Biosynthesis of 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone and Derivatives in in Vitro Grown Strawberries

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The biosynthesis of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (Furaneol) and its methyl ether and glucoside derivatives has been studied in strawberries. An in vitro system was used for growing this fruit, showing that the presence in the incubation medium of sucrose or hydroxyquinoline hemisulfate has no effect on the bioformation of these compounds. Strawberries in vitro grown showed an increase in furanone content with time, especially between the second and fourth days, to the same extent as field-grown fruits but at a higher rate. Among the precursors added to the incubation medium, D-fructose gave rise to an increase in furaneol and its glucoside derivative of 42.6% and 26.3%, respectively. D-fructose 6-phosphate seems to be the precursor of furaneol in strawberries since, when present in the incubation medium, it produced an average increase of 125% in all furanones contents with respect to control fruits.

Keywords: *2,5-Dimethyl-4-hydroxy-3(2H)-furanone and derivatives; biosynthesis; strawberry*

INTRODUCTION

The flavor of cultivated strawberries is mainly determined by a complex mixture of esters, aldehydes, alcohols, and sulfur compounds which have been extensively studied during the past 30 years (McFadden et al., 1965; Dirinck et al., 1977, 1981; Schreier, 1980; Hirvi and Honkanen, 1982; Pérez et al., 1992). Among the most important aroma compounds reported in strawberries are 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (Furaneol, trademark from Firmenich, Inc., Geneva, Switzerland) (Re et al., 1973; Pickenhagen et al., 1981) and its methyl ether, 2,5-dimethyl-4-methoxy-3(2*H*)-furanone (mesifurane) (Pyysalo et al., 1979). Glycosidically bound aroma compounds have an important role as flavor precursors (Williams et al., 1989). Mayerl et al. (1989) isolated and identified the β-glucoside of furaneol from strawberry juice. Furaneol and mesifurane are considered by most authors as the most important aroma constituents of cultivated strawberries (Hirvi, 1983; Douillard and Guichard, 1989, 1990; Herrmann, 1991). Both compounds have strong, sweet, and pleasant odors. Furaneol imparts caramel burnt sugar notes at high concentrations and becomes fruity at lower concentrations (Re et al., 1973). Mesifurane is described as having a more sherrylike aroma (Hunter et al., 1974). Larsen and Poll (1992) found that a mixture of furaneol and ethyl butanoate presented a strawberry-like odor.

Different studies have identified the presence of Furaneol, mesifurane (Hirvi and Honkanen, 1982; Douillard and Guichard, 1990), and Furaneol glucoside (Mayerl et al., 1989) in strawberries and have studied the evolution of these compounds along ripening and shelf life (Sanz et al., 1995; Pérez et al., 1996), but these furanones have not been found in all cultivars. Factors such as the furaneol water-soluble nature and thermal instability could well account for the failure of some authors to detect these compounds.

Although studies on Furaneol biosynthesis have been undertaken recently in strawberry callus (Zabetakis and Holden, 1996) and the yeast *Zygosaccharomyces rouxii* (Hecquet et al., 1996), the biosynthetic pathway is still unknown.

Strawberry is classified as a nonclimateric fruit on the basis of a lack of increased respiration and ethylene production as the fruit changes color, texture, and flavor (Abeles and Takeda, 1990). Direct study of nonclimateric fruits such as strawberries has been limited because ripening generally does not continue normally following detachment. In this sense, Perkins-Veazie and Huber (1992) provided an in vitro system to study nonclimateric fruit development that allows incorporation of substances to study fruit ripening (Perkins-Veazie et al., 1995, 1996; Pérez et al., 1997). On the basis of this in vitro approach, we have studied the biosynthesis of Furaneol in strawberry through the incubation of fruits with different possible precursors suggested from a literature survey on this subject.

EXPERIMENTAL PROCEDURES

Materials. Strawberry fruits (*Fragaria* × *ananassa* Duch.), cultivar Camarosa, were grown in field plantings in Torreagro (San Bartolomé de la Torre, Huelva, Spain). Strawberries at the pink stage were harvested with intact peduncles, placed in plastic bags, and transported on ice to the laboratory. Fruits were carefully matched according to color and weight. Ten fruits were randomly sampled and used for day 0 determinations, and others were immediately prepared for the in vitro growth experiments.

In Vitro Growth Experiments. The in vitro growth experiments were carried out using the system developed by Perkins-Veazie and Huber (1992). Fruit peduncles were trimmed to an uniform length of 60 mm and immersed in an autoclaved headspace vial (20 mL, 23 × 75 mm) containing 18 mL of growth solution. Each fruit was accommodated on a perforated rubber stopper that closed the vial. The growth

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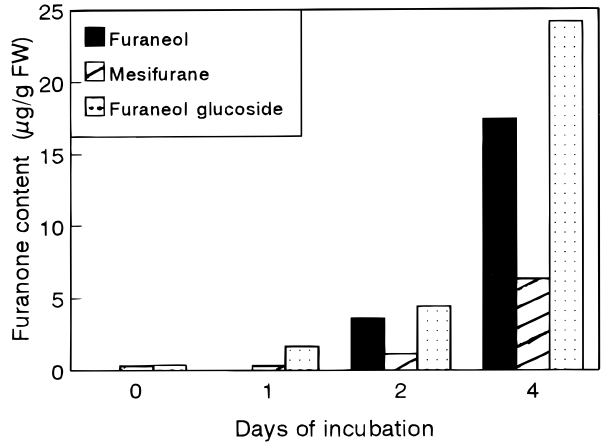

Figure 1. Evolution of furanone contents along 4 days of *in vitro* incubation of strawberries in 88 mM sucrose.

Table 1. Effect of Sucrose and HQS on Furanone Biosynthesis in in Vitro Developed Strawberries during a 4-Day Period

	furanone content increase (μg/fw)			
treatment	Furaneol	mesifurane	Furaneol glucoside	tot. furanones
+sucrose/+HQS	3.64	3.88	9.68	17.20
−sucrose/+HQS	4.01	4.07	9.49	17.56
−sucrose/−HQS	6.11	4.45	5.52	16.08
+sucrose/−HQS	4.18	6.7	7.37	18.25

solution consisted of autoclaved distilled water containing 1 mM hydroxyquinoline hemisulfate (HQS) and/or 88 mM sucrose as described for the study of the effect of these components. For the rest of experiments, control fruits were grown with 88 mM sucrose, while different substrates at a concentration of 25 mM were added to the growth solution of the treated strawberries. Ten fruits were used for each treatment in triplicate. All fruits were grown in a growth chamber, temperature 25/15 °C day/night, 16 h photoperiod, 300 μE m^{-2} s^{-1} light, and 85% relative humidity. Every day fruit peduncles were recut (1−2 mm). Strawberries were sampled at days 1, 2, and 4 for the evolution of furanone contents studies and at day 4 for production of furanones in every treatment.

Preparation of Sample for HPLC. Strawberries were cut symmetrically in four portions, and 10 portions of 10 different fruits were used for analytical determination of Furaneol, mesifurane, and Furaneol glucoside. Fruit portions were ground with distilled water in a Waring blender at 0−4 °C. Celite 545 was added and after mixing allowed to settle for 5 min. The mixture was filtered through filter paper Whatman No. 1, washed three times with distilled water, and again filtered through a 0.45 μm nylon membrane before HPLC analysis.

HPLC Analysis. Quantitative HPLC analysis was carried out according to a method described by Sanz et al. (1994) with slight modifications. A liquid chromatograph, Beckman Golden System, equipped with an ODS (2 mm × 250 mm) 5 μm column was used. The UV detector was selected at 280 nm, and the injection volume was 20 μL. The mobile phase utilized for the separation of furanones consisted of two eluents: 0.2 M sodium acetate/acetic acid, pH 4 (solvent A), and methanol (solvent B). Chromatographic conditions were the following: 0−8 min, isocratic 5% B; 8−9 min, gradient 5−15% B; 9−20 min, isocratic 15% B; 20−21 min, gradient 15−80% B (cleaning process).

RESULTS AND DISCUSSION

Previous work in our laboratory (Pérez et al., 1997) has shown that the system of in vitro grown strawberries developed by Perkins-Veazie and Huber (1992) is a potentially useful technique for studying the ripening physiology of fruits that exhibit limited capacity for continued development following harvest, such is the case of strawberries. Due to our current interest in establishing the origin of Furaneol and derivatives mesifurane and Furaneol glucoside, also known as furanones, we have tried this in vitro approach for furanone biosynthesis studies in strawberry. In this sense, Roscher et al. (1997) demonstrated by means of this technique that strawberries were able to assimilate and convert Furaneol into mesifurane and furaneol glycosides in Elsanta strawberries. They also found in this work that *S*-adenosyl-L-methionine is the source of the methyl group of the methoxy-substituted carbon in mesifurane. To establish the best conditions for strawberries developed in vitro in terms of Furaneol biosynthesis, we studied first the influence of sucrose and the bacteriostat HQS in the incubation medium in relation to the production of furanones. Table 1 shows the effect of sucrose and HQS in the incubation medium on furanones production. Although some differences were found among individual furanones, total furanones formed during a 4-day period incubation were quite similar. Thus, studies on Furaneol biosynthesis seemed not to be affected by the presence of sucrose and/or HQS. Since sucrose is necessary in the incubation solution for a more physiological fruit development and ripening as reported by Perkins-Veazie and Huber (1992), this sugar was added to this solution in further experiments. HQS was not added since it did not have any significant effect on furanone biosynthesis and because some of the possible Furaneol precursors tested give rise to a precipitate when combined with HQS according to preliminary experiments.

Figure 1 shows the evolution of individual and total furanones when strawberries were grown in vitro using fruits at the pink stage as starting material and sucrose as a carbon source. A clear increase in all the furanones was noticed, particularly between the second and fourth days. This increase in furanone production takes place to the same extent as field-grown fruits but at a higher rate according to our previous results (Sanz et al., 1995). It was observed that the response of in vitro grown

Table 2. Furanone Production from Individual Possible Precursors Added at 25 mM by in Vitro Cultivated Strawberries during a 4-Day Period

	furanone content increase (μg/fw)			
compd addedto the medium	Furaneol	mesifurane	Furaneol glucoside	tot. furanones
control	3.482 ± 0.231	1.264 ± 0.201	13.014 ± 0.638	17.760
D-fructose	4.964 ± 0.728	1.403 ± 0.313	16.442 ± 1.466	22.809
D-fructose 6-phosphate	6.634 ± 0.206	3.070 ± 0.531	31.375 ± 3.203	41.079
D-fructose 1,6-bisphosphate	3.642 ± 0.325	1.701 ± 0.279	12.527 ± 1.356	17.870
ascorbic acid	6.528 ± 1.347	1.686 ± 0.301	14.514 ± 0.947	22.728
rhamnose	3.367 ± 0.768	1.408 ± 0.427	12.026 ± 0.966	16.800
fucose	4.311 ± 0.599	1.938 ± 0.501	12.732 ± 0.951	18.981
sedoheptulose	2.809 ± 0.319	1.199 ± 0.023	11.334 ± 1.858	15.342

strawberries to furanone biosynthesis is greatly dependent on fruit maturity. Thus, further experiments were carried out with white fruits so that incubation solution uptake and fruit development are optima (Perkins-Veazie et al., 1992; Pérez et al., 1997).

Once established that strawberries can be cultivated in vitro using sucrose, and without HQS, in the incubation medium in order to study furanones, and especially Furaneol, biosynthesis, we tried different possible precursors of Furaneol as shown in Table 2. Pisarnitskii et al. (1992) reported that Furaneol content in strawberry is directly related to the methylpentoses rhamnose and fucose contents in this fruit. These authors stated that the former is the most probable precursor of Furaneol and that carbonyl−amine reactions at the final stages of fruit ripening are the cause for its formation. Table 2 shows that incubation of strawberries with these two methylpentoses did not produce any significant increase in Furaneol or Furaneol derivatives compared to control fruits. Thus, this hypothesis of a purely chemical reaction forming furaneol in strawberries is arguable and it should be assumed a possible biochemical pathway involved in this compound formation.

Recently, Sasaki (1996) proposed the pentose phosphate cycle intermediates as substrates for the biosynthesis of the Furaneol homologue 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2*H*)-furanone (HEMF), a character impact compound in shoyu. This biosynthesis is carried out by many kinds of yeasts. More specifically, this author proposed D-xylulose 5-phosphate from this cycle as the precursor of HEMF, although based on the number of carbons of HEMF; the presumed substrate should be sedoheptulose 7-phosphate as indicated by the results reported by this group earlier (Sasaki et al., 1991). Incubation of strawberries in a medium containing sedoheptulose did not give rise to Furaneol or HEMF (Table 2). More recently, Hecquet et al. (1996) found working with *Zygosaccharomyces rouxii*, one of the yeasts used by Sasaki's group, that D-fructose 1,6-bisphosphate is the precursor of furaneol. These authors also found that neither D-fructose nor D-fructose 6-phosphate added to these yeast cultures produced significant levels of Furaneol with respect to control cultures. We incubated strawberries with these three sugars as shown in Table 2. D-Fructose 1,6-bisphosphate did not produced higher significant levels of furanones. Incubations with D-fructose produced an increase of 28.4% in total furanones, with an especially significant increase for Furaneol (42.6%) and Furaneol glucoside (26.3%). Contrary to the results found by Hecquet et al. (1996) in yeast, D-fructose 6-phosphate incubations showed an average 125% increase in all furanones respect to control fruits. These data seem to point out this sugar phosphate as the true precursor of Furaneol in strawberries.

Loewus et al. (1956) demonstrated that ascorbic acid content increases during strawberry ripening. We have observed in our previous work (unpublished results) this fact and that this increase is concomitant with the rise in furanones contents. On the basis of the work by Velisek et al. (1976) on the degradation of L-dehydroascorbic acid in model systems, where formation of Furaneol-related compounds were reported, we have also incubated strawberries with ascorbic acid. Results showed a significant increase only in Furaneol content when incubated with this acid, although data presented a higher than average deviation standard, so it is not clear enough whether this acid could be involved in Furaneol biosynthesis.

CONCLUSIONS

Data from this work seem to demonstrate that D-fructose 6-phosphate is the presursor of Furaneol in strawberries, contrary to what was found by Hecquet et al. (1996) in yeast. This Furaneol arising from D-fructose 6-phosphate would be rapidly converted into mesifurane and Furaneol glucoside as it was demonstrated by Roscher et al. (1997). Moreover, the slight increase in furanones showed by D-fructose incubations and the similar to control furanone production when D-fructose 1,6-bisphosphate was used could indicate that the enzymatic system forming Furaneol would need as a requirement a phosphorylated fructose molecule at carbon 6. In this sense, the physiological source of D-fructose 6-phosphate in strawberry could be the pentose phosphate cycle, as stated by Sasaki's group for HEMF (Sasaki, 1996; Sasaki et al., 1991), since D-fructose enters in the glycolytic pathway through its conversion into D-fructose 6-phosphate by the action of fructokinase, and only a slight increase in furanone contents was found when incubating strawberries with this sugar. On the other hand, the formation of D-fructose 6-phosphate from D-fructose 1,6-bisphosphate is carried out by the enzyme fructose bisphosphatase in a less probable gluconeogenic way, and the enzyme from the glycolytic pathway converting D-fructose 6-phosphate into D-fructose 1,6-bisphosphate, phosphofructokinase, seems to act irreversibly in this sense.

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