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Original Article

Effect of salicylic acid on phenols and flavonoids content in callus culture of Iranian sodab (Ruta graveolens): A threatened medicinal plant of north of Iran

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Ruta graveolens locally known as "Iranian sodab or sadab " (Rutaceae), is a valuable medicinal and culinary plant that grows in Mediterranean zone and north of Iran. The plant is a real factory to produce secondary metabolites such as alkaloids, flavonoids, lignins, glycosides, coumarins and phenolics compounds. The present study investigated effect of salicylic acid on phenols and flavonoids content in callus culture of R. graveolens. Among different PGRs used in this study, MS media containing concentration of NAA (3 mg/l) are the most effective one for callus induction from internode explans. The results revealed that salicylic acid (0.5 mg/ml) showed as maximum as 10.65-fold improvement in total phenolic content (225.32 mg gallic acid equivalent/g of extract powder) and salicylic acid (20 mg/ml) showed 1.98-fold enhancement in total phenolic content (438.75 mg gallic acid equivalent/g of extract powder). Flavonoids content decreased at lower concentration of salicylic acid treatment (8.32 quercetin equivalent/g of extract powder) but increased at higher concentration (29.41 mg/g). From this study, it is concluded that salicylic acids can be used as elicitors to enhance the secondary metabolites in callus culture of R. graveolens. This study shows that the elicitation depends on elicitor dose and type of compound to be elicited.

Keywords: Salicylic Acid, Phenolic Content, Flavonoids Content, Callus Culture, Ruta Graveolens

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Introduction

Plants have many natural products such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other secondary metabolites which are valuable in antioxidant activity [1,2]. Ruta graveolens locally known as "Iranian sodab or sadab " (Rutaceae) is an evergreen erect, glabrous and glaucous perennial herb with 30 - 80 cm in height [3]. Ruta graveolens is native medicinal plant in Mediterranean zone and north of Iran [3,4]. It has many therapeutic uses such as treatment of inflammatory conditions, eczema, ulcers, arthritis, fibromyalgia, antidote for venoms, insect repellent, and as an abortifacient [5]. It has many chemical compounds such as flavonoids, phenols, furanocumarins, carotenoids, chlorophyll, and furanoquinolones [5]. Studies have shown that flavonoids show antioxidant activity and they have considerable effects on human nutrition and health. The mechanisms of action of flavonoids are through scavenging and chelating process [6,7].

In vitro cultures of R. graveolens are promising methods for the bio prospection of natural products [8-10]. In the investigate for alternatives to

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biosynthesis of valuable secondary metabolites from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of phytochemical molecules [11-14]. Salicylic acids are molecules known to be suitable elicitors for an extensive range of natural products from different plant origins and it is reported that the gene regulation related to the production of natural products can be induced in plants by SA [15]. The effect of SA on secondary metabolite production has been investigated in plant systems like Andrographis paniculata and Jatropha curcas [16,17].

There is no report of pharmaceutical studies in the literature about effect of salicylic acid on phenols and flavonoids content in callus culture of Iranian sodab (Ruta graveolens), a threatened medicinal herb of north of Iran. The present study seeks to enhancement of phenols and flavonoids content production of R. graveolens through callus culture.

Materials and Methods

Chemicals

Gallic acid and Quercetin were purchased from Merck and Fluka companies. All other chemicals and reagents used were of the highest commercially available purity.

Plant material preparation

The whole parts of Ruta graveolens were collected from Noshahr (Kheirudkenar forest) in Mazandaran in July of 2015.

Inoculation and culture condition:

For surface Sterilization, stem explants were rinsed with running tap water for 20 minutes, placed at 70% ethanol for 30 seconds, and then washed with sterilized distilled water three times. Later on, they were submerged in 1.5% sodium hypochlorite for 20 minutes and repeatedly washed three times with sterilized distilled water.

For in vitro callus induction of Sodab, Internode explants were cultured on MS (Murashige and Skoog, 1962) medium supplemented with various concentration of BAP (0.5, 1, 2 and 3 mg/l), 2, 4-D (0.1, 0.2, 0.5, 1 mg/ml) and NAA (0.5, 1, 2 and 3 mg/l). Induced calli was subcultured to MS media containing different concentration of SA (0.5 and 1 mg/ml). The control treatment was cultured on MS medium without any elicitor being added. The control and treated callus were removed from culture and dried under laminar flow without light [14].

Culture conditions

The pH of all the cultures was adjusted to 5.6–5.8 before autoclaving. The media were sterilized in an autoclave for 20 minutes at 121°C; cultures were incubated at 25 ± 2°C under a 16 hours photoperiod with cool white fluorescent illumination (100 lmol m_2 s_1 PFD).

Methanolic extracts preparation

Materials dried under laminar flow without light before extraction. Each part was extracted at room temperature by percolation method using methanol. The resulting extract was concentrated over a rotary vacuum until a crude solid extract was obtained.

Determination of total flavonoid content

Colorimetric aluminum chloride method was used for flavonoid determination [7]. Briefly, 0.5 ml solution of each plant extracts in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water, and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with a double beam Perkin Elmer UV/Visible spectrophotometer (USA). Total flavonoid contents were calculated as quercetin from a calibration curve. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 mg/ml in methanol.

Determination of total phenolic content

Total phenolic compound contents were determined by the Folin-Ciocalteau method [7]. The extract samples (0.5 ml of different dilutions) were mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) for 5 min and aqueous Na2CO3 (4 ml, 1 M) were then added. The mixture was allowed to stand for 15 min and the phenols were determined by colorimetric method at 765 nm. The standard curve was prepared by 0, 50, 100, 150, 200, and 250 mg / ml solutions of Gallic acid in methanol: water (50:50, v / v). Total phenol values are expressed in terms of Gallic acid equivalent (mg/g of dry mass), which is a common reference compound.

Statistical analysis

Experimental results are expressed as means. All measurements were replicated three times. The data were analyzed by an analysis of variance ($p<0.05$) and the means separated by Duncan's multiple range tests (by SAS software).

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Results and Discussion

Effect of auxins (2, 4-D, NAA) and cytokinin (BAP) on callus induction on internode as explants:

The effect of different PGRs on callus induction of Ruta graveolens has been shown in Table 1.

Callogenic response was observed in all the combinations of growth regulators used except on MS media containing BAP. Among different PGRs used in this study, MS media containing concentration of NAA (3 mg/l) are the most effective one for callus induction from internode explans. So that the higher volume (1.164 mg/l), maximum (100%) frequency, wet (1.118 mg) and dry (0.855 mg) weight of callus induced with culturing internode on MS medium containing 3 mg/l NAA (Figure 1.). Calli induction observed just 6 days after culturing and the induced calli had better quality (light-green, compact) in comparison with other PGRs used in our study (Table 1).

Auxins are reported to be the best plant growth regulators for callus induction when compared to others [18]. The finding is in accordance with earlier studies on this plant [19-24].



Fig 1. Callus induction from leaf explant on MS medium supplemented with 3 mg/l NAA after 30 days of culture.

Effect of SA on Phenols and flavonoids content

Tissue culture methods employing elicitors has been extensively utilized to increase the biosynthesis of natural products. Biotic elicitors such as salicylic acid (SA) are utilized in the tissue culture media for the maximum level of biosynthesis of natural products.

Total phenol compounds, as determined by Folin-Ciocalteau method, are reported as mg gallic acid equivalent/g of extract powder, by reference to standard curve (y = 0.0063x, r2 = 0.987). The total flavonoid contents are reported as mg quercetin equivalent/g of extract powder, by reference to standard curve (y = 0.0067x + 0.0132, r2 = 0.999). The results revealed that salicylic acid (0.5 mg/ml) showed as maximum as 10.65-fold improvement in total phenolic content (225.32 mg gallic acid equivalent/g of extract powder) and salicylic acid (20 mg/ml) showed 1.98-fold enhancement in total phenolic content (438.75 mg gallic acid equivalent/g of extract powder). Phenolic contents production decreased to higher level at 10 mg/ml SA followed by 20 mg/ml SA treatments compared to control (21.15 mg/g) (Table 2).

Flavonoids content decreased at lower concentration of salicylic acid treatment (8.32 quercetin equivalent/g of extract powder) but increased at higher concentration (29.41 mg/g). Phenolic contents increased with increasing concentration of salicylic acid. Flavonoids content decreased with increasing salicylic acid concentration ranging from 8.32 to 29.41 mg quercetin equivalent/g of extract powder compared to control (23.75 mg/g).

In this study, it was noticed that when callus cultures of R. graveolens were exposed to of SA secondary metabolites (phenolic and flavonoids) increased. SA induced increase in the natural products is observed earlier in several other medicinal plants like Jatropha curcas [16]. Similarly, andrographolide content in cell suspension culture of Andrographis paniculata (Burm. f.) Nees. is reported to be enhanced after 24 h. of treatment with 0.05 mM SA [17]. It is reported that when SA is employed to the cell culture of Salvia miltiorrhiza [25] and grape cell cultures [26]. The study shows that the elicitation depends on elicitor dose and type of compound to be elicited.

Table 1. Distribution of items related to knowledge and its relation to gender and year of entry among dental students about the prevention, early diagnosis, and referral of patients with oral cancer in 1397

Morphology	Time taken for callus initiation	Callus induction (%)	Callus dry weight (mg)	Callus fresh weight (mg)	Callus volume (mg/l)	Explant	Treatment (mg/l)
Light-green, compact	6	83.33	0.844	0.917	0.981	Internode	1 NAA
Light-green, compact	6	66.22	0.802	0.95	0.797	Internode	2 NAA
Light-green, compact	6	100	0.855	1.118	1.164	Internode	3 NAA
Yellow, watery	18	66.22	0.714	0.724	0.756	Internode	0.1 2,4-D
Yellow, watery	18	33	0.709	0.738	0.755	Internode	0.5 2,4-D
Yellow, watery	15	65.66	0.725	0.766	0.812	Internode	1 2,4-D
	0	0	0	0	0	Internode	1 BAP
	0	0	0	0	0	Internode	2 BAP
	0	0	0	0	0	Internode	3 BAP

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Table 2. Effect of SA on synthesis in total phenolic and flavonoids content in callus culture of Ruta graveolens

Treatment (mg/ml SA)	total phenolic content (mg/g)	flavonoids content (mg/g)
0	21.15	23.75
0.5	225.32	8.32
1	41.99	29.41

Conclusion

From this study, it is concluded that salicylic acids can be used as elicitors to enhance the secondary metabolites in callus culture of R. graveolens. Also, this study shows that the elicitation depends on elicitor dose and type of compound to be elicited.

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