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STSM Topic: Study of strawberry (*F. ananassa*) and *M. extorquens* cells for the biosynthesis of strawberry flavour

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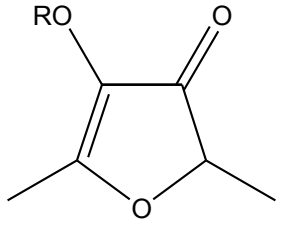
1. State of the art

Strawberry flavour is one of the most popular fruit flavours worldwide with numerous applications in food industry. Therefore, the biosynthetic origin of the most important strawberry flavour components is a challenging research area. The complexity of the strawberry flavour with its *ca* 350 components (Zabetakis and Holden, 1997) renders it an even more interesting research project. The application of sensory evaluation methods has aided to identify the most important components of strawberry flavour. The three most important flavour compounds in strawberries were identified as 2,5-dimethyl-4-hydroxy-2*H*-furan-3-one (DMHF), ethyl butanoate and ethyl hexanoate (Zabetakis and Holden, 1997).

The extensive commercial value of DMHF is underlined by the fact that two companies produce DMHF and trade it under two different commercial names (Fraision® by Vioryl SA in Greece or Furaneol® by Firmenich SA in Switzerland). Combining these two commercial names, useful information is gathered about the nature and the origin of DMHF, i.e. it is a furanone with a double carbon-carbon bond and a hydroxyl group and probably the most important flavour compound in all cultivars of either cultivated (*Fragaria* x *ananassa*) or wild (*Fragaria vesca*) “fraises” (strawberries in French).

2,5-Dimethyl-4-hydroxy-2*H*-furan-3-one occurs in nature in four forms: the free aglycone (**1**), 2,5-dimethyl-4-methoxy-2*H*-furan-3-one (mesifuran, **2**), 2,5-dimethyl-4-hydroxy-2*H*-furan-3-one β-D-glucopyranoside (DMHF-glucoside, **3**) and 2,5-dimethyl-4-hydroxy-2*H*-furan-3-one 6'-O-malonyl-β-D-glucopyranoside (DMHF malonyl-glucoside, **4**) (Fig. 1) (Zabetakis *et al.*, 1999a).



1: R=H

2: R=CH_3

3: R=ß-D-glucopyranose

4: R=6-O-malonyl ß-D-glucopyranose

Figure 1. Chemical structures of DMHF and derivatives.

Given the biosynthetic capacity of plant tissue culture (PTC), it has been studied the biosynthesis of DMHF in strawberry using callus cultures. Methylpentoses were the reported precursors of furanones in fruits so it was decided to provide our strawberry calli with 6-deoxy-D-fructose. Once this deoxy sugar was added to the nutrients, 4 weeks later DMHF-glucoside was analysed in the calli (Zabetakis and Holden, 1996). It was reported that all the deoxy analogues of D-fructose may act as substrates in the transglycosylation reaction of sucrose biosynthesis catalyzed by sucrose synthase; however, it is the 6-deoxy analogue that reacts more rapidly than all the deoxy analogues. It was thus proposed that 6-deoxy-D-fructose reacted with UDP-glucose in strawberry calli forming a sucrose deoxy analogue which was then reduced to DMHF-glucoside (Zabetakis and Holden, 1996).

Bacteria of the genus *Methylobacterium*, often referred to as PPFMs (pink-pigmented facultative methylotrophs) belong to α-proteobacteria. These bacteria can grow on methanol, methylamine, and various C2, C3, and C4 compounds. The *Methylobacterium* spp. are regularly found in the soil and on surface of leaves (Chistoserdova ***et al.,* 2003)**

Methylotrophic bacteria were initially discovered as covert contaminants of plant tissue cultures and were named as pink-pigmented facultative methylotrophs (PPFM) (Holland and Polacco, 1994).

Several studies have shown the positive effect of methylotrophic bacteria on plant growth. Some strains produce plant growth hormones, suggesting that their positive effect on plant growth is through phytohormone production (Holland and Polacco, 1994; Ivanova *et al.*, 2001).

Other beneficial effects proposed for *Methylobacterium* spp. in plant tissue include enhancing plant nitrogen metabolism, production of vitamin B_{12}, and removal of methanol and other metabolic wastes (Holland and Polacco, 1994)

Methylobacterium extorquens by CABI (former International Mycological Institute) (UK) (registration number: IMI 369321) has been cultured and suspension cultures were established. Being a facultative methylotroph, this bacterium grows more rapidly when methanol (0.25-0.5 % v/v) is present in the nutrient medium. Given that *Methylobacterium extorquens* possesses alcohol dehydrogenase(s) (ADHs) that can oxidize a wide range of alcohols to the corresponding aldehydes and ketones (e.g. 2-propanol to acetone) (Haber *et al.*, 1983), our group focused on studying the growth of this bacterium and how externally provided 1,2-propanediol was microbially oxidized to lactaldehyde.

The growth of *Methylobacterium extorquens* in a liquid medium containing 0.75% (v/v) 1,2-propanediol, 0.25 % (v/v) methanol and 1% (w/v) peptone (as a nitrogen source) and the parallel bioformation of lactaldehyde were screened over the course of 10 days. The bacteria followed a rather typical exponential growth phase followed by a stationary growth phase. The exponential growth from day 2 to day 4 was rather sharp. The maximum levels of lactaldehyde were obtained when the bacterial growth had reached the stationary phase (day 7). After this day, the levels of lactaldehyde decreased (Koutsompogeras *et al.*, 2006).

By linking the presence of 1,2-propanediol in strawberry (Zabetakis and Gramshaw, 1998) to the oxidative capacity of *Methylobacterium* sp. (Haber *et al.*, 1983), we co-cultured the strawberry calli with *Methylobacterium extorquens* and after 4 weeks of culturing, the formation of DMHF and mesifuran was observed (Zabetakis, 1997). This exciting result was explained on the basis that strawberry cells provided 1,2-propanediol to bacteria and the latter oxidised just the primary hydroxyl group, i.e. *Methylobacterium extorquens* converted 1,2-propanediol to 2-hydroxy-propanal or lactaldehyde (fig. 2, step 1). Verginer et al. (2010) reached the same result observing increased concentration of DMHF in strawberry tissues which were treated with *Methylobacterium extorquens*.

In another series of experiments, lactaldehyde was fed to strawberry calli and the bioformation of DMHF and DMHF-glucoside was observed (Zabetakis *et al.*, 1999b). Aldolase enzyme(s) are *omni* present in any living cell and participate in both glycolysis and gluconeogenesis (Stryer, 1997). Therefore, it can be proposed that lactaldehyde and dihydroxy-acetone-phosphate (DHAP) may undergo an aldol condensation catalysed by strawberry aldolase and yield 6-deoxy-D-fructose (fig. 2, step 2).

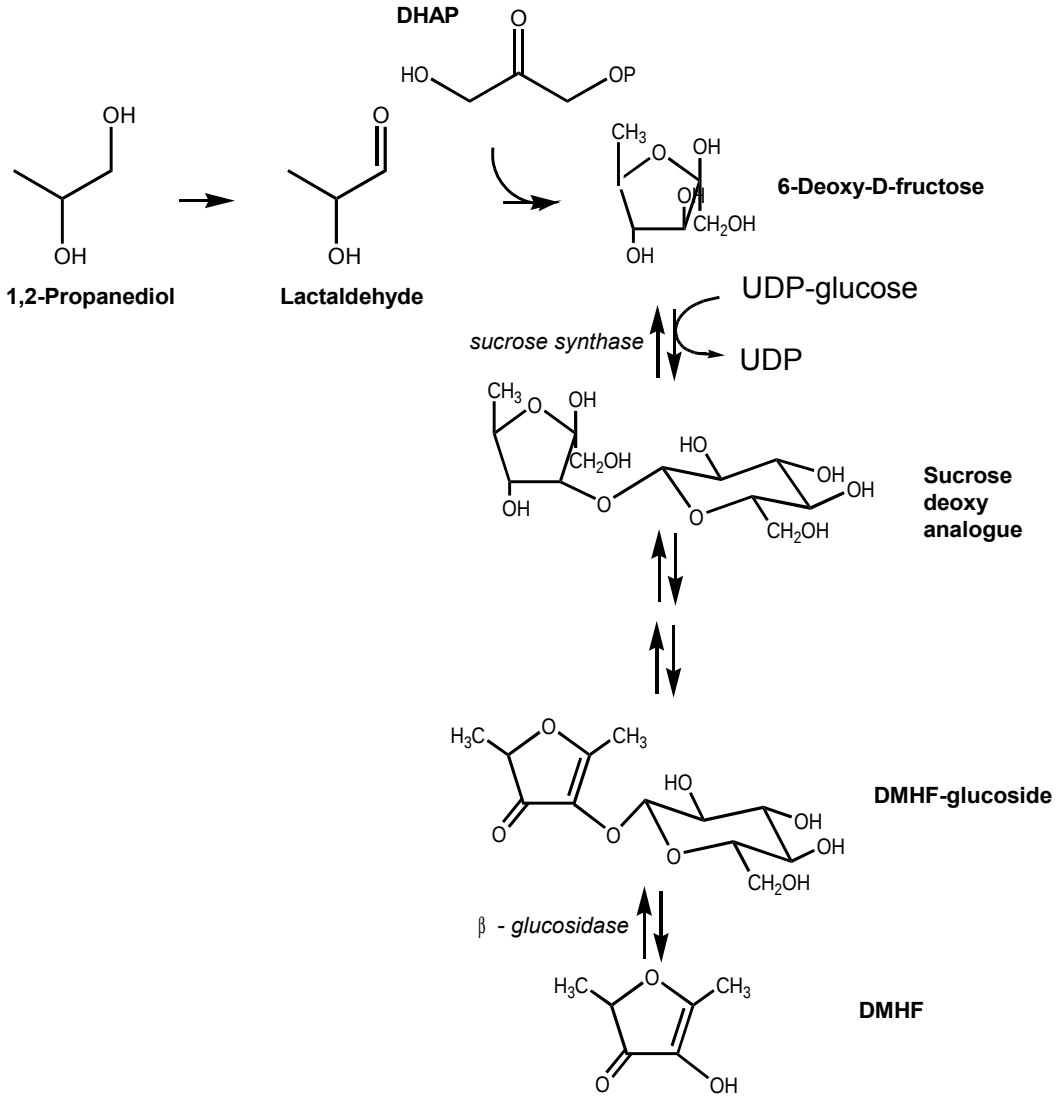


Figure 2. Proposed biosynthetic pathway of DMHF in strawberry callus / *Methylobacterium extorquens*.

2. Purpose of the STSM

The scope of this STSM was to study the symbiotic relationship of plant and microbial cells in the biosynthesis of plant flavour. The most important component of strawberry flavour is 2,5-dimethyl-4-hydroxy-2*H*-furan-3-one (DMHF). Some precursors of DMHF are biosynthesised by *Methylobacterium extorquens*. According to scientific data when this bacterium was cultured in liquid medium containing 0.75% (v/v) 1,2-propanediol, the formation of 2-hydroxy-propanal was observed, highlighting the role of alcohol dehydrogenase (ADH) enzymes of *Methylobacterium extorquens* in the biogenesis of DMHF's precursors. Thus the aim of the current STSM was to further study the bacterial ADH genes involved with DMHF biosynthesis. The significance of this work is that it allows deepening the understanding on the interaction of endophytes with plant cells with respect to secondary metabolism and jointly produced compounds. For example, many endophytic fungi are known to play a role in the secondary metabolism of the plant host. Such collaboration could be more common than anticipated among endophytes and one crucial factor defining the plant-endophyte interaction.

3. Description of work carried out during the STSM

Expression of the bacterial methanol dehydrogenase subunit 1 (CP001510; Mxa) and the plant DMHF biosynthesis gene (GB No. AY158836; FaQR) were determined in the strawberry tissue (raw and ripe) by in situ hybridization.

Probes specific for the aforementioned sequences were developed, and these as well as bacteria-specific probes (eubacterial E11 and methylobacteria-specific MB; Pirttilä *et al.* 2000) were labelled with digoxigenin. Specifically, the probe sequences of the methanol dehydrogenase (Mxa) and *Fragaria x ananassa* quinone oxireductase (FaQR) genes were amplified from the genomic DNA of *Methylobacterium extorquens* and strawberry leaves by PCR. The resulting PCR products were cloned into pGEM T-easy vector and labeled with digoxigenin-UTP by *in vitro* transcription with SP6 and T7 RNA polymerase (DIG RNA Labeling Kit (SP6/T7), ROCHE).

The strawberry samples were collected from ripe and raw fruits. Surface sterilized fruit samples were fixed in fixing solution at 4°C overnight, dehydrated, cleared through an ethanol-*t*-butanol series and embedded in paraffin. The paraffin sections were attached on silane-coated slides by baking at 55°C overnight. The

hybridization was performed at 42°C overnight using hybridization solution containing 0.5 ng/ml of each probe.

Summarizing the steps of the procedure were: a) the design and labeling of probes b) fixation of plant samples, c) sectioning, d) hybridization on slides and e) detection. The labels were detected by light microscopy and by confocal microscopy.

4. Description of the main results obtained

The most important results obtained were the following:

- The signal of bacterial ADH gene and plant DMHF biosynthesis gene were detected at the same locations in the plant tissue as the signal of MB and E11.
- The signal of bacterial ADHs gene and plant DMHF biosynthesis gene were mainly detected in the intercellular spaces of receptable cortical tissue, vascular tissue and intracellularly in the tissues of achenes.
- The ripe plant cells exhibited mainly unspecific signal, as the samples treated with controls (sense probe, no probe) were stained as well.

5. Foreseen publications from the STSM

The results obtained during the STSM are going to be submitted as a scientific article at the Scientific Journal: "Applied and Environmental Microbiology".

6. Confirmation by the Host Institution of the successful execution of the STSM

The analysis of biosynthesis of DMHF genes was successful as the main results were gained as hypothesized. An obstacle was the strong background in ripe tissues, which could be due to degraded DNA and/or alkalic phosphatase activity present. The fellow was very active and dedicated to getting the results within the schedule as planned. Further analyses could be the study of other developmental stages of the strawberry receptable and use of fluorescent label to confirm the results, if requested by the reviewers.

7. Other comments

Participation at COST FA1103 - Endophytes: from discovery to application, presenting the results obtained during the STSM.

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