), and antioxidant and antigenotoxic effects of its extracts are observed in *Salmonella typhimurium* TA98 and HepG2 cells (Zegura et al., 2011).

In food industrial, essential oils is coming from natural plant, so, are often used as spices and anticorrosive additives. The compounds of *R. officinalis* L. (such as Flavonoid, Phenolic, Piperitone, alpha-pinene, Limoene and 1,8 -Cineole from) are various due to the plant species and the harvest time, especially in the flowering stage (Papageorgiou et al., 2008; Rasooli et al., 2008). Gómez-Estaca et al (2010) have done an antimicrobial research about that the effect of essential oil from *R. officinalis* L. on 18 genera of bacteria, the result showed that it had antibacterial function. Rasooli et al (2008) have demonstrated that the natural products of *R. officinalis* L. could inhibited the



aflatoxins which were produced by *aspergillus flavus* and harmful to human beings and livestock. When extracts and vitamin C were used together, it appeared stronger oxidation resistance (Djenane et al., 2003). Furthermore, *R. officinalis* L. can be used for soil and water conservation, city landscaping and family potted (as mozzie buster). Therefore, it's a kind of economic plant that has prosperous exploiting and utilizing value (Li et al., 2006).

With the increasing demand of *R. officinalis* L. on food processing, medical, beauty and health care, this not only enhances the *R. officinalis* L processing industry, but also the rise of effective and high quality cultivation, therefore, it requires higher quality breeding and cultivation of rosemary.

Usually, there are three propagation methods: seed breeding, and cutting propagation. However, seed germination rate is quite low (about 13%) that it's not easy to survival by cutting propagation. Although laying propagation has high survival rate, there are no enough plantlets for large area cultivating (Chen and Kang, 2009).

Despite of controversial breeding, some significant biotechnologies which are beneficial to improvement of economic production and food crops, such as genetic engineering, cell engineering, haploid induction and somatic mutation, largely depended on the techniques that explants can regenerate and become intact plants (Vijendra et al., 2005). We adapt *in vitro* culture techniques, select the rosemary as the explants in this research, induces the leaves to dedifferentiation and become callus, later becomes adventitious buds and finally grow to intact plant. This not only offers the callus and intact plants to rosemary improvement by modern biotechnologies, but also quickly obtain large amount of rosemary materials that the genetic

traits are similar for large-scale cultivation. Meanwhile, it establishes the rosemary callus cultivation system in order to produce secondary metabolism product by cell engineering techniques.

1 Results and Analysis

1.1 Screening the optimal sterilization reagents

The treatment of different sterilizing reagents showed that 0.2% HgCl₂ (w/v, 5 min) was the optimal treatment (Table 1). From the table 1, we knew that, with 75% (v/v) ethanol, the contamination rate of explants was low as well as the survival rate and sometimes all of them were dead. It was estimated that when the alcohol-soluble substances of *R. officinalis* L may be dissolved, thus, the leaves were dead. Therefore, the explants sterilization cannot use 75% (v/v) ethanol, which will result in large amount of explants dead.

1.2 Callus induction of *R. officinalis* L. leaves

The mix proportion test of different concentration of sucrose and hormone indicated that MS medium with 6-BA 0.5 mg/L, NAA 0.5 mg/L, and 50 g/L sucrose was proved to be the optimal medium for the production of calli, which was able to start to dedifferentiation after 6 days. There are more explants that was successful to form callus (88%). However, it needed 9 days that the MS 30 g/L sucrose medium with start to dedifferentiation. From the table 2, we knew that the speed of callus dedifferentiation was much faster in 50 g/L sucrose medium than that in 30 g/L sucrose medium and the growth status of callus was better (Table 2; Figure 1).

1.3 Regeneration of *R. officinalis* L. callus

After the dedifferentiation, the leaves became callus, some callus were used to subculture for proliferation subculture, the other callus removed to dedifferentiation medium. The adjustment of cytokinins and auxin ratio

Table 1 Effect of different sterilizing reagents on *Rosmarinus officinalis* L. explants

Treatment	Sterilizing reagents	Holding time	Observed results			
			No. of explants	Rate of contamination	Survival rate	Death rate
1	75% ethanol+0.2% HgCl ₂	30 s+5 min	40	25% (75% sterile)	10%	90%
2	75% ethanol+2% NaClO	30 s+15 min	40	50% (50% sterile)	15%	85%
3	2% NaClO	15 min	40	80% (20% sterile)	65%	35%
4	0.2% HgCl ₂	5 min	40	7.5% (92.5% sterile)	95%	5%

Note: Death rate = No. of *R. officinalis* L. leaf sterile (but dead) / No. of sterile×100%

Table 2 Effects of mediums with different concentrations of sucrose for callus induction

Treatment	Medium formula	No. of explants	Growth status of calli		
			Rate of callus induction (%)	Day of callus initiation (d)	Status of calli
1	MS+sucrose 30 g/+KT 0.5 mg/L + 6-BA 0.5 mg/L+NAA 0.5 mg/L	100	78	9	Pale-yellow, dense
2	MS+sucrose 30 g/L+6-BA 0.5 mg/L +NAA 0.5 mg/L	100	79	9	Pale-yellow, dense
3	MS+sucrose 50 g/L+KT 0.5 mg/L +6-BA 0.5 mg/L+NAA 0.5 mg/L	100	82	6	Yellow green, loose
4	MS+sucrose 50 g/L+6-BA 0.5 mg/L +NAA 0.5 mg/L	100	88	6	Yellow green, loose

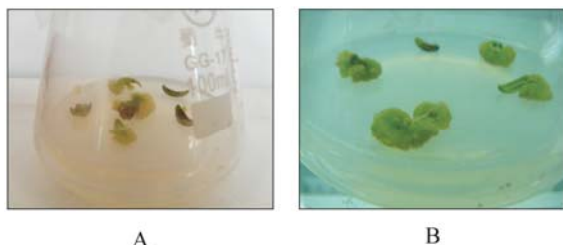


Figure 1 Callus induced from *R. officinalis* L. leaf explants
 Note: A: Calli on the medium with treatment 2 after 20 days; B: Calli on the medium with the treatment 4 after 20 days

in medium was able to dedifferentiate to adventitious buds. The results demonstrated that the MS medium with 6-BA 1.5 mg/L, KT 0.5 mg/L and NAA 0.5 mg/L was proved to be the optimal medium with 50 %

regeneration rate, and the number of adventitious buds ranks first place (Table 3).

1.4 Propagating of adventitious shoots

In order to reduce the teratogenic effect on the tube seedlings by the plant hormones accumulation, it was beneficial to reduce the cytokinins and auxin concentration (Table 4). Results indicated that MS medium with 6-BA 0.8 mg/L and NAA 0.5 mg/L was proved to be the best medium and the efficiency can reach 356% (Figure 2).

1.5 Rooting induction for *R. officinalis* L. shoots

When obtaining enough adventitious buds, it was suitable to reduce the plant growth regulators concentration,

Table 3 Regeneration of *Rosmarinus officinalis* L. callus

Treat-ment	Medium formula	No. of the explants	Observed result	
			Rate of budding (%)	The total No. of budding
1	MS+KT0.5 mg/L+6-BA 1.0 mg/L+ NAA 0.1 mg/L	40	10	4
2	MS+KT 0.5 mg/L+6-BA 1.5 mg/L+ NAA 0.1 mg/L	40	20	15
3	MS+KT 0.5 mg/L+6-BA 2.0 mg/L+ NAA 0.1 mg/L	40	15	9
4	MS+KT 0.5 mg/L+6-BA 1.0 mg/L+ NAA 0.3 mg/L	40	20	23
5	MS+KT0.5 mg/L+6-BA 1.5 mg/L+ NAA 0.3 mg/L	40	35	58
6	MS+KT 0.5 mg/L+6-BA 2.0 mg/L+ NAA 0.3 mg/L	40	25	32
7	MS+KT 0.5 mg/L+6-BA 1.0 mg/L+ NAA 0.5 mg/L	40	25	26
8	MS+KT 0.5 mg/L+6-BA 1.5 mg/L+ NAA 0.5 mg/L	40	50	72
9	MS+KT 0.5 mg/L+6-BA 2.0 mg/L+ NAA 0.5 mg/L	40	35	50

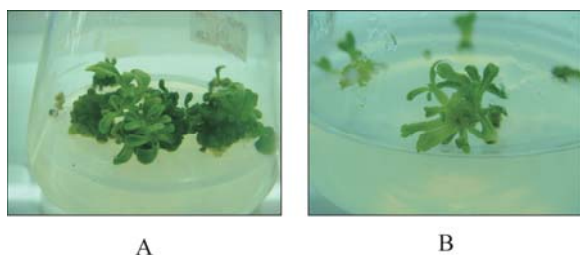


Figure 2 Adventitious buds propagating
 Note: A: The buds on the medium with treatment 2 after 30 d;
 B: The buds on the medium with treatment 6 after 30 d

which will induce the shoot to a complete plant (Table 5). Among these mediums, the result of the 0.1 mg/L NAA MS medium with the 65% rooting rate was better. However, the length of adventitious buds needed to be longer than 1.5 cm in order to obtain better root, whilst the length of adventitious buds was shorter than 1.5 cm, the roots grew fewer and slower, so did the whole plantlet (Figure 3).

2 Discussion

With the increasing of people living standard, natural extracts and essential oils of *R. officinalis* L. were widely used in terms of their aromatic, antiseptic antioxidant effects. Thus, the requirement of rosemary cultivation and breeding was predicted to increase day by day. Either the controversial methods or modern biotechnologies, such as genetic



Figure 3 Effect of different bud's length on rooting
 Note: A: Roots induced from 2.5 cm length shoots; B: Roots induced from 1.0 cm length shoots

Table 4 Propagation for adventitious shoots

Treatment	Medium formula	Observed results		
		No. of cultured shoots	No. of proliferating shoots	Rate of proliferation (%)
1	MS+6-BA 0.5 mg/L + NAA 0.5 mg/L	50	112	224
2	MS+6-BA 0.8 mg/L + NAA 0.5 mg/L	50	178	356
3	MS+6-BA 1.0 mg/L + NAA 0.5 mg/L	50	119	238
4	MS+6-BA 1.2 mg/L + NAA 0.5 mg/L	50	64	128
5	MS+6-BA 1.5 mg/L + NAA 0.5 mg/L	50	100	200
6	MS+6-BA 1.8 mg/L + NAA 0.5 mg/L	50	111	222
7	MS+6-BA 2.0 mg/L + NAA 0.5 mg/L	50	127	254

Table 5 Rooting induction for *Rosmarinus officinalis* L. shoots

Treatment	Medium formula	Observed results		
		No. of shoots	Rate of rooting (%)	Average No. of roots
1	MS+NAA 0.1 mg/L	40	65	3
2	MS+NAA 0.15 mg/L	40	45	2
3	MS+NAA 0.2 mg/L	40	40	2

engineering, cell engineering, haploid induction and somatic mutation, was likely to obtain the good quality rosemary, but biotechnological approaches in breeding and cultivation largely depended on an efficient regeneration of intact plants through *in vitro*

systems (Vijendra et al., 2005). This research utilized the leaves as explants, through the Plant *in vitro* culture techniques, induced the leaves to callus and dedifferentiate to adventitious buds, which will become intact plants. This not only applied good



receptor which makes use of enhancing the rosemary quality by modern biotechnologies, but also quickly produced large amount of plants with similar genetic traits. This became the theoretical basis for rosemary *in vitro* experiment. Simultaneously, this also provided the theoretical basis that extract the effective ingredient from callus directly.

The results also showed that 75% ethanol (30~40 s) with other sterilizing reagents could result in large explants death, which suggested that there were abundant ethanol-soluble components in *R. officinalis* L. leaf. If utilizing ethanol sterilizing reagents, the content will be dissolved and the cell die. Therefore, the 75% ethanol mixed sterilizing reagents is not suitable for this research. Meanwhile, MS medium with higher sucrose concentration (50 g/L) will promote rosemary leaves to dedifferentiation. This suggested that the rosemary leaves contain lots of essential oil, higher carbon source ratio that is beneficial to enhance the growth of rosemary with Alcohol-soluble substances and lipid. All of results provided the theory for the plant *in vitro* culture.

3 Materials and Methods

3.1 Materials

Leaf explants from spring shoots of *R. officinalis* L.; Basal medium of Murashige and Skoog (MS) (Murashige and Skoog, 1962) with 3%~5% (w/v) sucrose, 0.7% Agar, pH 5.8 and different concentration of plant growth regulators; sterilizing these mediums under 121°C, 0.1-0.5 kPa 20 min; distilled water; 75% (v/v) ethanol; 0.2% HgCl₂ (w/v); 2% sodium hypochlorite (NaClO, v/v).

3.2 Comparative study of the sterilization effects of the explants primary cultivation

R. officinalis L. leaves and spring stems (3~5 cm) bought from market, washed by running water for 0.5~1 hours, sterilized by different sterilizing reagents (Table 1), washed by sterile still water for 3~5 times, and cultured. The temperature is (25±2)°C with 70%~80% relative humidity. The light intensity is 2 500 Lux with 14 hours in the daytime and 10 hours at night. After cultured 3 d, 6 d, the status of explants were observed and then record the data every 10 days. or without pre-sterilized in 70% (v/v) ethanol for 30~40 s, and then in commercial bleach (2% sodium

hypochlorite) for 15 min, or 0.2% HgCl₂ (w/v) for 5 min (Table 1), and then washed 3 to 5 times in distilled water. Leaves were excised from young stems and placed on solid agar sterile medium for callus induction. They maintained in the culturing room at (25±2)°C and in a 14 h light (2 500 Lux), 8 h dark photoperiod.

3.3 Callus induction of *R. officinalis*

To investigate the callus induction medium, this research designed mixture of 30 g/L source, 50 g/L source with cytokinins and auxin, separately (Table 2). Note: callus induction (%) = No. of calli from leaf / No. of cultured leaf × 100%; Start-up period (unit; d): The days are from the cultivation to 50% leaf blades that began to differentiate and form calli.

3.4 Regenerating of *R. officinalis* L. calli

When calli expanded to 1.5~2.0 cm², some of them continued to subculture in the original medium, while the other induce to dedifferentiate to roots with different cell division concentration. This research has designed various cytokinins and auxin ratio to investigate the callus dedifferentiation conditions. Note: budding rate (%) = No. of budding calli (piece of) / No. of culturing calli (piece of) × 100%.

3.5 Propagation of *R. officinalis* L. adventitious buds

Adjusting the auxin concentration in medium, the rosemary **adventitious buds** is predicted to induce the lateral buds propagation. The auxin compose and densities is in Table 4. Note: Proliferation rate = No. of shoots / No. of transferring shoots × 100%.

3.6 The rooting and the formation of complete plants

In the process of plant tissue culture, medium inducing shoots would inhibit the root growth, and vice versa. Therefore, The adventitious buds that coming from callus didn't have root. It needed to reduce cytokinins and auxin concentration to grow into an intact plantlet. Select the adventitious buds with 2~5 cm in length to rooting and set up three different NAA concentration; When roots came into 3~6 with about 2~4 cm in length, they could transfer into the soil to grow up.

Author Contributions

YMD and ZNL conceived the overall study, performed the



experiment designs, and drafted the manuscript. CQ, BGL and RLD took part in some experiments. YTL supervised the projects and revised the whole written-up. All authors have read and approved the final manuscript.

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