Cellular and Molecular Biology

Darvishi *et al. Cell. Mol. Biol.*2016, 62 (3): 7-9
ISSN: 1165-158X
doi: 10.14715/cmb/2016.62.3.2

Original Research

In vitro induction of α-pinene, pulegone, menthol, menthone and limonene in cell suspension culture of pennyroyal (*Mentha pulegium*)

E. Darvishi[1], D. Kahrizi[1*], S. Bahraminejad[1], M. Mansouri[2]

[1] Department of Agronomy and Plant Breeding, Faculty of Agriculture, Razi University, Kermanshah, Iran.
[2] Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran

Abstract: Medicinal plants are known as important sources of secondary metabolites. Because of the economic value of pennyroyal [*Mentha pulegium* L. (Lamiaceae)] in food industries, propagation of this valuable plant has special importance. Plant cell suspension culture can increase some produced components. The aim of this research was performing cell culture for induction of some secondary metabolites of *M. pulegium* and compares it with native one. The MS medium was used for suspension culture. To investigate quantitative materials, 4 levels of yeast extract elicitor (20, 40, 60 and 80 mg/L) and salicylic acid in 4 levels (2, 4, 6 and 8 mg/L) were used. Obtained extracts were analyzed by GC-MS. Statistical analysis showed that the amount of limonene, menthone, menthol and α-pinene were more than mentioned compounds in natural plant as control. The maximum amount of this metabolites were obtained as limonene (in 60 mg/l yeast extract), menthone (in 40 mg/l yeast extract and 2 mg/l salicylic acid), menthol (in 6 mg/l salicylic acid) and α-pinene (in 4 mg/l salicylic acid) in the *M. pulegium* cell culture. The Pulegone was fond more in natural plants than cell culture mass. The most important secondary metabolites were increased by cell culture containing of salicylic acid and yeast extract elicitors in *M. pulegume*.

Key words: Salicylic acid, yeast extract, elicitor, secondary metabolite.

Introduction

The medicinal plants are known as valuable sources for production of secondary metabolites. Secondary metabolites have many applications in food industries, sanitary and pharmacy fields (1-6). Some important medicinal components are found in low concentrations in different medicinal plant species. Then researchers always have attempted to increase them. Plant biotechnology has appropriate methods, cell culture, tissue culture, genetic engineering, molecular markers which can increase gene expression and production to produce drug (1, 7-9).

In recent years, plant tissue culture techniques are used as powerful tools to the micro-propagation and breeding of many plant species (10).Plant tissue culture has numerous applications in the field of medicinal plants; such as rapid and mass plant multiplication, pathogen-free plants production, enhance performance and yield, protect endangered species and the *in vitro* production of secondary metabolites (7, 10, 11).

In vitro conditions, not only has increased the plants medicinal component production but new product also produced as compared natural conditions. Secondary metabolite can be produced by tissue culture methods under sterile and controlled conditions, and also purer and safer compounds are produced. Actually cell culture method is the best and the most economical method to produce these metabolites (10, 12).

Pennyroyal [*Mentha pulegium* L. (Lamiaceae)] is an important medicinal plant and has an important position in food industries, sanitary and pharmacy fields. The pennyroyal plant is herbaceous and shrub height to 60 cm that grows in around rivers and all part of plant have medicinal trait. The main components of the essential oil of this plant are α-pinene, β-pinene, limonene, 3-octyl acetate, *p*-cymene, menthone, isomenthone, menthol, pulegone, caryophyllene, lauric acid, myristic acid, palmitic acid, salicylaldehyde etc (13).

Menthol is the most important secondary metabolite in pennyroyal. Actually the cooling taste which is felt during eating comes from menthol. Pennyroyal can be consumed as a pain killer, antibacterial, antifungal, anti-bloat, anti-inflame, etc (13).

The few investigators were reported on *M. pulegium* cell culture. The aim of this research was performing cell culture for investing secondary metabolites of *M. pulegium* and compares it with those in native one.

Materials and Methods

As the abundant calli are necessary to cell suspension culture, then the white and delicate calli were obtained in MS medium with 1 mg/L 2,4-D. Furthermore this medium was used for cell culture as liquid medium. Two grams of calli was inoculated to 100 mL liquid media with different concentrations of elicitor. Four levels of yeast extract elicitor (20, 40, 60 and 80 mg/L) and salicylic acid in 4 levels (2, 4, 6 and 8 mg/L) were used. These elicitors were filtered to media after autoclaving. Then they were placed on incubator shaker with 100 round per minute in 25±1˚C. This study was performed in completely randomized design (CRD) with nine treatments (two elicitors each one in four levels

Received January 10, 2016; **Accepted** March 15, 2016; **Published** March 20, 2016

*** Corresponding author:** Danial Kahrizi, Associate Prof., Department of Agronomy and Plant Breeding, Faculty of Agriculture, Razi University, Kermanshah, Iran. Email: dkahrizi@yahoo.com

Table 1. Mean squares for the percentage of secondary metabolite in *Mentha pulegium.*

Source of variation	df	Limonene	Menthone	Menthol	Pulegone	α-pinene
Elicitor	9	0.42**	2.185**	17.362**	16.454**	1.597**
Error	20	0.004	0.009	0.006	0.048	0.003
CV%		3.73	1.828	1.056	1.918	2.088

**significant differences in the level of 0.01

with one control) and three replications. After ten days, cell masses were filtered by filter paper and were dried by freeze dryer and then were extracted by micro-Clevenger. Obtained extracts were analyzed by GC-MS to determine the amount of secondary metabolites in cells.

Results and Discussion

According to Table 1, the impact of the different concentrations of elicitor in the MS media culture was investigated on secondary metabolites and it was found that significant differences exists between different concentrations of elicitor in the MS media and the percentage of secondary metabolite (P≤0.01). According to Table 2 and Fig. 1, it was defined that percentage of Alpha-pinene as a secondary metabolite in salicylic acid 4 mg/L was more than other concentrations of elicitors, and also the amount of this metabolite in the cell culture was more than natural plant. The percentages of this metabolite decreased with increasing concentrations of yeast extract elicitor. Yadegari and Shakeri (2014) increased amount of this metabolite in cell culture of *Salvia officinalis* L. by adding of salicylic acid elicitor which it has conformity with current research results (14). Amount of pulegone as a secondary metabolite was more in the natural plant than the cell suspension culture, and amount of this metabolite was very low under the suspension culture without elicitor and with 20 mg/L yeast extract elicitor. In other words, percentage of pulegone increased with increasing concentrations of yeast extract elicitor (Table 2 and Fig. 2).

Regression analysis showed that these two variables have regression equation Y=6.382+0.993X

and coefficient showed that 98% of changes in this metabolite relates to the concentrations of yeast extract elicitor, and also regression equation Y=9.826+0.813X was obtained for percentage of pulegone increase with increasing concentrations of salicylic acid elicitor and its coefficient showed that 66% of changing this metabolite relates to the concentrations of salicylic acid elicitor. Menthone is one of the important secondary metabolites in pennyroyal (*Mentha pulegium*).The maximum amount of menthone was in the cell culture containing salicylic acid 2mg/L (6.41%) and in the cell culture containing yeast extract elicitor 40 mg/L (6.46%). The minimum amount of this metabolite (3.77%) was found in the natural plant. In other words, amount of menthone decreased with increasing concentrations of salicylic acid elicitor. About limonene, it was observed that the maximum amount of this metabolite was in the cell culture containing yeast extract elicitor 60 mg/L and the minimum amount was in the cell culture containing

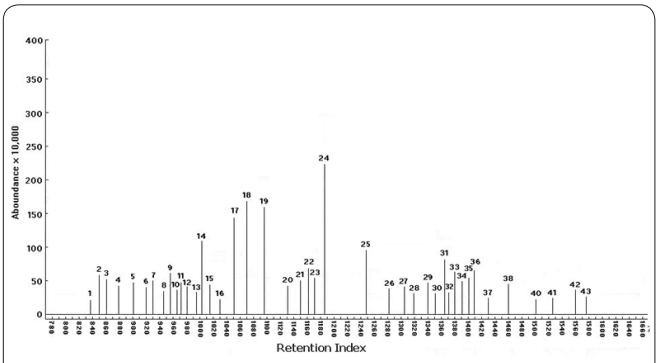

Figure 1. GC-Mass results of the effect of salicylic acid on secondary metabolites in *Mentha pulegium* α-pinene (3), limonene(11), menthone (17), menthol (19), pulegone (24).

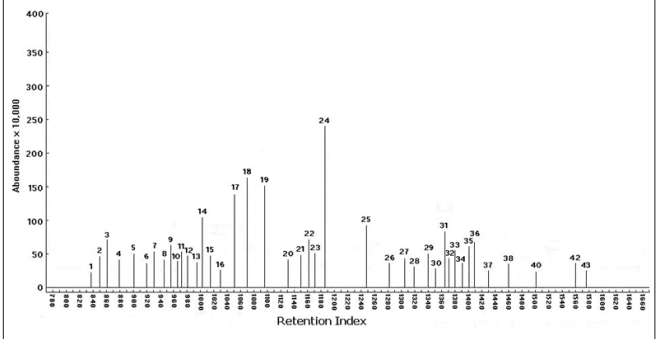

Figure 2. GC-Mass results of the effect of yeast extract on secondary metabolites in *Mentha pulegium* α-pinene (3), limonene(11), menthone (17), menthol (19), pulegone (24).

Table 2. Mean comparison for the effect of the different concentrations of elicitor on the percentage of secondary metabolites in *Mentha pulegium.* Where SA is salicylic acid.

Elicitor (mg/ml)	α-pinene	Pulegone	Menthol	Menthone	Limonene
Control	3.18 bc	8.39 g	1.43 i	5.60 b	1.49 f
Yeast (20)	3.29 b	7.97 g	6.35 h	4.60 d	1.60 f
Yeast (40)	2.86 e	10.11 f	7.03 g	6.46 a	2.03 c
Yeast (60)	2.68 f	11.20 e	7.89 f	4.39 d	2.49 a
Yeast (80)	3.06 cd	13.30 c	9.04 c	5.30 c	2.20 b
SA (2)	2.21 g	10.21 f	7.00 g	6.41 a	2.02 c
SA (4)	3.81 a	12.27 d	8.50 e	5.10 c	1.68 de
SA (6)	2.59 f	14.06 b	9.78 a	5.76 b	1.20 g
SA (8)	3.02 d	13.03 c	8.79 d	5.20 c	1.66 de
Natural plant	1.11 h	14.79 a	9.24 b	3.77 e	1.80 d

salicylic acid 6 mg/L. Percentage of limonene increased with increasing concentrations of yeast extract elicitor and it decreased with increasing concentrations of salicylic acid elicitor. Yadegari and Shakeri (2014) reported that limonene cannot be produced by cell culture *in Salvia officinalis L.* which this result could be obtained when applies for other plants (14).

Menthol is one of the most important secondary metabolite in the pennyroyal that creates coolness in mouth by eating it. The maximum amount of menthol obtained in the cell culture containing salicylic acid 6 mg/L which it was more than natural plant and the minimum amount of this metabolite was observed in the cell culture without elicitor (Table 2). According to regression equation Y=5.347+0.99X, amount of menthol increased with increasing concentrations of yeast extract elicitor, and determination coefficient showed that 98% of changes in this metabolite relates to the concentrations of yeast extract elicitor, therefore high concentrations of yeast extract elicitor in the medium is being recommended. In a study, Chakraborty and Chattopadhyay (2008) reported that menthol can be increased in *M. piperita* by cell culture (15).

Conclusion

The most important secondary metabolites in *Mentha pulegume* were increased in cell culture presence of salicylic acid, citric acid and yeast extract elicitors.

Acknowledgments

Special thanks to Dr. Yari, Zagros Bioidea Lab, Razi University Incubator for critical reading of this manuscript. The Razi University of Kermanshah, Iran, has financially supported this research project.

Special thanks to Dr. Ram Chandra Suthar (Department of Biotechnology, P. S. Science and H. D. Patel Arts College, Kadi-382517, S.V. Campus, KSV University, Gujarat, India) for critical reading of this manuscript.

References

1. Kumar J, Gupta PK. Molecular approaches for improvement of medicinal and aromatic plants. Plant Biotechnol Rep 2008; 2: 93-112.
2. Mazid M, Khan T, Mohammad F. Role of secondary metabolites in defense mechanisms of plants. Biol Med 2011; 3: 232-249.
3. Molsaghi M, Moieni A, Kahrizi D. Efficient protocol for rapid Aloe vera micropropagation. Pharma Biol 2014; 52: 735-739.
4. Raoa SR, Ravishankar GA. Plant cell cultures: Chemical factories of secondary metabolites. Biotechnol Adv 2002; 20**:**101–153.
5. Verpoorte R, Memelink J. Engineering secondary metabolite production in plants. Curr Opin Biotechnol 2002; 13: 181-7.
6. Wink M. Functions and Biotechnology of Plant Secondary Metabolites. Second edition. Inc. New Delhi, India 2010; 20-30.
7. Mulabagal V, Tsay HS. Plant cell cultures an alternative and efficient source for the production of biologically important secondary metabolites. J Appl Sci Eng Tech 2004; 2: 29-48.
8. Oksman-Caldentey KM, Inzé, D. Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. Trends Plant Sci 2004; 9: 433-440.
9. Taravat E, Zebarjadi AR, Kahrizi D, Yari K. Isolation, cloning and characterization of a partial novel aro A gene in common reed (*Phragmites australis*). Pharma Biol 2014; 52: 1-5.
10. Kahrizi D, Arminian A, Masumi Asl A. *In vitro* Plant Breeding. Razi University. Press. 2011.
11. Tripathi L, Tripathi JN. Role of biotechnology in medicinal plants. Trop J Pharm Res 2003; 2: 243-253.
12. Bourgaud F, Gravot A, Milesi S, Gontier E. Production of plant secondary metabolites: a historical perspective. Plant Sci 2001; 161: 839-851.
13. Özgen U, Mavi A, Terzi Z, Yıldırımb A, Coşkunc M, Houghtond P.J. Antioxidant Properties of Some Medicinal Lamiaceae (Labiatae) Species. Pharma Biol 2006; 44: 107-112.
14. Yadegari M, Shakerian A. The effect of salicylic acid and jasmonic acid foliar applications of essence and essential oil of *Salvia officinalis* L. J Appl Sci Agri 2014; 9: 1578-1584.
15. Chakraborty A, Chattopadhyay S. Stimulation of menthol production in *Mentha piperita* cell culture. In Vitro Cell Dev-Pl 2008; 44: 518-524.