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Recognition of Sulfur Compounds in Tissue Culture Different Organs of Persian Shallot (*Allium hirtifolium* Boiss) by GC/MS

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ABSTRACT

Background & Aim: Persian shallot (*Allium hirtifolium* Boiss) with common name of "Musir" is one of the endemic medicinal plants in Iran. Leaves and bulbs with antibacterial, antifungal, anticancer, anti-diabetes and anti-obesity effects are edible parts of this plant. The tissue culture is an influential method to multiply plant productions in order to meet demands of pharmacy and food industries. Therefore this article will focus on levels and structures of sulfur compounds in seeds, grassland bulbs and compare them with those of leaves, roots, bulbs and callus obtained from the tissue culture using the headspace- Gas chromatography-Mass Spectrophotometry (GC/MS) method.

Experimental: At the first of the study, seeds and stem-discs of grassland bulbs were cultured in MS media. After 90 days seeds, grassland bulbs, seed-leaves, seed-bulbs, leaves and roots of seed-bulbs, stem-disc bulbs, and callus of stem-discs analyzed by GC/MS-headspace method.

Results & Discussion: The results of analysis showed the highest variety of sulfur compounds was in seed bulb roots, while lowest levels were found in seeds, seed bulb leaves and callus. And also the highest level of sulfur compounds was discovered in stem-disc bulbs (28.31%), seed bulb roots (18.61%) and grassland bulbs (6.23%).

Industrial and practical recommendations: Our findings has been shown Persian shallot sulfur compounds have mono, di and tri sulfur in structures and the best source for these metabolites were roots and stem-disc bulbs producing by *in vitro* culture method.

1. Introduction

Alliums with more than 800 species are the biggest monocotyledon genus (Li *et al.*, 2010). This genus has hundreds medicinal plants specie, and it is one of the necessary medicinal and life supporting drugs resources (Gaitant *et al.*, 2010). Allium plants have antibacterial, antifungal, antiviral effects and are used in remedies of diabetes, stress, arthritis, hemorrhoid, colds and flu (Azadi *et al.*, 2008; Taran *et al.*, 2006). *Allium hirtifolium* Boiss belongs to Alliaceae family and is one of the important endemic Alliums that grows wildly in cold mountains of Iran (Ebrahimi *et al.*, 2009). This plant are propagated by seeds and bulbs (Etemadi *et al.*, 2011; Mohammadi *et al.*, 2010). The storage tissue of Persian shallot is usually a single main bulb like, yellow with oval white skinned (Ebrahimi *et*

al., 2009). The shallot is a perennial, herbaceous and aromatic plant which is in danger of extinction (Asili *et al.,* 2010). It is a valuable vegetable to provide dry products such as shallot powder. Moreover, Persian shallot has linoleic acid, linolenic acid and other important elements including Potassium(k), Iron(Fe), Copper(Cu), Zinc(Zn) and Manganese (Mn) (Diaz *et al.,* 2011). This herb with reducing polymerization microtubules protein has a crucial role in treated HeLa (cervival cancer) and Mcf-7 (human, caucasion, breast, and enocarcinoma) (Azadi *et al.,* 2009). In addition, by affecting activity of glycosynase enzyme, hidroalcholic extract of Persian shallot is useful for delaying the development and disorders of diabetes (Hoseini *et al.,* 2011). Mousavi et al. (2013) reported that *Allium hirtifolium* Boiss have more beneficial effects on the treatment of atherosclerosis. Also Ismail et al. (2013) mentioned Persian shallot hydro methanolic extract is effective against some pathogenic bacteria such as *Staphylococous aureus*, *Streptococous peneumoniae*, *Escherichia coli* and *Salmonella typhimunium*. The tissue culture technique is being used for producing primary and secondary metabolites as early as 1950's (Saxena and Roy, 2007). Some of the secondary components are important to medicinal properties of plants and the tissue culture is an effective method for increasing secondary metabolites in different organs of plants. Persian shallot with (-SH) groups of sulfur compounds can oxidize the lipid synthesizing enzymes and this activity may lead to the reduction or even inhibition of lipid synthesis (Nasim *et al.,* 2009). Since there is insufficient data to investigate the effects of tissue culture on sulfur compounds of Persian shallot, this study aims to examine levels and structures of sulfur compounds in seeds, grassland bulbs and compare them with those in leaves, roots, bulbs and callus obtained from the tissue culture by Gas Chromatography-Mass Spectrophotometry (GC/MS) with the headspace method.

2. Materials and Methods

2.1. Seed Culture

Seeds of Persian shallot were surface sterilized with 70% ethanol for 1 min and 25% (v/v) Sodium hypochlorite solution with two drops of Tween 20 for 15 min. Then the seeds were washed three times by sterile distilled water in side an aseptically flow cabinet. The seeds were cultured in half Murashig and Skoog (MS) (1962) with 2 mg/l N6-benzyl adenine (BA) medium and placed at 4°C temperature. After generation all cultures were transferred to a growth chamber at 22°Ctemperature and 16/8h photoperiod.

2.2. Stem-disc Culture

Bulbs of Persian shallot, coming from the grassland were stored at 4°C for 5 months. For internal disinfection roots and outer peels were removed from bulbs which were washed with tap water and then put in hot water (45°C) for one hour. The bulbs were surface sterilized with 70% ethanol for 1 min and sodium hypochlorite 25% (v/v) with two drops of Tween20 for 25 mins. After rinsing three or four times with sterile distilled water stem-discs were cut into 0.5mm diameter and cultured in Murashig and Skoog (1962) medium without hormones. All cultures were incubated in a growth chamber with white light, 16/8h photoperiod and 22°C temperature.

2.3. GC/MS Analysis Preparation

After 90 days seeds produced bulbs with leaves and roots and, callus and bulbs were generated from stem disc explants (Fig 1).

For Gas chromatography-Mass Spectrophotometry (GC/MS) analysis seeds, grassland bulbs, seed-leaves, seed-bulbs, leaves and roots of seed bulbs, stem-disc bulbs, and callus of stem-discs were dried at the room temperature (25°C) with sterile conditions. In the end the volatile compounds of 0.1 g of each sample were evaluated using the headspace method.

2.4. GC/MS Injection Condition

Qualitative analysis of the samples was performed by an Agilent model 6890 GC interfaced to a 5975 mass selective detector. The separation was achieved using 30 m Hp-5MS capillary column with inner diameter of 0.25 mm and 0.25 µm tick stationary phase film. Oven temperature was programmed to initiate at 40 °C for 5 minutes, then the temperature was raised to 270 °C at rate of 5 °C min^{-1}, and finally increased up to 280 °C and held there for 5 °C. The total run time was about 60 minutes.

3. Results and discussion

After GC/MS separation sulfur compounds were confirmed by kovats index calculated with help of

standard carbons peaks that had been injected to GC/MS and match quality factors of Wiley library (Table 1). Dry matters of seeds and grassland bulbs of *Allium hirtifolium* Boiss were analyzed using the GC/MS headspace. The GC/mass showed that the grassland bulbs had four sulfur compounds (6.23% grassland bulb) comprising Disulfide dimethyl, Dimethyl trisulfide, 2,3,5-trithiahexane and N-butyl-Benzene sulfonamide and seeds only have one (0.1% seed) sulfur component namely N-butyl-Benzenesulfonamide (Fig. 2). Study of tissue culture samples only identified N-butyl-Benzene sulfonamide in the seed bulb leaves (2.58%) and callus (1.82%) (Fig. 3).

Table 1. Names and chemical information of sulfur compounds

Compounds name	Molecular Formula	Kovats index (KI)	Retention time (RT)
Disulfide, dimethyl	$C_2H_6S_2$	722	3.299
Dimethyl trisulfide	$C_2H_6S_3$	962	11.175
2,3,5-trithiahexane	$C_3H_8S_3$	1119	17.217
Chloromethyl methyl sulfide	$C_3H_8S_2$	1120	17.218
4-Mercaptopyridine	C_5H_5NS		23.348
2,4-Dithiapentane	$C_3H_8S_2$	1506	26.606
N-butyl-Benzene sulfonamide	$C_{10}H_{15}NO_2S$	1774	31.669

Also we found three other sulfur compounds including N-butyl-Benzene sulfonamide (1.98%), Chloromethyl methyl sulfide (1.70%) and Disulfide dimethyl (1.53%) in dry matter of seed bulbs. Analysis of seed bulb roots and stem-disc bulbs driven from the tissue culture method showed both samples had Disulfide dimethyl, Dimethyl trisulfide and 2,3,5-trithiahexane and seed bulbs roots contained N-butyl-Benzene sulfonamide and 4-Mercaptopyridine. Examination of sulfur compounds samples revealed a large variety of sulfur compounds in seed bulb roots, grassland bulb and stem-disc bulbs. In grassland bulb, in addition to 4-Mercaptopyridin, there are the same sulfur compounds as there are in roots (Table 2).

Seed bulb leaves, callus and seeds with 2.58%, 1.82%, and 1.11% respectively of N-butyl-Benzene sulfonamide have less variety and levels of sulfur compounds. Results showed maximum amount of sulfur compounds in stem-disc bulbs (35.67%), seed bulb roots (18.61%) and grassland bulb (6.23%) (Table 3).

Furthermore among sulfur compounds, Dimethyl disulfidein grassland bulbs and seed bulb roots with 64.55% and 11.91% respectively and 2,3,5-trithiahexane in stem-disc had the highest levels. Generally sulfur compounds of Persian shallot obtained from the headspace method were mono, di and tri sulfide in in vivo and in vitro samples (Table 2).

Many Allium species have different types of sulfur compounds such as Allin, a non-protein sulfur amino acid and etc. (Benkeblia and Lanzotti, 2007). Sulfur is required for the synthesis of other compounds such as secondary sulfur compounds (glocosinolates, phytochelatins, allins), thiol (glutathione) and sulpholipids, which play important roles in the physiology, adaptation and protection of plants against pests and stress. Proteins have sulfur; cysteine and methionine residues which are highly significant in the conformation, function and structure of proteins are good illustrations (Durenkamp and Kok, 2004). Organosulfur compounds are known for anticancer, anti HIV and antifungal properties, so diets that are generally rich in plants from family of Alliaceae have beneficial health effects (Haq and Ali, 2003).

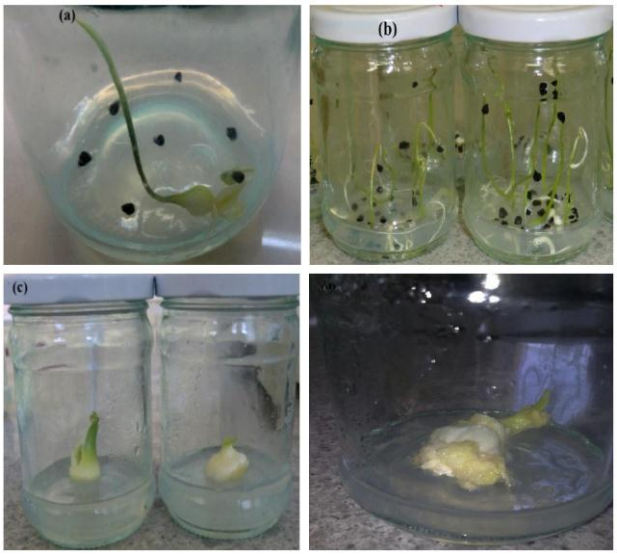

Fig 1. (a) Seed bulb; Seed leaves (b); Stem-disc bulbs(c); Callus induction(d)

The volatile compounds of Allium genus mainly represented by thiosulfinate are very unstable. Depending on the species and under different conditions, thiosulfinates of Allium species can be decomposed to additional sulfur constituents including methyallyl, diallyl, diethyl mono,di-, tri-, tetra-, penta and hexasulfieds (Shams, 2003). Cell suspentions, callus and other tissue cultures have been used successfully for study of allin (s-allyl-L cysteien) synthesis in garlics and isoallin in onions (Hughes *et al*, 2005). The result in this paper argued that the tissue culture had very important effects on sulfur components of Persian shallot because the highest level of sulfur compounds are found in seed bulb roots growing in Murashig and Skoog medium with five different compounds such as Disulfide, dimethyl, Dimethyl trisulfide, 2,3,5-trithiahexane, N-butyl-Benzene sulfonamide and 4 –Mercapto pyridine that constitute 35.67% of total volatile.

Table 2. Sulfur compounds of samples; Seeds(A), Grassland bulbs(B), Stem-disc bulb(C), Seed bulb(D), Seed leaves(E),Seed bulb leaves(F), Seed bulb roots(G), Callus(H).

Sulfur compounds name	**Samples**							
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Disulfide, dimethyl	-	+	+	+	+	-	+	-
Dimethyl trisulfide	-	+	+	-	-	-	+	-
2,3,5-trithiahexane	-	+	+	-	-	-	+	-
Chloromethyl methyl sulfide	-	-	-	-	+	-	-	-
N-butyl-Benzenesulfonamide	+	+	-	+	+	+	+	+
2,4-Dithiapentane	-	-	+	-	-	-	-	-
4-Mercaptopyridine	-	-	-	-	-	-	+	-

Table 3. Data analysis of different sample; Disulfide, dimethyl(DD), Dimethyl trisulfide(DT), 2,3,5-trithiahexane(TTH), Chloromethyl methyl sulfide(CMS), N-butyl-Benzenesulfonamide(NBS), 2,4-Dithiapentane(DP), 4-Mercaptopyridine(MP)**.**

		Mean Square						
Fd		**DD**	**DT**	**TTH**	**CMS**	**NBS**	**DP**	**MP**
Seed	7	0.00^{f}	0.00^{d}	0.00^{d}	0.00^{b}	0.09^{d}	0.00^{b}	0.00^{b}
Grassland bulb	7	4.87^{c}	0.95^{c}	0.49^{c}	0.00^{b}	0.02^{d}	0.00^{b}	0.00^{b}
Stem-disc bulb	7	8.09^{b}	3.01^{a}	19.47^{a}	0.00^{b}	0.00^{d}	3.88^{a}	0.00^{b}
Seed bulb	7	0.92^{e}	0.00^{d}	0.00^{d}	1.07^{a}	1.41^{c}	0.00^{b}	0.00^{b}
Leaf seed	7	2.20^{d}	0.00^{d}	0.00^{d}	0.00^{b}	2.40^{a}	0.00^{b}	0.00^{b}
Leaf seed bulb	7	0.00^{f}	0.00^{d}	0.00^{d}	0.00^{b}	2.58^{a}	0.00^{b}	0.00^{b}
Root	7	12.00^{a}	2.66^{b}	0.98^{b}	0.00^{b}	1.85^{b}	0.00^{b}	1.43^{a}
Callus	7	0.00^{f}	0.00^{d}	0.00^{d}	0.00^{b}	1.89^{b}	0.00^{b}	0.00^{b}
Error	16							
Total	23							

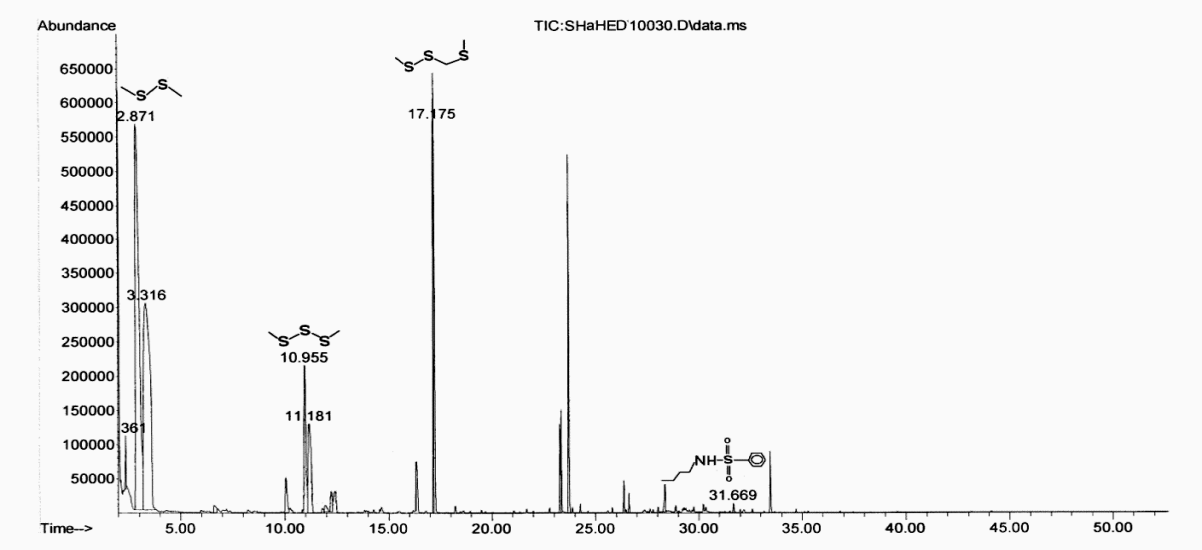

Fig 2. GC/MS peak of Grassland bulb sulfur compounds.

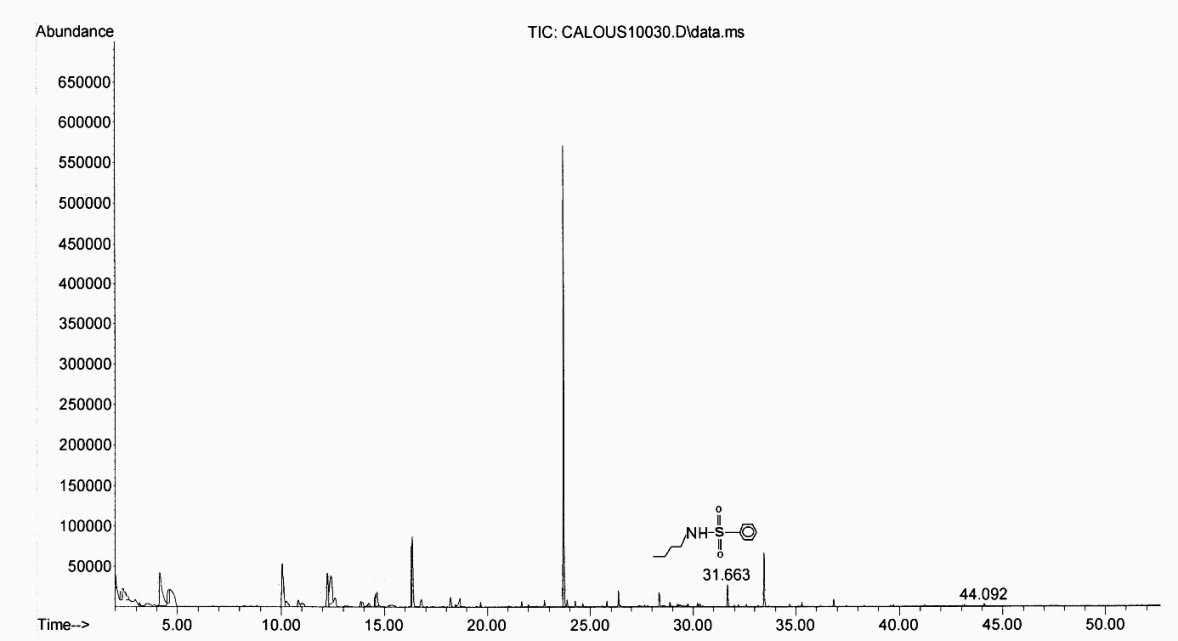

Fig 3. GC/MS peak of Callus sulfur compounds.

The active volatiles compounds in *Allium resoum* identified by Gas Choromatography Mass Spectophotometery with the headspace sampling include dimethyl disulfide, disulfide allyl dimethyl, trisulfide dimethyl, sulfide dimethyl, disulfide diallyl, trisulfideallyl dimetyl, trisulfideallyl methyl, trisulfidediallyl 2-perpenyl and tetrasulfideallyl dimethyl (Najja *et al.*, 2011). Our findingshave showed N-butyl-Benzenesulfonamide was the only sulfur compound which was found in all samples except the control sample where 2,4-Dithiapentane was observed. The major sulfur compounds of chive flowers and onions were 3,4-dimethyl thiaphene, methyl n-propyl disulfide and dimethyl trisulfide (Grzeszczuk *et al.*, 2011) .

Volatile analysis of chive by GC/MS with the headspace method indicated this plant had di and trisulfur compounds (Mann *et al*, 2011). Melluki *et al*.

(1994) reported that the essential oil of callus sulfur compounds of chives (*Allium schoenoparsum* L.) were less than that of leaves. Allin (s-allyl-L-cysteineSulfoxide) is one of the sulfur compounds in lily family (Melluki *et al.*, 1994). Nasim *et al*. (2009) demonstrated that the most amount of Allin was in leaves between different organs of garlics such as leaves, roots, embryos, and callus. Allin, Isoallin, s-methyl- and s-propyl-L-systeine-s-oxide are commonly present in Allium plants that can decompose to other sulfur compounds with allinase enzymes (Benkeblia and Lanzotti, 2007). In this study sulfur compounds of callus (1.825%) were less than those of seed leaves (4.47%) and seed bulb leaves (2.58%). Also the most important compounds of grassland bulbs in other sections of Persian shallot were 2,3,5-trithiahexane (Roshan *et al.*, 2012) and trisulfid dimethyl (Sham, 2003). Diaz *et al*. (2011) reported essential oil of Leaves and roots of *Allium schoeoprasum* L. analyzed by GC/MS, consisted of bis-(2-sulfhydryethyl-) disulfide with the major components in both samples (72.06% leaves, 56.47% roots)(Diaz *et al.*, 2011). Also they found 2,4,5-trithiahexan in leaves (5.54%) and roots (15.90%) (Ebrahimi *et al*., 2008). *Allium ascalonicum* L. growing in Thailand and *Allium tuberosum* Rottlerin China had diallyl mono, di, tri. In this study, the chemical analysis has identified sulfur compounds obtained from GC/MS were mono, di and tri sulfide. The thiosulfinate can break down to disulfide and trisulfide that are toxic to insect herbivores (Mohammadi *et al.*, 2010).

4. Conclusion

Our findings by Gas chromatography-Mass Spectrophotometry with headspace method has been shown Persian shallot (Musir) sulfur compounds has mono, di and tri sulfur in structures and the best source for these metabolites were roots and stem-disc bulbs producing by invitro culture method. Significantly, the data obtained from the present study suggests that the tissue culture is an effective method of increasing sulfur compounds in *Allium hirtifolium* Boiss.

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