# PRODUCTION OF VOLATILE SULPHIDES IN *ALLIUM PORRUM* CELL CULTURES

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## ABSTRACT

Production of volatile sulphides in cell cultures of *Allium porrum* is described. *Allium porrum* calluses were initiated from whole seedlings. The high growth rate of *Allium porrum* callus was achived in Murashige and Skoog media containing only 1ppm 2,4-Dichlorophenoxy acetic acid. The routine method of solvent extraction of volatile sulphides was used for *Allium porrum* and the concentrated extract was subjected to capillary GC and GC-MS. Dipropyl disulphide and 4-methyl thiazolethanol were identified in *A. porrum* aggregated suspension cells.

**Key words:** *Allium porrum*, Cell cultures, Dipropyl disulphides, Thiaminase

## INTRODUCTION

*Alliums* which belong to the Lilaceae family have been grown for many centuries for their characteristic pungent flavour and medicinal properties. Fenwick and Hanley (1) have reviewed the therapeutic effects of *Allium* species. The characteristic odour of *Alliums* is due to its volatile oil constituents, which consists of a large number of sulphur compounds. Among the various constituents of food flavours, sulphur compounds posses powerful and characteristic odours, which contribute to the flavours of many foods (2). The constituents of *Allium* species have been the subjects of many publications. Naturally occurring sulphur containing compounds which are found especially in garlic, onions and leeks, may be important in reducing the risk of artherosclerosis. The volatile components of *Allium cepa* (Onion), *A. sativum* L. (Garlic), *A. schoenoprasum* L. (Chive), and *A. porrum* (Leek) have been extensively investigated (3-6).

Successful establishment of onion callus cultures without assessment of its flavour levels (7), detection and analysis of its volatiles which were comparable with the whole plant, (8) as well as effects of hormonal treatment, nutritional factors, and precursors (9) has been described. Also production of volatile sulphides in several allium species except of *Allium porrum* has been reported. This article presents the results of investigation on establishment of *Allium porrum* callus and effects of suspension cultures for production of the biologically active substances.

## MATERIALS AND METHODS

*The Source of Plant Materials and Germination of Seeds*: The seeds of *Allium porrum* which were obtained from Esfahan Botanical Research Center, Iran were germinated as follows. The seeds were first surface sterilized by shaking in 30% (w/v) aqueous hydrogen peroxide solution containing 1% (v/v) Tween 80 for one to five minutes. They were then incubated in 10 x 90 mm sterilized glass petri dishes lined with two sheets of filter paper which contained approximately 20 ml of distilled water. The petri dishes were kept in the dark at a temperature of 27±2°C until the seeds germinated and then were transferred into twelve hourly light/dark conditions for a strong seedling. The seedlings obtained from the aseptically-germinated seeds were repeatedly cut with sterile scalpel and were aseptically transferred to screw-cap wide-mouth bottles containing 50 ml of solid media which contained various concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) and kinetin. These were then left at a temperature of 27± 2°C under a twelve hourly light/dark cycle.

*Preparation of Solid and Liquid Media*

Murashige and Skoog (Sigma, 4.4 g), was slowly added to the distilled water, (750 ml) with gentle continuous stirring and heating until it was completely dissolved. Sucrose (BDH, 30 g) and ascorbic acid (BDH, 5 mg) were then added. For the preparation of solid media rather than liquid media, the solidifying agent, agar (Agar-agar, Sigma, 12 g), was slowly added. The mixture was

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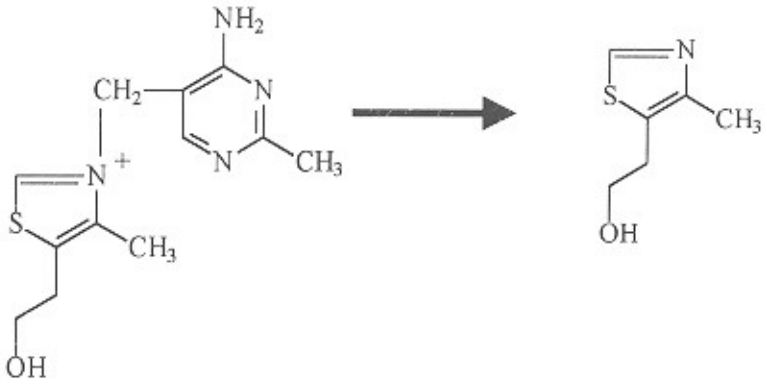

Figure 1: The proposed cleavage of thiamine

continuously heated and stirred to produce a clear solution, but boiling was avoided. The required growth hormones (2,4–dichlorophenoxy acetic acid and kinetin) and coconut water (5 ml) were added and then the pH was adjusted to 5.7 by the use of sodium hydroxide 1N or hydrochloric acid. The final volume was then made up with distilled water. Then liquid media was poured into 250 ml Erlenmeyer flasks that were then covered with aluminum foil, and solid media, before solidification, were poured into screw-capped wide mouth bottles. The amount of medium poured into each container was 50 ml. Finally, the containers were autoclaved at 121°C /15 psi for 15 min. and the medium was allowed to cool and stored at room temperature until the time that were used.

*Preparation and Maintenance of Callus Tissue and Suspension*

Calluses were usually initiated from the whole seedlings. The established culture was maintained by subculturing every 2-6 of weeks, depending on the growth rate of cells. When the callus had reached adequate size (4-5 $cm^3$), or the media showed signs of exhaustion and dehydration, the callus was divided into several healthy looking pieces of about 1 $cm^3$ and transferred onto new fresh media. Suspension cultures were made by transferring a healthy 3-4 $cm^3$ size callus to sterile 250 ml Erlenmeyer flasks containing 50 ml of liquid medium. The callus was then agitated, by a magnetic stirrer until a suspension of free cells was formed. The flasks were then placed on a rotary shaker running at 100 rpm. The suspension cultures were maintained by subsequent subculturing. Both types of culture were incubated, at 27±2 °C under 12 hours / day photoperiods unless otherwise mentioned. Light was supplied from cool white fluorescent tubes. Initiation and all other manipulations were carried out under aseptic conditions, using a flow laminar cabinet with vertical one way (up to down) flow of sterilized air.

*Extraction*

The routine method of solvent extraction of volatile sulphides was used for *Allium porrum*. This involved the homogenization of 2 x 5 g prechilled and crushed fresh callus material or filtered cells in 10 ml of 0.1 M sodium pyrophosphate buffer, pH 9.0 in a screw-capped test tube for 10 minutes. After centrifugation at 2000 g for 5 minutes, the supernatant was removed and extracted with 2 ml re-distilled diethyl ether on a shaker for 5 minutes. The ethereal extract was similarly centrifuged and the solution was dried over anhydrous sodium sulphate, concentrated under $N_2$ and analyzed by gas chromatography (GC).

*GC Analysis of Volatiles*

One µl of the reduced extract prepared above was subjected to capillary GC. A Hewlett Packard 5890 GC, fitted with a flame ionization detector, was used for the analysis of volatiles. The column used was a high performance fused silica cross-linked methyl silicon megabore capillary column, the film

thickness was 3.0 μm. The length of the column was 30 m and its diameter 0.31 mm. The results were recorded on a Hewlett Packard 3392A integrator. The operating conditions were as follows: The temperature program used was 70-280°C at 10°C/min, injector and detector temperature 270 °C.

*Identification of Components by Gas Chromatography -Mass Spectrometry*

Determination of mass spectra was carried out using a Kratos Concept 25 spectrometer at 70 eV equipped with a Sun Mash 3 computer data output. The operating conditions were similar to those of GC analysis, but with Helium as the carrier gas. Mass spectrometer conditions were: ionization current, 1A; ionization potential, 70 eV; source temperature, 150; resolution, 1000; scan speed, 1 sec/decade. Identification was based on retention data, electron-impact-mass spectra (EI-MS) data and comparison of chemical ionization spectral data with those relevant reference samples and literature (5, 10, 14).

## RESULTS AND DISCUSSION

*Cell Cultures*

The high growth rate of *Allium porrum* callus was obtained on media containing only 1 ppm 2,4-D. The media supplemented with 1 ppm 2,4-D and 0.1 kinetin also produced adequate callus (table 1). The calluses were white or cream in colour and friable in appearance. *A. porrum* suspension cell cultures were white-coloured and granular.

**Table 1.** Effect of growth regulators on *Allium porrum* callus induction

| Treatment | Callus growth |
|---|---|
| 0.1 ppm K + 1 ppm 2,4-D | $2^+$ |
| 1 ppm 2,4-D only | $3^+$ |
| 0.25 ppm K + 2.5 ppm 2,4-D | $1^+$ |
| 2.5 ppm 2,4-D only | $1^+$ |

K= Kinetin; 2,4-D= 2,4-Dichlorophenoxyacetic acid, $1^+$= slight growth; $2^+$= moderate growth, $3^+$= profuse growth.

*Secondary metabolites*

Two secondary metabolites were identified in *A. porrum* cell suspension cultures. Their mass spectra fragmentations are presented in table 2. Although several volatile sulphides have recently been identified in callus cultures of onion (*Allium cepa* L.)(11), no sulphide compounds were detected in *Allium porrum* calluses over 3 generation. In this study, dipropyl disulphide was obtained from *A. porrum* aggregated suspension cells formed in hormone-free medium. Formation of aggregated cells induced the synthesis of this disulfide. Similarly, the formation of small roots has been reported to be essential for disulfide production in *A. cepa* callus cultures (12).

**Table 2.** GC-MS Identification of *Allium porrum* suspension culture volatiles

| Rt (min) | Compound | EI* | M+ | Ref. |
|---|---|---|---|---|
| 4.53 | Dipropyl, Disulfide | 43, 150, 41, 108, 66, 39, 74, 47, 45 | 150 | 5 |
| 6.89 | 4-methyl thiazolethanol | 112, 85, 45, 113, 143, 59, 71 | 143 | 14 |

*Major fragments in order of decreasing m/z.

In addition, an unusual compound was found in cells and media of *A. porrum* suspension cultures, which was identified as 4-methyl thiazolethanol. The isolation of this compound from *Panax ginseng* plant has been described and it has been claimed that it was isolated for the first time from a natural source (13). This compound appeared to be a cleavage product of thiamine. It was assumed that the formation of this compound arises from the attack of thiaminas on the C-N+ bond of thiamine (Figure 1). In an attempt to support this idea, the suspension cultures were fed with high levels of thiamine and it was found that the presence of 1000 ppm of thiamine led to production of methylthiazolethanol as the only detectable component in cells and medium. This finding suggests that suspension cells of *A. porrum* contain thiaminase, which hydrolyse thiamine. It is the first evidence for the existence of thiaminase in plant cell cultures. Consistent with this result is the report that cultures of *Saccharomyces cerevisiae* contain thiaminase, which transform thiamine to methyl thiazolethanol (14), and reports on the presence of methyl thiazolethanol in three strains of the fungus *Ligninolytic basidiomycetes* (15). Although thiaminase has been reported from various bacteria, its physiological function is unknown (15). The production of methyl thiazolethanol in cell cultured of *A. porrum*, may be of special interest and further studies are required to explore the enzymatic reactions occurring in this culture.

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