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ANALYSIS OF PRENYLLIPIDS IN THE CHLOROPLASTS OF THE LIVERWORT *HETEROSCYPHUS PLANUS*

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ABSTRACT

Liverworts belonging to the Class Hepaticae of the Division Bryophyta, are an excellent source of lower terpenoids. They possess intracellular oil bodies which are composed of lipophilic terpenoids and aromatic compounds. In this study, an attempt was made to identify the prenyllipid component that is localized to the chloroplasts of the liverwort, *Heteroscyphus planus* (Mitt.) Shiffner. The prenyl diphosphates were extracted by stirring the intact chloroplasts overnight in methanol, without rupturing the chloroplasts in liquid nitrogen. The chloroplasts were found to contain farnesyl diphosphate and geranylgranyl diphosphate which were detected after hydrolysis by alkaline phosphatase to their respective isoprenols, farnesol and geranylgeraniol. Hydrolysis with KOH yielded phytol and geranylgeraniol which were present in the chloroplasts as esters with chlorophyllide (chlorophyll *a* and geranylgranyl diphosphate-chlorophyllide respectively). Only phytol was detected at high level, whereas, geranylgranyl diphosphate and geranylgranyl diphosphate-ester were at very low levels. The *cis* and *trans* isomers of farnesol were separately identified, but in very low quantities. These results were further confirmed by GC-MS analysis. Linoleic acid, palmitic acid, methyl ester of palmitic acid and the ester of phthalate acid were identified as other lipids localized to the chloroplasts of *H. planus.*

Keywords: terpenoids, lipophilic, farnesol, geranylgeraniol, phytol

INTRODUCTION

The isoprenoids make up the largest family of natural products comprising more than 22,000 known compounds. Isoprenoids or isoprenoid-derived compounds play a vital role in all living organisms. They include various primary metabolites, such as, sterols, carotenoids, growth regulators and quinones. The most numerous isoprenoids are, however, considered as secondary metabolites as they are not essential for the viability of the organism. These terpenoids are involved in important interactions mediating plant-plant, plant-insect and plant-pathogen interactions (Harborne, 1991). The functions of the vast majority of the known isoprenoids are still largely undiscovered.

The basic building block of all isoprenoid natural products is isopentenyl diphosphate (IPP). Three separate cell compartments, the plastids, the mitochondria and the cytosol, have been shown to be capable of converting IPP into various other terpenoids (Gray, 1987, Kleinig, 1989). Generally, the biosynthesis of farnesyl diphosphate (FPP) and its derivatives, sesquiterpenes and tritepenes including phytosterols takes place in the cytosol and the cytosol/endoplasmic reticulum boundary (Feron *et al.*, 1990; Hugueney and Camara, 1990). The monoterpenes and the diterpenes are exclusively synthesized in the plastids (Kleinig, 1989). Carotenoids and chlorophylls are the major prenyllipids synthesized in the chloroplasts (McGarvey and Croteau, 1995). In addition, chloroplasts contain prenylquinones (plastoquinone-9, phylloquinone and α-tocopherol) (Lichtenthaler, 1993). Some of these plastidprenyl-lipids include essential vitamins such as, vitamin A (β-carotene), vitamin E (α-tocopherol) and vitamin K1 (phylloquinone) (Douce and Joyard, 1996).

Cultured cells of liverworts have proven to be a useful model for studying the biosynthesis of isoprenoids due to the production of a variety of lower terpenoids by these cells. The intracellular oil bodies of liverworts are composed of various terpenoids and other aromatic compounds. This is a unique feature of the liverworts among all other bryophytes such as mosses (Class Musci) and hornworts (Class Anthoceratae) (Asakawa, 2001). Moreover, they produce lower terpenoids, which are very similar qualitatively and quantitatively to those of higher plants. The composition of oils from the

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suspension culture, the redifferentiated plant, and the parent plant has shown to be comparable (Takeda and Katoh, 1981). Many different types of isoprenoids have been identified in cultured cells of the liverwort *Heteroscyphus planus* (Nabeta *et al*., 1993), however, they have not been identified in relation to the cell compartments that they are localized.

The objective of this study was to identify the prenyllipid component of the chloroplasts of the liverwort *H. planus*. Intact chloroplasts were isolated from *H. planus* and protocols for extraction and hydrolysis of the pyrophosphates from the chloroplasts were established. The prenyllipids were identified using GC and GC-MS.

MATERIALS AND METHODS

The origin of intact plants, induction and subcultures of suspension cultured cells of *H. planus* have been reported previously (Nabeta *et al*., 1993). The cultures of *H. planus* were grown in 75 ml of MSK (modified Murashige and Skoog) medium with 4% glucose (Katoh, 1988). Twenty grams of fresh cells of *H. planus* from 28-30-day old suspension cultures were harvested using suction and ground twice in (v/w) chloroplast isolation buffer (0.33 M sorbitol, 5 mM $MgCl_2$, 10 mM $Na_4P_2O_7.10H_2O$, pH 6.5, 2 mM sodium isoascorbate) for 1 min in a mortar. The brei was filtered through 20 μM nylon mesh and the residue was again ground in the same amount of buffer and filtered. The filtrates were pooled and centrifuged at 2500 g for 50 sec and the supernatant was discarded. The resultant pellet was washed with isolation buffer (added the same buffer and centrifuged at 2500 g for 50 sec) to remove broken chloroplasts that adhered to the surface of the intact chloroplasts. Preparation and isolation of chloroplasts were carried out at 4^0 C.

The isolated chloroplasts were confirmed intact as described previously (Karunagoda, 2008). The intact chloroplasts were stirred overnight in methanol at room temperature, to extract the prenyl diphosphates from the intact chloroplasts. The methanol extract was divided into two equal fractions. One fraction was concentrated *in vacuo* and treated over night with 10 μl bacterial alkaline phosphatase enzyme (EC 3.1.3.1, Sigma) at 30^0 C to hydrolyze the diphosphates in a reaction mixture of 10 mM Tris, pH 7.8, 1 mM $MgSO_4$ and 100 mM KCl in a total volume of 2.5 ml. The resultant isoprenols were extracted three times, each time with 10 ml of ether, which contained a known amount, *i.e*. 0.01 mg of an internal standard (IS), stearyl alcohol; $CH_3(CH_2)_{17}OH$) in order to quantify the isoprenols. As extraction with ether was carried out three times, the total amount of IS present in the ether extraction was 0.03 mg. The ether extracts were combined, dried over dry Na_2SO_4 and concentrated *in vacuo* to yield isoprenols. The concentrated isoprenol sample was dissolved in 10 μl ether and 1μl was used for gas chromatographic (GC) analysis. The other half of the MeOH extract was concentrated and hydrolysed for 2 h with 2.5% KOH in MeOH under reflux (Ahrens *et al*., 1977) and extracted two times with 10 ml of hexane, containing 0.01 mg of internal standard (stearyl alcohol $CH_3(CH_2)_{17}OH$)/10ml hexane) in order to quantify the esters. Therefore, the total amount of IS present in the hexane extraction was 0.02 mg. The hexane extracts were combined and dried overnight with dry Na_2SO_4, concentarted *in vacuo* to yield phytols and dissolved in 10 μl ether and 1 μl was injected for GC analysis.

GC analysis was carried out for each of the hydrolyzed samples and authentic samples of farnesol (FOH), geranylgeraniol (GGOH) and phytol, in a Shimadzu GC 17-A Gas Chromatograph. The conditions were as follows: column- J & W Scientific DB-1, i.d. 0.25 mm X 60 m; initial temp. 60^0 C, initial temperature was kept for 5 min, progress rate at 2^0 C/min, final temp. 220^0 C; carrier gas- He, flow rate at 1.2 ml/min; detector- FID. The GC peak areas obtained with the chromatograms of FOH, GGOH, geranylgeranyl chlorophyllide (GG-chlorophyllide) ester and phytol were compared with the peak areas of the IS to estimate the quantities of each of them as follows.

GC-MS analysis was carried out in a Hitachi M-80B Gas Chromatograph for both of the above samples to identify any other lipid localized to the chloroplasts. The conditions were as follows. Column- DB-Wax (i.d. 0.25 mm X 60 m, J & W Scientific); initial temp. 60^0 C for 5 min, progress rate 2^0 C/min, final temp. 220^0 C for 20 min; carrier gas- He, flow rate at 1.1 ml/min; detector- FID; injection port- 220^0 C; ionization voltage- 70 eV, Scanning- 1 sec/scan & rescan duration 0.25 sec.

RESULTS

The objective of this study was to identify the prenyllipids present in the chloroplasts of *H. planus*. Therefore, it was important to obtain the intact chloroplasts of the liverwort and then extract the chloroplastidic prenyllipids. The chloroplasts isolated from the *H. planus* suspension cell cultures were confirmed intact. Overnight extraction of intact chloroplasts was sufficient to extract the prenyllipids in the chloroplasts. The methanol extract of the intact chlroplasts of *H. planus* cells contained the isoprenoid diphosphates and were subjected to enzyme hydrolysis to yield respective isoprenols and KOH hydrolysis to yield esters. Hydrolysis produced the isoprenols which were analyzed by GC and GC-MS and the isoprenols were identified using authentic samples.

The retention times of the authentic samples of FOH, GGOH and phytol were 11.39, 28.90 and 24.41 min respectively (Fig. 1). The isoprenols and the esters that resulted from hydrolysis were analysed using GC under the same conditions (Fig. 2 A & B). Based on the retention time of authentic samples, farnesol, gernylgeraniol, geranylgeranyl ester and phytol were identified by GC analysis. Quantities of each isoprenol and phytol obtained by the above methods were determined by GC, using the amount of internal standard (stearic acid) (Table 1). To identify the other lipids of the isolated chloroplasts of *H. planus*, suspension cells were analyzed by GC-MS. Linoleic acid, palmitic acid, palmitic ester and phytol were identified in the chloroplast fraction hydrolysed with KOH (Figs. 3 and 4 a, b, c & d).

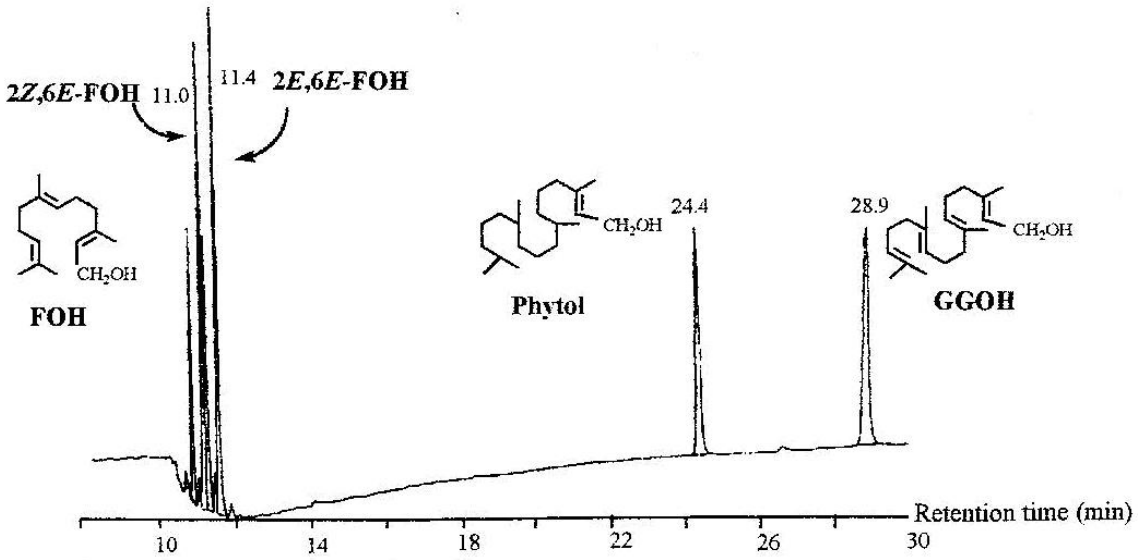

Figure 1. GC Profiles of Authentic Farnesol, Geranylgeraniol and Phytol. (Shimadzu GC 17-A Gas Chromatograph, column- J & W Scientific DB-1, i.d. 0.25 mm x 60 m; initial temp. 60^0 C for 5 min, progress rate; 2^0 C/min, final temp. 220^0 C; carrier gas- He, flow at 1.2 ml/min).

Table 1. Quantity of each isoprenol and phytol obtained from hydrolysis of the methanol extract of intact chloroplasts from 20 g of *H. planus* cells, as estimated by GC peak areas.

Isoprenoid	Quantity (mg)
2*E*, 6*E* Farnesol	1.1
2*Z*, 6*E* Farnesol	1.5
Geranylgeraniol	0.4
Phytol	16.4
Geranylgeranyl ester	0.25

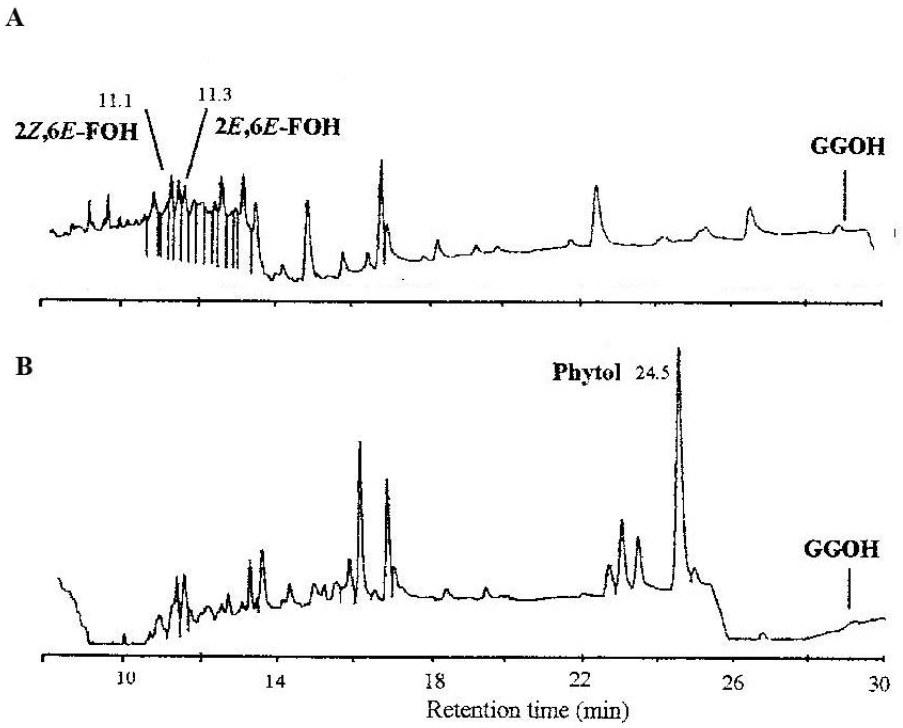

Figure 2. GC profiles of the methanol hydrolysate from intact chloroplasts of *H. planus.* (Shimadzu GC 17-A Gas Chromatograph, column- J & W Scientific DB-1, i.d. 0.25 mm x 60 m; initial temp. 60^0 C for 5 min, progress rate; 2^0 C/min, final temp. 220^0 C; carrier gas- He, flow at 1.2 ml/min). A) Enzymatic hydrolysis (hydrolysed with 10 μl bacterial alkaline phosphatase) and B) KOH hydrolysis (hydrolysed for 2 hrs with 2.5% KOH in methanol under reflux)

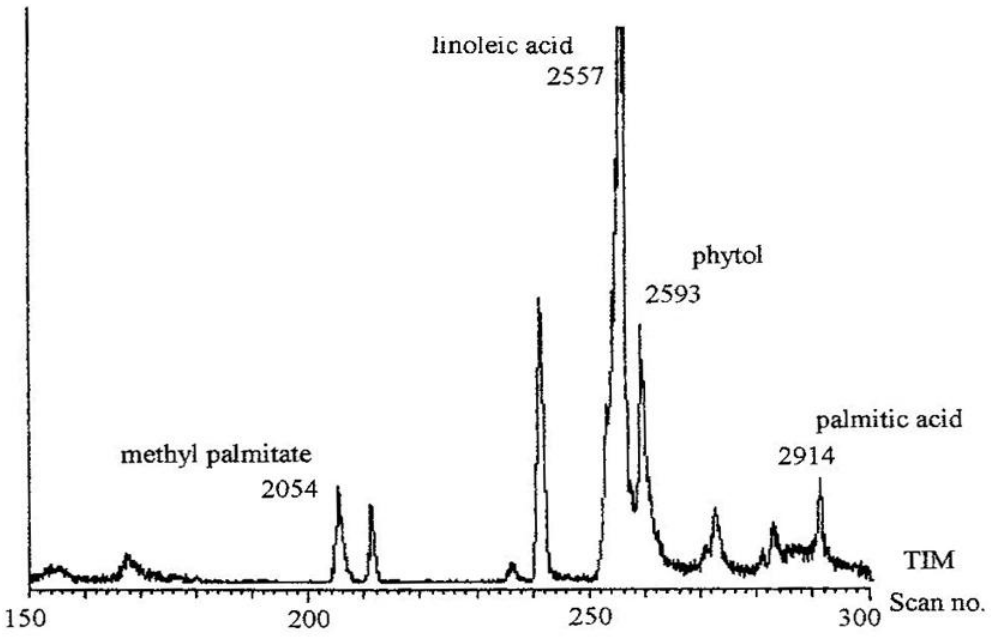

Figure 3. GC-MS profiles of the KOH hydrolysed extract of *H. planus* intact chloroplasts. (Hitachi M-80B Gas Chromatograph Column- DB-Wax (i.d. 0.25 mm X 60 m,); initial temp. 60^0 C for 5 min, progress rate 2^0 C/min, final temp. 220^0 C; carrier gas- He, flow rate at 1.1 ml/min).

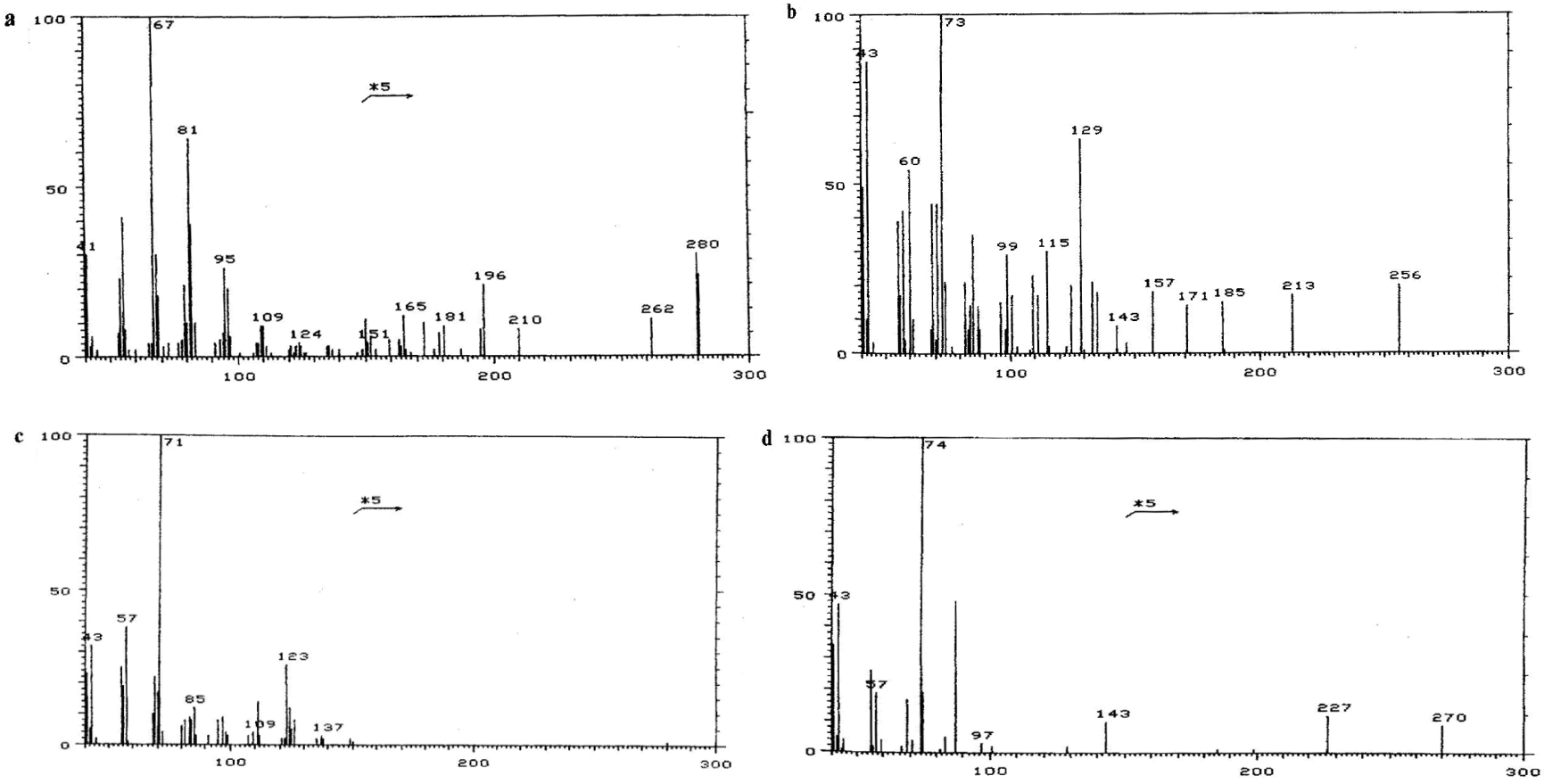

Figure 4. Mass spectra of hydrolysed chloroplast extract of *H. planus.* a) linoleic acid, b) palmitic acid, c) phytol and d) methyl palmitate. (Hitachi M-80B Gas Chromatograph Column- DB-Wax (i.d. 0.25 mm X 60 m,); initial temp. 60^0 C for 5 min, progress rate 2^0 C/min, final temp. 220^0 C; carrier gas- He, flow rate at 1.1 ml/min;)

DISCUSSION

The study was carried out to identify different terpenoids localized to the chloroplasts of liverworts. The intact chloroplasts were isolated from cultured cells of the liverwort, *H. planus*. The prenyl diphosphates and chlorophylls were extracted in methanol and hydrolysed by either KOH or alkaline phosphatase enzyme. The alkaline phosphatase enzyme hydrolyzes and removes the diphosphate groups of prenyl diphosphates resulting in the respective isoprenols. This enzyme does not hydrolyze the chlorophylls as there are no phosphate groups attached to the chlorophyll molecule. Therefore, methanolic alkali hydrolysis was used to hydrolyze chlorophyll *a* to afford phytol. The resultant isoprenols and phytols were analysed using GC. The enzymatic hydrolysis resulted FOH and GGOH, whereas, phytol and a low amount of GGOH (produced by hydrolysis of GGOH-ester) was formed by KOH hydrolysis.

Phytol is the most prominent of all isoprenoids in the chloroplasts of *H. planus* (16.4 μg for total fresh weight of 20 g) while the geranylgeranyl diphosphate (GGPP) and geranylgeranyl-ester (GG-ester) are the lowest, 0.4 μg and 0.25 μg respectively. Geranylgeranyl ester or GGPP-chlorophyllide is synthesized in the chloroplast membrane by esterification of chlorophyllide to GGPP, instead of phytyl diphosphate. The two isomers of FPP; 2*E*, 6*E* and 2*Z*, 6*E* FPP are present in the chloroplasts in comparable amounts, ranging from 1.1-1.5 μg. Phytol was the only isoprenic alcohol to be identified in a significant amount in the chloroplasts of liverworts. A previous study carried out by analyzing the crude ether extract of the air-dried thalli of the liverwort, *Marchantia polymorpha* by GC-MS also showed only phytol as a chloroplastidic isoprenoid (Suire *et al*., 2000). FOH and GGOH may have been present at too low levels to be detected by the GC-MS. These results are in agreement with the results of the present study which showed very low levels of FOH, GGOH and GG-ester in *H. planus* chloroplasts.

Extraction of prenyl diphosphates by stirring the intact chloroplasts overnight in methanol, adequately recovered prenyl diphosphates and chlorphylls present in the chloroplasts. It was not necessary to use harsh conditions, such as, rupturing the chloroplasts in liquid N_2, to extract prenyl diphosphates. The two hydrolysis methods, KOH hydrolysis and the alkaline phosphatase enzyme hydrolysis, were sufficient to hydrolyse the extracted diphosphates to afford respective alcohols for the estimation of chloroplastidic diphosphates and phytol. The lipids present in the chloroplast of liverworts other than prenyl lipids, were identified as linoleic acid, palmitic acid, methyl ester of palmitic acid and the ester of phthalic acid, using GC-MS.

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