

In vitro Propagation Response of *Rosmarinus officinalis* L. to Biotic and Abiotic Elicitors on Phenolic Content and Photosynthetic Pigments

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Abstract

Micropropagation protocol was modified to evaluate the best procedure to induce multiple shoots from *Rosmarinus officinalis* L. sterilized seedlings obtained from seeds which vitality is always very low. Aseptic shoots (1-1.5 cm) were cultured on full strength Murashige and Skoog medium modified with several growth regulators (abiotic elicitors): Benzyladenine (BA), Kinetin (Kin) and coconut water (biotic elicitors). Data was indicated that BA at the concentration of 3 mg/L encouraged shoot multiplication. The highest number of leaves and plant length also was obtained with medium modified with 3 mg/L BA and coconut milk at 5 ml/L. All treatments were significantly different from the control. Total phenolics content, anthocyanins, chlorophylls were extracted and spectrophotometrically determined as secondary products. Data was concluded the highest content of phenolic 10.45 (mg/g) and chlorophyll b 0.67 (mg/g) when BA was 3.0 mg/L. Whereas chlorophyll a reached to 0.64 mg/g in presence of 5.0 mg/L BA and 5.0 ml/L coconut milk. Anthocyanin scored high level when BA was 3.0 mg/L combined with 5.0 ml/L coconut milk.

Keywords: *Rosmarinus officinalis* L., *in vitro* propagation, shoot multiplication, plant growth regulators, and secondary products

1. Introduction

Medicinal and aromatic plants play an important role in all areas of the world. The family Lamiaceae took the oral wide acclaim According to the content of a range of medicinal plants, each of which is in itself a treasure such as Rosemary. It is a perennial woody, aromatic herb, common in the Mediterranean region, but is now grown worldwide. *Rosmarinus officinalis* L, commonly known as rosemary, is a woody, perennial herb with fragrant, evergreen, needle-like leaves and white, pink, purple, or blue flowers. It is a member of the mint family Lamiaceae, which includes many other herbs. The name “rosemary” is derives from the Latin for “dew” (*ros*) and “sea” (*marinus*), or “dew of the sea” (Room, 1988).

The plant is also sometimes called anthos and it is a considerable source of secondary metabolites, especially essential oils, which have antiviral, antimicrobial, antispasmodic, antioxidant, diuretic, anticonvulsant, analgesic hepatoprotective, and anti-carcinogenic properties (Bozin et al., 2008; Saltan & Ozaydin, 2013). It is also being used in cosmetic industry and food (Wang et al., 2008). The essential oil of rosemary consists of flavonoids as carnosic acid, carnosol, rosmarinic acid, and volatile oils (Okamura et al., 1994). Carnosic acid and Carnosol constitute 3.8-4.6% of the extracted material, and they are considered as constituents in rosemary that show pharmacological properties (Huang et al., 1994). Several flavonoids and phenolic compounds such as hispidulin, cirsimaritin, apigenin, genkwanin, naringin, caffeic acid and rosmarinic acid are also present in rosemary extracts (Jasim, 2017). It has been considered as one of the most effective herbs for treating inflammatory diseases, headaches, poor circulation, headaches, and physical and mental fatigue (Eilyad et al., 2012). Rosemary extracts and its components have anti-lipid peroxidant activities and free radical scavenging by various *in vitro* antioxidant assays (Bilto & Alabdallat, 2015).

Due to shining virtues of rosemary and its poor seed germination (30%), there is a need for new propagation methods which can increase its production (Nourin & Humera, 2014). Plant tissue culture has been used as a tool

for rapid micropropagation and, the conservation of medicinal plants and also for providing a source of secondary metabolites which has an important medical aspect such as phenolic compounds, anthocyanin and their glycosidic forms (anthocyanins) acted as strong antioxidants (Tokusoglu & Yildirim, 2012).

2. Materials and Methods

This study was carried out in Plant Tissue Culture Laboratory, Faculty of Science, Taif University, Saudi Arabia during the January to March season 2016.

2.1 Sterilization Procedure

Seeds of *R. officinalis* L. were sterilized by washing in tap water supplemented with soap and small drops of Tween 20, then immersed in ethanol 70% for 1 min, washed with sterile distilled water and in 15% commercial Clorox solution for 10 min. Seeds were washed three times with sterile distilled water in laminar air flow hood.

2.2 Culture Media

Sterilized seeds were cultured on (MS medium 1962). After germination, seedlings about 1-1.5 cm high also were cultured in (MS medium 1962) modified with (abiotic elicitors) Kinetin (Kin) at 0.0, 3.0, and 5.0 mg/L and, Benzyl amino purine (BA) at 0.0, 3.0, and 5.0 mg/L in addition of (biotic elicitors) 5.0 ml/L Coconut water. Sucrose was used at 30 g/L and agar at 8 g/L. The media were distributed into sterilized jars. Each of which contained 30 ml of MS medium. The pH medium was adjusted to 5.7-5.8 before autoclaving for 15 min at 121 °C, 1.5 kg/cm³. Jars were incubated in the growth chamber at 26±2 °C and 16 hr light/day under constant fluorescent light of 1500 Lux.

2.3 Experimental Treatments

There were nine treatments; each treatment included ten jars with (three explants in each) as follows:

- (1) 0.0 mg/L BA + 0.0 mg/L Kin + 0.0 ml/L coconut milk (control)
- (2) 3.0 mg/L (BA)
- (3) 3.0 mg/L BA + 5.0 ml/L (coconut milk)
- (4) 5.0 mg/L (BA)
- (5) 5.0 mg/L BA + 5.0 ml/L (coconut milk)
- (6) 3.0 mg/L Kin
- (7) 3.0 mg/L Kin + 5.0 ml/L (coconut milk)
- (8) 5.0 mg/L Kin
- (9) 5.0 mg/L Kin + 5.0 ml/L (coconut milk)

2.4 Statistical Analysis and Experimental Design

A complete randomize design was used. Data obtained were subjected to analysis of variance and the means of treatment were compared using least significant differences (L.S.D.) test as outlined by Snedecor and Cochran (1972).

2.5 Data Recorded

After 4 weeks, data from culture media were collected as follows:

- (1) Average length of shoots (cm).
- (2) Average number of shoots per explant.
- (3) Average number of leaves.

2.6 Biochemical and Physiological Measurements

2.6.1 Content of Photosynthetic Pigments

Chlorophyll a (*Chl a*) and chlorophyll b (*Chl b*) contents were determined spectrophotometrically according to Metzner et al. (1965):

$$\text{Chlorophyll } a \text{ } (\mu\text{g/ml}) = 10.3 \times E_{663} - 0.918 \times E_{644}$$

$$\text{Chlorophyll } b \text{ } (\mu\text{g/ml}) = 19.7 \times E_{644} - 3.87 \times E_{663}$$

These pigment fractions finally, were calculated as fresh weight (mg/g).

2.6.2 Total Anthocyanin Determination

Anthocyanin total content in the extracts was measured according to the described procedure by Alberto and Rabino (1975) after anthocyanin extraction with methanol supplemented with HCl 1%, and centrifugation, absorbance was determined at 530 and 657 nm. The formula $A = (A_{530} - 0.25 \times A_{657})$ was applied to determine chlorophyll contribution and its degradation products to the absorption at 530 nm. The Expression of anthocyanin content was as milligrams of Cyanidin-3-glucoside equivalent per mg FW.

2.6.3 Total Phenolic Content

The determinations of total phenolic were done using Folin-Ciocalteu reagents (Singleton & Rossi, 1965). Standard solution of gallic acid (2.0 mg/ml) was prepared by accurately weighing 0.01 g and dissolving in 50 ml of distilled water. Absorbance was measured at 765 nm after standing for one hour at room temperature. The results were expressed in (mg/g) gallic acid equivalents (GAE).

3. Results and Discussion

3.1 Number of Shoots

Data presented in Figures 1 and 2 showed the improving effect of several concentrations of Kin and BA with or without coconut water on production of shoots of *R. officinalis*. Almost all treatments increased shoots number per explant than the control and in almost cases it was insignificant increment. The highest shoots number 7 was obtained from MS medium with 5.0 mg/L Kin as shown in Figure 5 when 3.0 mg/L Kin combined with 5.0 ml/L coconut milk. Malik et al. (1992) also illustrated that applied kinetin induced more branching in lentil and, enhancing formation of multiple shoot by applying cytokinin in *Pisum sativum*. Li et al. (2017) investigated the effect of Kin added to MS medium cultured with Arabidopsis plants and concluded that Kin recognition could be affected by plant competition conditions that are the result of growing distance, and that the integrated performance of individuals were suitable to evaluate Kin interactions of plant species.

3.2 Shoot Length

The same figure showed that seedlings grown on MS medium including different levels of growth regulators showed significant differences. It is clear that the concentration of 5.0 mg/L BA significantly induced the shortest shoots of *R. officinalis* (1.6 cm). However, inclusion of 3.0 mg/L BA in presence of coconut milk 5.0 ml/L in the medium elongated the shoots significantly. Differences on shoot length happened because different genotype gives various results on MS medium with numerous levels of plant growth regulator Shimelis et al. (2014). The difference is also due to variation of genotype and the number of days taken for culturing Sharma (2005). Addition of Coconut water increased the frequency of explants showing shoot regeneration, and elongation growth. Coconut water provides nutrients and is also a source of essential vitamins and growth hormones like zeatin (γ -alyl aminopurine), and inositol besides reducing nitrogen compounds Mondal et al. (2015).

3.3 Number of Leaves

Highest number of leaves (29.3 leaves) was observed in culture medium modified with 3.0 mg/L BA in presence of 5.0 ml/L coconut milk ml/L. On the other hand 5.0 mg/L BA gave a remarkable significant reduction of leaf number 4 as shown in Figure 1. Razdan (2003) concluded that the medium supplemented with 25% coconut water only supported viability, shooting; shoot elongation, rooting, root elongation and leaf formation. Coconut water as an organic substance in culture media had significant effect on shoots production and growth. Ge et al. (2004) reported the presence of zeatin-O-glucoside and dihydrozeatin-O-glucoside, a cytokinin in coconut water.

3.4 Total Phenolic Content

Figures 1 and 2 show that the treatment of 3.0 mg/L BA enhanced the content of total phenolic to (10.45 mg/g) compared with all other treatments except control which achieved (9.87 mg/g). The same result (9.87 mg/g) was also achieved from the level of 5.0 mg/L BA in absence of coconut milk. However, the level of 5.0 mg/L Kin + 5.0 ml/L (coconut milk) gave a significant reduction of total phenolic content (2.91 mg/g). The effect of benzyladenisne on the phenolic content was reported by Abdel-Al et al. (1988) who showed that cytokinins increased phenolic content in cotton plants.

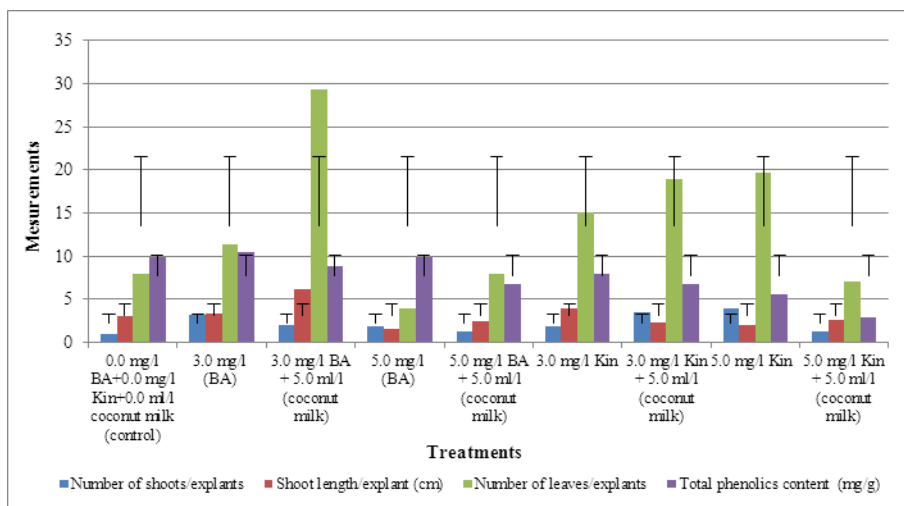


Figure 1. Effect of growth regulators on growth performance and total phenolics content of *R. officinalis* shoot

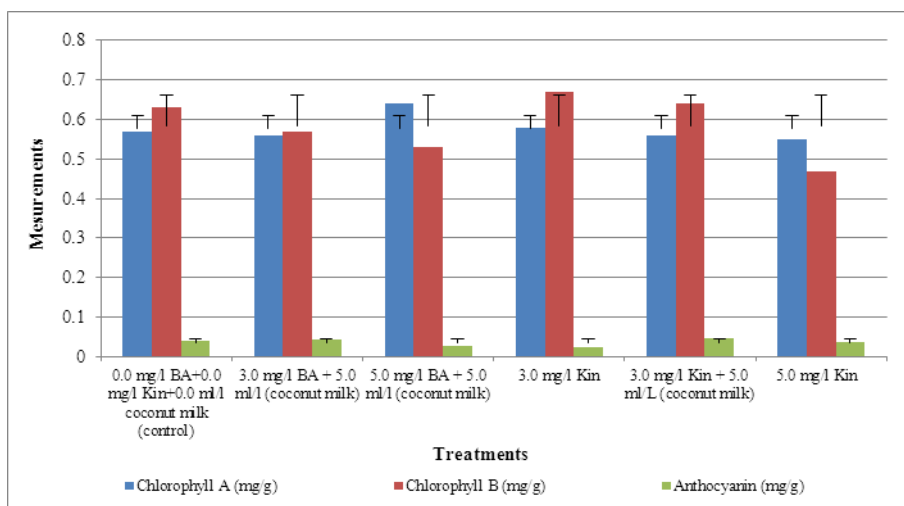


Figure 2. Effect of growth regulators on chlorophyll A, chlorophyll B and, anthocyanin content of *R. officinalis* shoots



Figure 3. The effect of Kin and BA in presence of coconut water on shoot elongation



Figure 4. The untreated seedling

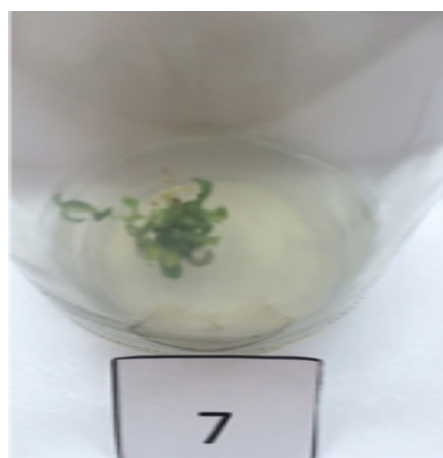


Figure 5. The effect of Kin on shoots formation

Data in Figure 2 showed that there were not significant differences between all treatments concerning chlorophyll a and chlorophyll b content of *R. officinalis* shoots. Anthocyanin content increased significantly 0.045 mg/g when Kin was 3.0 mg/L in the presence of coconut milk 5.0 ml/L followed by 0.043 mg/g anthocyanin obtained from 3.0 mg/L BA with 5.0 ml/L coconut milk. The presence of Kin in the level of 3.0 mg/L gave the lowest content of anthocyanin 0.022 mg/g. BA appeared to store the potential of anthocyanin production by subsequent subculture and also gave small reduction of callus growth. Similar results were obtained in the wild *Vaccinium bahalae* suspension cultures when the ability of anthocyanin production was restored as 20 μ M BA was introduced to culture medium as a substitution of Kin (Yemin et al., 1998). This report confirms the reverse relation between anthocyanin production and cell growth. There is a different phytohormone combination which stimulates the production of anthocyanin in several plants. Largado-Valler (2016) concluded that in the absence of BA in the culture medium anthocyanin production can be negligible. Anthocyanins are secondary metabolites in higher plants which contribute to the colors of fruits and flowers. They are, water soluble glucosides of anthocyanidins vacuolar pigments that appear purple, red or blue due to pH. They belong to flavonoids synthesized by phenylpropanoid pathway. They spread in all tissues of higher plants, including flowers, leaves, roots, and stems (Hershey, 2001).

4. Conclusion

The level of 5.0 mg/L Kin gave the highest number of shoots/explants., Tallest shoots and the highest numbers of leaves were obtained in 3.0 mg/L BA with 5.0 ml/L coconut water. The highest total phenol content obtained from the level of 3.0 mg/L BA. On the other hand, coconut water at 5.0 ml/L with Kin 3.0 mg/L achieved the highest anthocyanin content. Concerning chlorophyll a and chlorophyll b, all previous treatments gave non-significant increment.

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