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REVIEWS

Aroma Production by Tissue Cultures

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Although plant tissue cultures have been in use for the past hundred years, adapting them for the production of aroma compounds started only in the 1970s. The use of tissue cultures in aroma production has its advantages, because plant cells, unlike whole plants, are not limited to geographic locations or the seasons. Cell mass can be doubled relatively rapidly and can be induced for the production of compounds in a coordinated manner. Compounds can be isolated from cells or the medium with relative ease. Therefore, it would seem to be ideal to use plant cell cultures for the production of aroma compounds. Cell cultures, however, also have some problems. The production of aroma compounds or their precursors is in relatively low amounts, and thus this production method is expensive. Additional expenses are the cost of the medium and the purification of the compounds for food use. Also, cell cultures can only be used effectively in systems for which the biochemical pathway of the aroma compounds is known. In this paper the results of experiments for the use of tissue cultures in the production of vanilla, raspberry, strawberry garlic, and onion aromas is discussed.

Keywords: (+)-*S*-(1-Propenyl)-L-cysteine sulfoxide; (+)-*S*-allyl-L-cysteine sulfoxide; (+)-*S*-methyl-L-cysteine sulfoxide; (+)-*S*-propyl-L-cysteine sulfoxide; alliin; aroma; benzalacetone synthase; callus; cell culture; chalcone synthase; chitosan; garlic; onion; phenylpropanoid pathway; *p*-hydroxybenzalacetone; *p*-hydroxybenzaldehyde; *p*-hydroxyphenylbutanone; raspberry; raspberry ketone; strawberry; tissue culture; vanilla; vanillic acid; vanillin

INTRODUCTION

The first experiment with plant tissue cultures was reported 102 years ago by the Austrian scientist Gottlieb Haberland (*1*). During the 100 years since this first report, the use of cell cultures increased rapidly. Cell and tissue cultures today are not only used for the propagation of plants but also evaluated for the commercial production of natural products, including flavors and fragrances. For those having a historical interest in this original publication, the article has been reprinted in both English and German in the book *Plant Tissue Culture—100 Years since Gottlieb Haberland* (*2*). Haberland's revolutionary vision and original experimentation pointed to the creation of artificial embryos, which are widely used today for plant propagation, and to callus and tissue cultures that are extensively used for the elucidation of biochemical pathways or production of some natural compounds.

The production of compounds by plant cell cultures for aroma, flavor, or pharmaceutical uses has advantages over production by conventional agricultural practices. Unlike the production of plants that is seasonally limited, cell cultures provide a system that can be used year-round and that is independent of the seasons, geographic location, and political situations. Cells can be induced in a coordinated manner to produce metabolites of interest, and these can be isolated from the nutrient medium or from the cells (*3*). Uptake and transport of nutrients is not an issue, because the cells or tissues are surrounded by the nutrient medium. Components or metabolites of the photosynthetic apparatus do not have to be dealt with, because the cells or tissues do not rely on energy produced by photosynthesis. Tissue or cell cultures, however, do pose some problems. The first requirement for establishing a cell culture is the development of a friable callus requiring tissue regeneration over a number of transfers, which may take time. Also, the cultures have to be kept under sterile conditions at all times, and this requires special handling and equipment. Tissue/cell cultures have to be scaled up and optimized for the production of metabolites of interest. The scaling-up process usually magnifies the production cost of a compound. These problems have been addressed in detail by Misawa (*4*) in the bulletin *Plant Tissue Culture: an Alternative for Production of Useful Metabolites* and are mentioned here only briefly.

Although certain similarities exist between microbial and plant cell cultures, there are important differences that have to be considered. Due to size differences and the composition of the cell wall, plant cell cultures are much more fragile and susceptible to shear than microbial cultures. Due to the large

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central vacuole, the water content of plant cells is significantly higher than that of microbial cells. Whereas the duplication time of microbial cultures is measured in hours, that of plant cells takes days. Product accumulation with microbial cultures is usually in the medium and takes hours, whereas that with plant cells or tissues can take place either in the vacuole of the cells or in the medium and may take weeks. The cost of the medium for plant cell cultures is ~10-fold that of microorganisms. An additional challenge of plant cell or tissue cultures is that they may produce a decreased amount of secondary metabolites compared with the parent plants. This behavior seems to be plant specific; a ~10-fold increase in rosmarinic acid accumulation has been reported in cell suspension cultures of rosemary (*5*).

A generally complicating factor in the production of flavor compounds in a tissue culture system is that most flavors are not the result of the presence of a single compound in the respective plant, but are due to the presence of multiple compounds. An example is the cultivated strawberry, in which 143 different compounds were identified (*6*). The slightest change in the composition of these compounds can cause significant flavor modification. This problem is exemplified by comparison of the aroma composition of the wild strawberry, for which the flavor is the result of the sum of 91 acids, alcohols, aldehydes, ketones, esters, lactones, furans, aromatic compounds, terpenes, and furan glucosides (*6*).

The best results toward the production of flavor compounds by cell cultures have been achieved in cases when the characteristic aroma of the fruit or plant is caused mainly or entirely by the synthesis and accumulation of a single compound or only a few compounds with similar structures and properties. This is the case with vanilla, for which the major flavor component is vanillin; with raspberries, for which raspberry ketone gives the characteristic flavor; and with onions and garlic, for which the characteristic flavor derives from alliin derivatives. The tissue cultures of these plants will be discussed below, together with the cost-effectiveness of cell cultures and the safety of the compound produced by tissue culture.

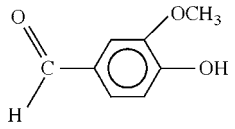
VANILLA

Vanilla is the most universally used flavoring ingredient in the world. Its history and agronomic production have been reviewed recently by Havkin-Frenkel and Dorn (*7*). Vanilla is produced by the plant *Vanilla planifolia*, an orchid native to the southern region of Mexico. Harvested pods or beans are exposed to a lengthy and laborious curing process, during which time they turn black and develop the characteristic vanilla flavor. The cured pods are marketed under the name vanilla. Any extract made from the pods is called vanilla extract and is sold under this name.

Prior to the invasion of southern Mexico by the conquistadors, vanilla was exclusively produced by the Aztecs, who called it "tlilxochitl", or black pod, and used it to flavor a drink made of honey and ground cocoa that they called "xocolatl". Vanilla derives from the Spanish vanillia, meaning a little pod. Until the mid 19th century vanilla was produced solely in Mexico. Although repeated attempts were made to grow the plants and produce the pods in Europe and elsewhere, they all failed due to lack of pollination, which was performed by a special fly whose range was restricted to Mexico's southern regions (*8*). Only after the introduction of an artificial pollination method did commercial production of vanilla start. Mexico's share in vanilla production today is almost insignificant (0.41%). Major vanilla-producing locations are in Indonesia (46%) and Madagascar (43.5%); Comoros (2.7%), Tonga (3%), French Polynesia (4.5%), and Costa Rica (0.3%) also produce vanilla (*7*).

The production of vanilla is labor intensive and takes a relatively long time. Because of the architecture of the flowers and the lack of natural pollinators, the pollination of the flowers has to be done manually. The time for pollination is crucial, because the flowers have to be pollinated within a narrow time window (<24 h) to bear fruits. Vanilla beans require 10−12 months to mature from the time of pollination. Mature beans are yellowish green, are bitter, and do not have the characteristic vanilla flavor, which develops only upon curing. The curing process is composed of three steps. The first step is killing, during which the green beans can be treated by a variety of methods: scalded with hot water, exposed to the sun, wilted in the oven, scarred, treated with ethylene gas, or frozen to disrupt tissue integrity. The cheapest, but most labor-intensive, methods are the scalding and sun- or oven-baking. Although tissue integrity of the beans is disrupted at this step, they still contain high amounts of moisture, which has to be removed. This takes place during the sweating process. During the sweating process, which requires 7−10 days, the moisture content of the beans is rapidly reduced to ~60−70%. During this process the beans turn dark brown and start to develop the characteristic vanilla flavor. To concentrate the flavor and reduce the chance of microbial spoilage, the beans are further dried to a moisture content of ~25−30%. This is followed by a conditioning step, when the beans are stored in closed containers to reach their highest flavor content. In most commercial processes either sun-drying (Mexican method) or hot water treatment (Madagascar or Bourbon method) is used.

The natural vanilla flavor is a mixture of compounds in which vanillin is the major component (86%), followed by *p*-hydroxy-



vanillin

benzaldehyde (8.6%), vanillic acid (4.3%), and *p*-hydroxybenzyl methyl ether (0.9%) (*8*). Because of the time-consuming and labor-intensive process used in its production, natural vanilla flavor is expensive, ca. U.S. $2000/lb, whereas synthetic vanillin produced from lignin or guaiacol is relatively cheap, costing around U.S. $6/lb. Because natural vanilla flavor is much more sought after than the synthetic product and due to its high cost and lengthy process of production, alternative processes, such as by tissue culture, seem to be an obvious choice.

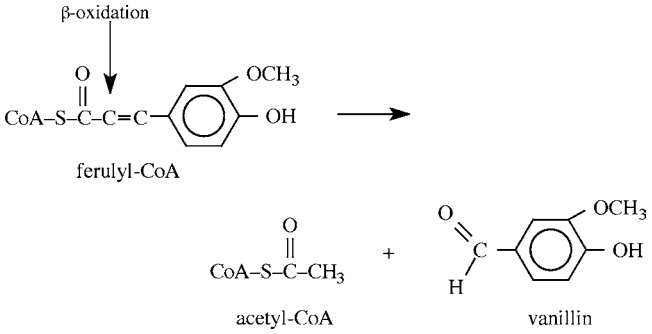
Establishment of vanilla cell suspension cultures was first reported by Funk and Brodelius (*9*) after preliminary experiments on *Nicotiana*, *Escholzia* (*10*), and glycine (*11*) to increase secondary metabolite formation. The cell cultures were established from green vanilla pods on MS media, supplemented with 2,4-D. The tissue culture conditions resulted in the formation of a fine suspension culture that reached a stationary phase in 8−9 days after inoculation. During the linear phase of growth of the cells, cell mass doubled approximately every 36 h. To investigate the effect of growth regulators on the cell suspension cultures, the investigators evaluated the role of 2,4-D, NAA, kinetin, and 2-benzyladenine on the cells. Essentially, they observed the same amount of growth with all four compounds, but HPLC analysis showed drastic influences on the phenylpropanoid metabolism under the different hormone regimes. Cells grown on 2,4-D containing medium did not produce any extractable phenolic compounds. This observation was supported by previous work on the phenylpropanoid pathway (*12*, *13*), during which decreased secondary metabolite production of cell cultures in

the presence of 2,4-D was also observed. Secondary metabolite production in the cell cultures was increased when NAA was substituted for 2,4-D. Maximal secondary metabolite production was obtained by a combination of NAA with cytokinins. The major phenolic compounds extracted from these cells were *p*-coumaric and sinapic acids, compounds that are precursors of the lignin pathway. There was also an increased amount of lignous material formation in cell cultures grown in the presence of cytokinins.

To investigate the effect of elicitation on the cell culture, the investigators added an elicitor isolated from yeast extract to vanilla suspension cultures grown in the presence of NAA. This medium was selected because cells grown in the presence of NAA produce extractable phenolic compounds without producing lignous material. The yeast elicitor preparation was used for the vanilla cell cultures, because this was successful in the previous experiments with *Nicotiana* and *Escholtzia* cell cultures. However, the yeast elicitor preparation did not work with the vanilla cell suspension cultures, and a chitosan preparation was substituted to induce enzyme activity of phenylalanine ammonia-lyase (PAL) and the production of phenolic compounds. Chitosan, however, is also known to be a permeabilizing agent, and to maximize phenolic compound production and minimize cell leakage, the system had to be fine-tuned for each batch of cells.

The concentration of lignin precursors such as ferulic and sinapic acids increased rapidly in the chitosan-treated cell suspension cultures, with ferulic acid production peaking after 32 h and sinapic acid after ~78 h. In untreated cells the two cinnamic acid derivatives, that is, ferulic and sinapic acids, also increased, but no production of vanillin and syringaldehyde could be observed after oxidative hydrolysis. In chitosan-treated cells vanillin and syringaldehyde could be readily detected under the same conditions. Although these first experiments with vanilla cell cultures did not result directly in the production of vanillin, they established certain criteria for further manipulation of the cells for vanillin production.

One of the restricting factors in the production of vanillin was the lack of understanding of the biosynthetic pathway of vanillin within the plants. Because the structure of vanillin indicates a close relationship to ferulic acid, a lignin precursor, Zenk suggested that vanillin may derive in the plant from this compound via a β-oxidation that is similar to the β-oxidation of fatty acids (*14*). In this mechanism, the phenylpropanoid pathway that leads



to the production of lignin would proceed to feruloyl-CoA, and part or all of this compound would be subjected to β-oxidation, which would produce acetyl-CoA and vanillin.

The suggested reaction scheme implied that if one can inhibit the phenylpropanoid pathway with inhibitors past the feruloyl-CoA ligase step, vanillin would accumulate in the cells. This idea was followed up on by Brodelius's group, who treated the vanilla cell suspension cultures with selected phenylpropanoid pathway inhibitors and monitored the accumulation of soluble phenolic compounds, especially vanillin and vanillic acid, in the treated cells (*15*). They found that addition of ^{14}C-labeled cinnamic acid to the nonelicited cell cultures resulted in the transient accumulation of *p*-coumaric, caffeic, and ferulic acids and coniferyl alcohol. Trace amounts of *p*-hydroxybenzoic acid were also detected in the cells. Elicitor-treated cells accumulated the same compounds, albeit at a much lower level. When ferulic acid, the putative precursor of vanillin, was added to the untreated cells, coniferyl alcohol and traces of vanillic acid accumulated. The accumulation of these compounds indicated that benzoic acid derivatives are formed from phenylpropanoid compounds, as suggested by Zenk's data (*14*).

Addition of inhibitors of the phenylpropanoid pathway interfered with the production of phenolic compounds at different steps in the pathway. Treatment of control and elicited cell suspension cultures with α-aminooxy-β-phenylpropinoic acid resulted in the shutdown of the phenylpropanoid pathway, and no extractable phenolic compound could be detected in the treated cells. This was to be expected, because the compound has been known for some time to be an inhibitor of the first enzyme of the pathway, PAL (*16*).

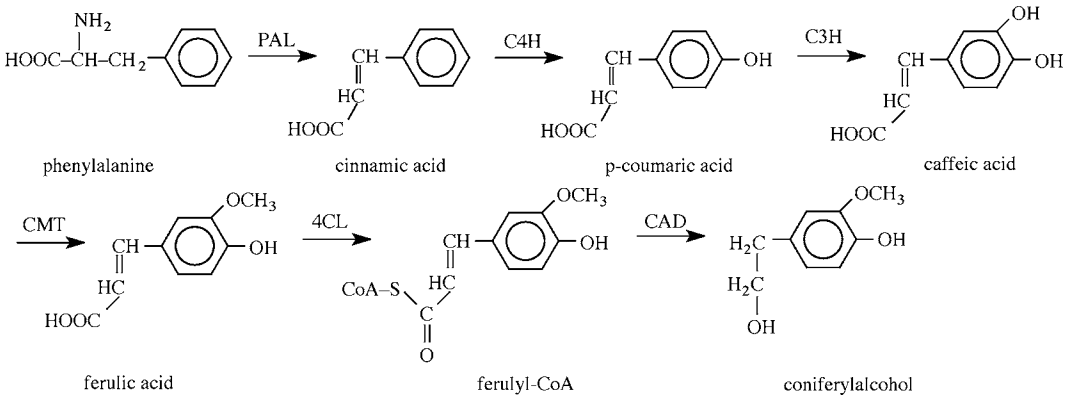
The second inhibitor used in the above studies, *N*-(*O*-aminophenyl)sulfinamoyltertiobutyl acetate, was used in similar studies; this inhibitor was reported to have a specific effect on cinnamyl alcohol dehydrogenase, a lignin pathway enzyme (*17*). Addition of the compound to the cell cultures inhibited the formation of lignin and soluble phenolic compounds. Apparently this inhibitor of cinnamic alcohol dehydrogenase also inhibits other, early pathway enzymes and shuts down the phenylpropanoid pathway (**Scheme 1**).

Addition of 3,4-methylenedioxycinnamic acid (DMCA) to the cell cultures resulted in the formation of soluble phenolic compounds, of which vanillic acid was the major component (*18*). Accumulation of vanillic acid started in the cells when the concentration of DMCA reached a high, steady-state level. Apparently, DMCA is an inhibitor of the enzyme 4-coumarate ligase (4CL), which prevents the further processing of ferulic acid into lignin. Thus, the accumulating ferulic acid, or one of its precursors, is channeled toward vanillic acid formation. Using radiolabeled cinnamic and ferulic acids, the research group deduced that cinnamic acid is incorporated into vanillic acid, whereas the more logical precursor, ferulic acid, is not. These results were contradicted by feeding experiments using living vanilla pods, in which label from ferulic acid was readily incorporated into vanillin (*19*), indicating the possibility that different mechanisms are functioning in cell suspension cultures and intact pods.

Another complicating factor in the elucidation of the biochemical pathway for vanillin formation was that 4-methoxyy-3,4-dimethoxycinnamic and 3,4,5-trimethoxycinnamic acids were readily converted to benzoic, vanillic, and syringic acids, respectively. The specific demethylation of the 4-methoxy substituent suggests that cinnamic acid derivatives with substitution patterns different from those participating in the phenylpropanoid metabolism may also be used in the biosynthesis of vanillin. In preliminary experiments the investigators detected the activity of a specific cinnamate 4-*O*-methyltransferase that was induced by kinetin (*18*). On the basis of these data, the investigators suggested that the biosynthetic pathway for vanillin branches off the main phenylpropanoid pathway at the level of caffeic acid, as illustrated in **Scheme 2**.

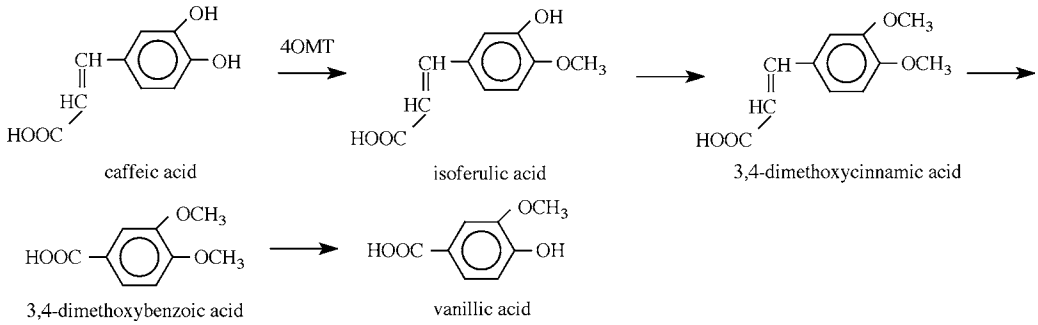
The proposed scheme seems to be cumbersome because it involves the addition and removal of an extra methyl group. However, it should be contemplated as one of the possibilities

Scheme 1. Biosynthetic Scheme of the Phenylpropanoid Metabolism[a]



[a] Enzymes in the pathway: PAL, phenylalanine ammonia-lyase; C4H, cinnamyl alcohol 4-hydroxylase; C3H, *p*-coumarate 3-hydroxylase; CMT, caffeate-3-*O*-methyltransferase; 4CL:CoA ligase, CAD:cinnamic acid dehydrogenase.

Scheme 2



until the biosynthetic pathway is established with certainty. A recent paper (*20*) argues against the above mechanism and, on the basis of demonstrated enzyme activity in vanilla cell suspension cultures, suggests a pathway by which vanillin biosynthesis follows the route from *p*-coumarate to *p*-hydroxybenzaldehyde to 3,4-dihydroxybenzaldehyde to vanillin, a more direct path to vanillin biosynthesis than the addition and removal of the methoxy group in the 4-position. This suggestion is supported by reports from other laboratories on the synthesis of *p*-hydroxybenzaldehyde from *p*-coumarate in *Lithospermum erythrorhizon* (*21*) and carrot cell cultures (*22*) and by the results of feeding experiments in *V. planifolia* cultures, in which *p*-hydroxybenzaldehyde accumulated after feeding of *p*-coumaric acid (*23*). That the use of the β-oxidation pathway by multiple plants to produce benzoic acid derivatives may be a common mechanism is suggested by a recent article (*24*) on the detection of the activity of a biphenyl synthase that uses benzoyl-CoA as substrate in the biosynthesis of biphenyl phytoalexins.

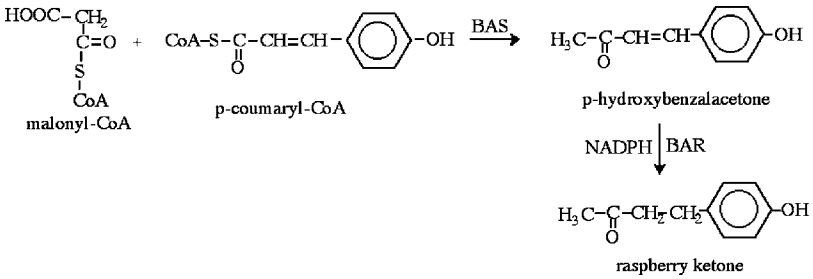
Another difficulty that one encounters in working with plant cell cultures is the scaling-up of the cultures for production. Most cell culture experiments were carried out in 200−2000 mL Erlenmeyer flasks as shake cultures, and this system, although adequate for biochemical studies, is not amenable for large-scale production. An interesting development in the 1990s was the design of a magnetically stabilized fluidized bed reactor (*25*), which permitted continuous growth of plant cells. In this system the cells are protected from shearing during their growth, and because they are immobilized on calcium alginate beds, they are constrained from moving through the fermenter. The magnetically stabilized fluidized bed system permits a good cell mass transfer, minimizes cell collision, and allows cell production while controlling the residence time of the cells. The pharmaceutical industry has ample experience with the propagation of microbial cultures on a large scale and the production of biologically active compounds. This aspect of production most likely will not be the crucial problem with the use of plant cell cultures. However, in my opinion, major progress in vanillin production by cell cultures will come only after the clarification of the biosynthetic pathway andidentification of the enzymes and genes involved in vanillin synthesis. A similar assessment was expressed in a recent review (*26*).

Production of vanillin by microbial cell cultures has also been suggested as a viable source of "natural" vanilla flavor. A recent paper suggests the use of the enzyme vanillyl alcohol oxidase obtained from the mold *Penicillium simplicissimum* to transform creosol (2-methoxy-*p*-cresol), a tar component, or vanillylamine, derived from capsaicin, the pungent principle of hot peppers, as substrate (*27*). In a review paper on the biotechnological production of vanillin, Priefert et al. (*28*) discuss a variety of approaches taken to synthesize vanillin from other chemicals. One suggested substrate is lignin, one of the most abundant natural products in plants, which may be converted to vanillin and other byproducts by fungal enzymes. Stilbenes, ferulic acid, eugenol, isoeugenol, and some aromatic amino acids were also suggested as possible substrates for biotransformations by microbial cell cultures. However, the economics of using these compounds and these approaches to production were not assessed. Although there may be some ideological justification in some consumer segments for using natural vanilla extract at the cost of $2000/lb and not the readily available synthetic vanillin at $6/lb as flavoring substance, the significance of producing vanillin from chemicals by unusual microorganisms should not be overlooked.

RASPBERRY KETONE

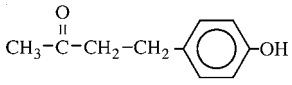
The aroma of raspberries is a complex mixture of volatile compounds of which over 230 have been identified (*29*). The

Scheme 3. Biosynthetic Scheme of Raspberry Ketone Formation[a]



[a] The short pathway consists of two steps: formation of the raspberry ketone precursor *p*-hydroxybenzalacetone from malonyl and *p*-coumaryl CoAs by the enzyme benzalacetone synthase (BAS) and reduction of the *p*-hydroxybenzalacetone in the presence of NADPH to the raspberry ketone by benzalacetone reductase (BAR).

major volatile compound in this complex mixture is *p*-hydroxyphenylbutanone, commonly referred to as the raspberry



raspberry ketone

ketone. The compound was first identified in raspberries as part of the aroma fraction by Schinz and Seidel in 1957 (*30*).

A detailed investigation of raspberry ketone content in raspberries from different geographic origins indicated levels of ketone between 7 and 200 μg/100 g of fruit (*31*, *32*). Our investigations of different raspberry cultivars in the United States showed large differences, with raspberry ketone content varying between 1 and 17 μg/100 g of ripe fruit (*33*). This investigation also showed a good correlation between organoleptic and chemical methods in detecting differences in raspberry ketone levels.

Raspberry ketone is used in the food industry to flavor beverages and confectionary and dairy products, and most of the compound used is of synthetic origin (*34*−*36*). Raspberry ketone is relatively stable at room and processing temperature, but the pure compound's aroma differs somewhat from the aroma of ripe raspberries, most likely because of the absence of other volatiles. To produce natural raspberry aroma, tissue culture approaches were established as a model system for the production of raspberry ketone (*37*). The establishment of raspberry cell suspension cultures was deemed to be essential for plant material that was available year-round without seasonal limitations. The cell suspension cultures from raspberry leaves were stable, grew well, and quadrupled their fresh weight in 10 days.

In fruits, having differentiated cells, raspberry ketone is produced during the ripening stage when sugars and pigments accumulate. In cell suspension cultures that contain largely dedifferentiated cells, the physiological processes of fruit ripening do not take place. Therefore, imitation of the ripening process has been attempted by using elevated levels of sucrose and methyl jasmonate (*38*). Raspberry ketone formation in the cells increased upon addition of jasmonic acid to the medium to a level of 20 μg compound/g of tissue. This yield, however, is deemed to be insufficient for a large batch-scale production of raspberry aroma by cell cultures.

The main problem with the cell culture production of raspberry ketone was that its biosynthesis was not understood, a fact that prevented the use of precursor substrates. Therefore, experiments were carried out to elucidate the raspberry ketone biosynthetic pathway (*39*). 2-^{14}C-labeled malonyl-CoA and *p*-coumaryl-CoA, precursors of the flavonoid biosynthetic pathway, were fed to raspberry cells or incubated with cell-free extracts obtained from raspberry fruits or cell suspension cultures. *p*-Hydroxyphenylbut-2-ene-3-one (*p*-hydroxybenzalacetone), the precursor of raspberry ketone was identified as one of the reaction products in both the raspberry cells and the cell-free reaction mixtures. The identity of *p*-hydroxybenzalacetone was established by chromatographic and mass spectroscopic methods and by cocrystallization of the reaction product to constant specific radioactivity. Further incubation of *p*-hydroxybenzalacetone with cell-free extracts in the presence of NADPH allowed conversion to the raspberry ketone. From these experiments the biosynthetic pathway was established as shown in **Scheme 3**.

One way to increase the production of raspberry ketone in the cells is by overexpressing the gene that controls the activity of the rate-limiting enzyme in the pathway, in this case the benzalacetone synthase. Therefore, work was carried out to isolate the benzalacetone synthase and determine its characteristics (*40*). Because benzalacetone synthase is a member of the aromatic polyketide synthase enzyme family (*41*), an enzyme family present in multiple forms of which all members are very similar in their protein structure and genetic sequence, the cloning of its gene promised to be challenging. To date, five raspberry polyketide synthase genes have been cloned, expressed in *Escherichia coli*, and their protein products characterized by their biochemical reactions (*42*). Two of these were determined to be chalcone synthases that are most likely part of the anthocyanin and flavonol glycoside pathways; one protein had no activity with the substrates tested, and one synthesized the open-chain product *p*-coumaryltriacetic acid. In addition to these genes, the presence of another 11 polyketide synthase genes was reported in red raspberries (*43*). Breakthrough with the identification of the benzalacetone synthase gene came from an unexpected direction. The gene was found in an unrelated plant that was investigated for its pharmaceutical properties (*44*), the Chinese rhubarb. Although the work on obtaining the BAS gene from raspberries is continuing, it is not likely to lead to rapid improvement in the production of the raspberry ketone. The high similarity of the polyketide synthase genes in the plant suggests that the manipulations to enhance raspberry ketone formation will most likely also influence the quantity of anthocyanin pigments in the plant either by increasing pigment levels by overexpression or decreasing them by cosuppression.

The situation with the production of the raspberry ketone by tissue culture techniques resembles that of vanillin. The chemical synthesis of the compound is relatively simple and economical, wheres the production by tissue cultures is time-consuming and costly, exceeding that of the chemical synthesis by orders of magnitude.

STRAWBERRY

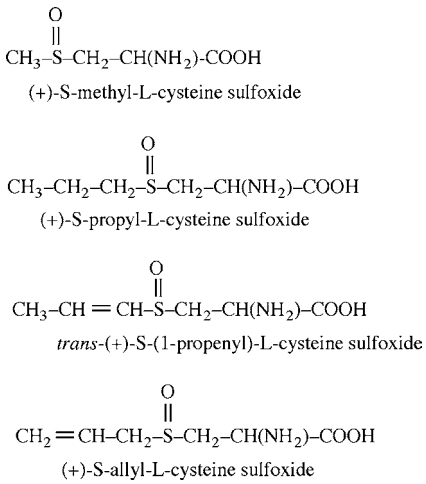
Strawberries, like raspberries, also belong to the Rosaceae, individual members of which produce most of our agriculturally

significant fruits. Strawberries are also used in the confectionary industry to produce pastries, ice cream, yogurt, and other products. Strawberry flavor is obtained conventionally by fractional distillation (*45*). However, due to the sensitivity to degradation of the strawberry volatiles, sometimes combined steam distillation−extraction is used. The aroma of strawberries is immensely complex: 278 volatile components were identified, among them 33 acids, 39 alcohols, 17 aldehydes, 14 ketones, 102 esters, 8 lactones, 15 acetals, 2 furans, 23 aromatic compounds, 6 sulfur compounds, 3-glucosides, and 11 terpenes (*6*). It would be extremely difficult to increase the formation of every compound group in such a complex system; therefore, the few experiments that were designed to enhance the formation of strawberry volatiles by tissue culture applications were focused on individual component groups. Because the largest group of volatile compounds in strawberry flavor is represented by esters, Harlander's laboratory investigated volatile ester production by strawberry tissue cultures (*46*). According to this and another paper (*47*), strawberry cell suspension cultures readily converted α-keto acids and short-chain fatty acids to volatile esters. Investigations on the production of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone and related compounds were also carried out with strawberry callus cultures (*48*, *49*). Feeding of the calli with 6-deoxy-D-fructose resulted in the production of the above furanone derivative and accumulation of its glucoside in the tissues. Formation of the same compounds was also detected in callus tissues when they were challenged with bacterial infections.

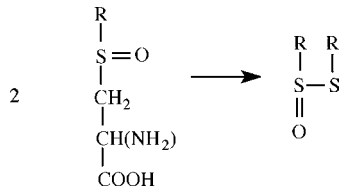
Strawberry tissue cultures were tamed in a number of laboratories; however, the main goal was to establish a system for genetic transformation (*50*) and to investigate somaclonal variations (*51*) and hormonal control of phenotypic behavior (*52*) or that of anthocyanin synthesis (*53*). To date, the production of strawberry flavoring agents from tissue cultures has not been reported.

GARLIC AND ONION

Garlic and onion are used as flavoring agents in food preparations worldwide. Both garlic and onion belong to the genus *Allium*, the outstanding feature of which is the accumulation of a large amount of sulfur-containing compounds. Interest in the sulfur compounds of both garlic and onion by 19th century chemists led to the isolation of some sulfides from garlic (*54*, *55*) and onion oils (*56*). However, systematic characterization and structure elucidation of the aroma precursors was carried out by Virtanen's group in the 1950s and 1960s (see ref *57*). Whitaker's review deals in detail with the chemistry and biochemistry of the onion and garlic volatiles; therefore, only the direct precursors of the onion and garlic flavor compounds are dealt with in this paper.



The major flavor precursors in the genus *Allium* are four alkyl- and alkenyl-L-cysteine sulfoxides: (+)-*S*-methyl-L-cysteine sulfoxide, *S*-propyl-L-cysteine sulfoxide, and *trans*-(+)-*S*-(1-propenyl)-L-cysteine sulfoxide are found in onions, whereas (+)-*S*-allyl-L-cysteine sulfoxide (alliin) is the major component of garlic flavor, at ∼2.4 g/kg of bulb tissue. The characteristic flavor of onions and garlic derives from these compounds when the tissues are crushed, by the action of the enzyme alliin lyase (*58*), which produces the corresponding thiosulfinates.



Because both garlic and onion contain a mixture of the cysteine sulfoxides, the corresponding sulfinates also occur as mixtures. Newer investigations have shown that the alliin lyase from garlic differs in K_m value, pH optimum, and isoelectric point from that of onions. There is also a major difference in molecular weight and subunit structure between the two enzymes: the garlic holoenzyme has a M_r of 85000 and consists of two subunits, whereas that from onions has a subunit M_r of 50000 and consists of four subunits (*59*).

Callus cultures of onion were first reported by Freeman et al. (*60*), who investigated their *S*-alkyl/alkenylcysteine sulfoxide content. Whereas differentiated callus tissues showing root formation had 57 μmol/g of dry weight of *S*-alkyl/alkenylcysteine sulfoxide content and gave the characteristic onion aroma when crushed, undifferentiated callus tissues showed a much lower content of the same compounds (21 μmol/g of dry weight) and did not smell like onions when crushed. The parent onion tissue in comparison had 102 μmol/g of dry weight of *S*-alkyl/alkenylcysteine sulfoxides. The authors attributed the diminished *S*-alkyl/alkenylcysteine sulfoxide content of the undifferentiated callus tissue to a diminished biosynthesis of the compounds in the undifferentiated callus tissue. Another investigation on flavor production in onion callus tissues reported flavor precursor concentrations at 10 μmol/g of fresh weight in intact onions, whereas that of the undifferentiated callus tissue was below the micromolar concentration (*61*). Investigations from other laboratories comparing cysteine sulfoxide levels in onions and in their callus cultures essentially confirmed these reports. Thus, Turnbull et al. (*62*) found cysteine sulfoxide levels of 9 μmol/g of fresh weight in dormant onions that approximately doubled during sprouting, whereas callus tissues contained these compounds at the nanomoles per gram of fresh weight levels. Interestingly, during root formation from callus (i.e., tissue differentiation), the level of the flavor precursors increased to 1.3 μmol/g of fresh weight; however, these compounds could not be detected in the newly formed shoots. That the formation of cysteine sulfoxides is a function of tissue differentiation in garlic was also affirmed in reports by Malpathak and David (*63*) and Ohsumi et al. (*64*). Experiments with chive tissue cultures gave similar results (*65*).

The tissue culture experiments with garlic, onion, and chive to produce aroma compounds give a strong indication that this may not be feasible for physiological reasons. Production of the flavor precursors was observed, albeit at a low level, in differentiated tissues but not in the dedifferentiated tissues of

the callus. Because callus cultures and cell suspension cultures produced from them contain mainly undifferentiated or dedifferentiated cells, in which the biosynthetic pathway is not induced, they do not seem to be amenable for the production of the various cysteine sulfoxides that function as precursors for chive, garlic, and onion flavors.

FUTURE PROSPECTS

Although the production of the majority of aroma compounds by plant tissue culture methods is presently not feasible for either economic or physiological reasons, future developments may hold promise. There is precedent for the commercialization of some secondary metabolites produced by plant tissue cultures. An example is ginseng (*Panax ginseng*), products of which have been produced commercially in Japan since the early 1990s by plant tissue cultures, and sales of these products reached U.S. $3 million in 1995 alone (*66*). Flavor and aroma compounds from plant tissue cultures may become available as we learn the details of their biochemical pathways and their genetic control. Flavor ingredients produced by short biochemical pathways are presently chief candidates, where the overexpression of one or two genes may increase product yield manyfold. Once these hurdles are overcome, the cell cultures may be scaled up, the cultures stabilized, and continuous extraction methods developed for the isolation of the flavor compounds. The safety of the compounds isolated from cell cultures should also be considered. The establishment and propagation of plant cell cultures require chemicals such as kinetin, 2-benzyladenine, and 2,4-D, which are usually not a part of the human diet and which will have to be removed during the isolation process. In some unique instances, feeding aroma precursors to plant cell cultures may also be a possibility for their production. In the past 10 years biology has entered a new phase, and, like cell cultures, this phase is expanding exponentially. Flavor compound production by plants or their tissue cultures is a biological process, and branches of modern biology such as genomics, proteomics, and metabolomics may radically revise our ability to manipulate these production systems.

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