

FLAVONOID ACCUMULATION IN TISSUE AND CELL CULTURE*

Studies in *Citrus* and Other Plant Species

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1. INTRODUCTION

The genus *Citrus* provides a number of economically important horticultural crops, including the orange, the lemon and the grapefruit. The flavonoid composition of these species are of interest biosynthetically, taxonomically, and because they affect the market quality of the fruit and its products. The work on flavonoid metabolism and function in citrus has been done with cell free extracts derived from whole plant tissues and some work using citrus cell cultures. This chapter will summarize some of the more important contributions of cell culture studies to the flavonoid research literature, summarizing the general work on flavonoid accumulation in plants, with a detailed overview of the work done in *Citrus* species.

The diversity of "secondary metabolites" produced by plants has served man with both fascination and utility for centuries. Secondary metabolites have been defined historically as naturally-occurring substances that do not seem to be vital to the immediate survival of the organism that produces them, and are not an essential part of the process of building and maintaining living cells. However, recent research indicates that secondary metabolites play pivotal roles in the chemical functionality of the plant, determining the role a particular species will play in the environment. In addition, research is beginning to show roles for these compounds at the cellular level as plant growth regulators and modu-

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lators of gene expression. Secondary compounds are crucial in the plant's response to stresses, such as changes in light or temperature, competition, herbivore pressure, and pathogenic attack. They appear to play critical roles in the plant's ability to survive and reproduce. Plants have also incorporated these secondary metabolites into specialized physiological functions such as seed production and intracellular signaling. These secondary metabolites are often functionally unique at the species level, and taxonomically discrete individual compounds are responsible for identical functions in separate species. One class of secondary metabolites, the flavonoids, which are derivatives of the phenylpropanoid pathway, has been implicated in all these roles and is an ideal system for study.

Much of this "emerging picture" for secondary metabolite biosynthesis and function has come about from studies initiated by the development and use of some key techniques in the years that followed the Second World War: 1) the widespread availability of radioactive isotopes of carbon, hydrogen, sulfur and phosphorus for research in the 1950s, 2) the development of new chromatography techniques for the isolation and quantitation of plant natural products in the 1950s and 60s, especially thin-layer chromatography (TLC), gas chromatography (GC), and later, in the 1970s, high pressure (or performance) liquid column chromatography (HPLC), 3) the development of protocols for the extraction of active enzymatic preparations involved in secondary metabolite biosynthesis from plant tissues in the 1960s, and 4) the development of culturing techniques for growing undifferentiated plant cells in the laboratory in the 1970s. With the advent of these tools, and the recent interest in the field of plant natural products, research in this field has exploded in recent years (Cordell, 1995).

This is especially true of the plant flavonoids. The flavonoids are derived from phenylalanine via the general phenylpropanoid biosynthetic pathway. This pathway is highly regulated and controlled by both normal growth and development as well as being induced by wounding and attack by pathogens. Flavonoids have attracted the attention of man for centuries. Certain plant flavonoids were among the first dyes used by man. While they have been systematically studied since around the turn of the century, only recently have their biochemical roles in plants been fully appreciated (Dakora, 1995; Shirley, 1996).

With the development of modern scientific techniques, these compounds have been identified and purified from their biosynthetic sources, and their modes of action, especially in mammalian health, are being examined and commercially exploited. By 1975, the study of flavonoid accumulation and function was well advanced, with careful biosynthetic studies underway. This ubiquitous class of phenolic compounds is found in nearly all vascular plants. Among the phenolics, flavonoids are of particular interest, since they appear to function in all roles in which plant secondary metabolites have been implicated (Dakora, 1995). They, and the other phenolics derived from the phenylpropanoid biosynthetic pathway, have been reported as having a wide variety of physiological effects in both plants and animals, serving as enzyme activators and inhibitors, metal chelators, antioxidants, free radical scavengers, transcription regulators, phytohormones, and as mutagenic, antimutagenic, carcinogenic, anticarcinogenic, cytotoxic, antineoplastic, anti-inflammatory and anti-allergenic substances. In plants they have been shown to function in protection from UV radiation damage; in mineral nutrition; in temperature and water stress; in pollination and seed dispersal (by their color properties); and as constitutive chemoprotective agents against other plants, microbial pathogens, fungi, insects, and herbivores. Flavonoids have also been shown to be naturally occurring auxin transport regulators (Jacobs and Rubery, 1988). Much of the early work was done in plant species other than *Citrus*.

As a specific result of the plant's interaction with changes in its the environment, specific flavonoids and other phenolic metabolites are produced and accumulated (Snyder and Nicholson, 1990; Laks and Pruner, 1989; Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995). These compounds have been postulated as being one of the earliest chemical classes evolved by plants for protection from the stronger UV radiation that struck the earth before the development of the ozone layer (Kubitzki, 1987). The biosynthesis of new flavonoids is constitutive in plants, being internally controlled during normal growth and development, such as new vegetative leaf growth and reproductive organ development (Heller and Forkmann, 1988). Flavonoid biosynthesis may also be induced by exogenous stimuli, such as changes in light and temperature (Hahlbrock and Scheel, 1989). Biosynthesis and/or modification of constitutive flavonoids can be triggered, or elicited, by damage to the plant caused by physical agents (wind, freezing, water stress, ozone, heavy metal ions, certain herbicides), herbivore attack (insects, grazing animals) and microbial invasion (bacteria, fungi) (Nahrstedt, 1990; Nicholson and Hammerschmidt, 1992; Ebel, 1986; Dixon and Paiva, 1995; Dixon et al., 1992; Chappell and Hahlbrock, 1984).

They are, as one reviewer has pointed out, "biological molecules for useful exploitation" (Dakora, 1995). An excellent series of books have been published on plant flavonoids. These include the first comprehensive work on the subject by Geisman (1962), a series of books edited by Harborne (Harborne et al., 1975; Harborne and Mabry, 1982; Harborne, 1994) and one by Stafford (Stafford, 1990). For general reviews of flavonoid biosynthesis in plants see Heller and Forkmann (1988); Hahlbrock and Scheel (1989); and Stafford (1990).

2. CELL CULTURE AND FLAVONOID RESEARCH

Cell culture provides a uniform mass of undifferentiated cells free from microbial contamination, which is available for study regardless of the season. The technique also provides unparalleled control of the environment through the manipulation of the cell media. And, most importantly, cell culture provides a means to induce the formation of secondary metabolites at higher levels and over shorter periods of time than occurs in differentiated plants. Through refinement of the technique, it was found that sterile cultures of undifferentiated cells could be procured from almost all species of plants.

Since the activity levels of many of the enzymes in the flavonoid biosynthetic pathway are low and/or transient in nature, much of the early work on this pathway was conjectural in nature. With the advent of plant cell culture, however, increased levels of some secondary enzyme activities could be attained by altering the media conditions. It is now well established that many cell cultures produce relatively large quantities of one or more of the secondary metabolites produced by the whole plant, but other cultures from the same species or even the same plant will produce trace amounts of secondary metabolites, different compounds or sometimes none at all. Some cultures will produce compounds not normally found in the whole plant. The reasons for the differences in metabolite production in the cell cultures are unknown. They may be due to the nature of cell culture, as they are undifferentiated cells which were initiated from an injury to a part of the whole plant (Becker et al., 1987; Charlwood and Rhodes, 1990). There are also disadvantages to the use of cell culture for these types of studies (Ellis, 1984). The possibility of genomic instability, the loss of morphological differentiation, the loss of the ability to accumulate all the compounds found in the tissues of the intact plant, and the loss of regulatory control.

In spite of the technique's limitations, many of the biosynthetic steps in plants have been elucidated mostly through the use of cultured cells. Flavonoids are synthesized from

phenylalanine via a biosynthetic route in plants termed the "general phenylpropanoid pathway." This important pathway generates a large number of compounds which have in common a phenyl structural element. Secondary metabolites derived from this pathway include (in addition to flavonoids) tannins, phenols, benzoic acids, stilbenes, cinnamate esters, and coumarins. A sequence of reactions converts phenylalanine into co-enzyme A (CoA) derivatives of substituted cinnamic acids. These are further converted to various other metabolites. Flavonoids are derived from the conjugation of *p*-coumaryl-CoA with three malonyl-CoA molecules to form naringenin chalcone, which is considered to be the precursor of all the flavonoids. Naringenin chalcone is rapidly converted to the flavanone form, naringenin. Naringenin can then be further modified enzymatically by reactions including reduction, oxidation, hydroxylation, *O*-methylation, *O*-glycosylation, *C*-glycosylation, acylation, sulfonation, rearrangement, and polymerization to form other flavonoids.

Flavonoids have generally been classified into 12 different subclasses by the state of oxidation and the substitution pattern at the C-2—C-3 unit (Harborne et al., 1975). These include flavanones, flavones, flavonols, chalcones, dihydrochalcones, anthocyanidins, aurones, flavanols, dihydroflavonols, proanthocyanidins (flavan-3,4-diols), isoflavones, and neoflavones. More than 10,000 flavonoids have been identified from natural sources and more continue to be identified at a rate of more than 10 per month (Dakora, 1995). Of particular interest is the use of flavonoids as taxonomic markers, as individual species within plant families often vary widely in flavonoid type and content (Seigler, 1981). In addition, individual plants within a species will produce and accumulate different flavonoids depending on several factors, such as plant growth stage, reproductive stage, the particular plant tissue involved, and the type of environmental stress or pathogenic attack involved (Dixon and Paiva, 1995).

The control mechanisms for complex modifications in flavonoid biosynthesis (such as B-ring hydroxylation, methylation, or glycosylation) have been studied in a small number of cultured cell suspensions and whole plants, including parsley (acylated flavone and flavonol glycosides) (Ebel and Hahlbrock, 1977), soybean (isoflavones) (Dixon et al., 1983), green bean (isoflavones) (Banks and Dewick, 1983; Ebel et al., 1984; Ebel et al., 1985; Ebel, 1986), alfalfa and chickpea systems (Heller and Forkmann, 1988; Hahlbrock and Scheel, 1989; Hahlbrock, 1977; Hahlbrock, 1976), and carrot (anthocyanins) (Cheng et al., 1985; Hinderer et al., 1984; Ozeki and Komamine, 1982; Ozeki and Komamine, 1985a; Ozeki and Komamine, 1985b)). Much of the recent work on the control of these pathways has been determined using new molecular biological techniques. Specific modification of plant genomes will allow researchers to delve further into the controls operating in this complex system. These pathways appear to be very tightly regulated and controlled by other metabolic, developmental, and stress-related factors. The enzymes in these pathways are often present as several different isozymes, indicating groups of tightly controlled metabolic chains which are driven by the formation of very specific end products (Douglas et al., 1992; Dixon et al., 1992; Stafford, 1990).

Induction has been studied extensively in cell culture systems. Phenylpropanoid pathway genes are activated in cells treated with either microbes or elicitors, resulting in the formation of newly synthesized flavonoids that inhibit microbial growth. In some cases, these same biosynthetic pathways can be induced by UV light or physical damage to the cells (Logemann et al., 1995; Nojiri et al., 1996; Negrel and Javelle, 1995; Liu et al., 1993a; Liu et al., 1993b). Modern genetic engineering techniques may be used to modify plant cell cultures to produce large amounts of flavonoids (Logemann et al., 1995; Nojiri et al., 1996; Negrel and Javelle, 1995; Liu et al., 1993a; Liu et al., 1993b; Kuc, 1995; Zenk, 1991). Several cell lines have already been shown to produce and accumulate fla-

vonoids that are different than those normally found in mature plants, some of which have interesting biocidal properties (Charlwood and Rhodes, 1990; Becker, 1987; Yamamoto et al., 1993; Yamamoto et al., 1992).

3. CITRUS FLAVONOIDS

The study of the flavonoids in *Citrus* species is of interest because they accumulate high concentrations of flavonoid glycosides. The most common glycosidic group attached to the flavonoids in citrus is the rhamnose-glucose diglycoside. This sugar group is present in two isomeric forms—neohesperidose and rutinose. The two sugars differ only in the position of the attachment of rhamnose to the glucose. The main flavonoids found in most cultivated citrus species are the flavanone glycosides. The flavanone rutosides, such as hesperidin and narirutin, are tasteless, while the flavanone neohesperidosides, such as naringin and neohesperidin, are bitter, with the bitterness intensity being relative to the composition of the flavanone aglycone structure. These compounds can account for up to 5 % of the dry weight of the leaf and fruit tissue. The accumulation of the naringin and neohesperidin in citrus species related to the pummelo causes these fruits to have a bitter taste. This includes the economically important sour orange and grapefruit varieties. Pummelo accumulates the neohesperidosides exclusively, while sour orange and grapefruit accumulate both the neohesperidosides and the rutosides found in other citrus species related to the citron and the mandarin (Albach and Redman, 1969; Horowitz and Gentili, 1977).

The biosynthesis of flavonoids in citrus is similar to other plant species (Hasegawa and Maier, 1981). The flavonoid glycosides are accumulated in young leaves and fruits during the cell division stage (Fisher, 1968; Berhow and Vandercook, 1989). The woody portions of the plant do not biosynthesize the characteristic flavonoids of the leaves and fruits, though these compounds are found in stem and root tissues (Berhow and Vandercook, 1991; Jourdan et al., 1985). The major flavanone glycosides are probably accumulated in the cell vacuoles. During the cell elongation and subsequent maturation of the leaves and fruit, there is little further biosynthesis. As the fruit reaches maturity, flavonoid concentrations in the fruit decrease due to dilution effects (Jourdan et al., 1985; Hagen et al., 1966; Castillo et al., 1992; Shaw et al., 1991; Vandercook and Tisserat, 1989).

Interestingly, however, the metabolic fate of the flavanone glycosides in the citrus plant tissues is not known, and there is as yet no clue as to what functional role these compounds play in the plant themselves. It may be that these compounds are accumulated constitutively in the developing leaves and fruits, glycosylated for storage purposes, and saved for utilization in response to stress, either as the flavanone aglycone or for further modification in response to the specific stress. Hence, citrus species may be accumulating large quantities of an "inactive" form of phenolics stored in a non-toxic form for future use. Several of the citrus flavanone aglycones, including naringenin and hesperetin, have been shown to have biological activities in other plant and animal systems, yet their function in citrus has yet to be demonstrated.

Flavonoid composition in citrus has been extensively examined. The most comprehensive reviews on the composition of the flavonoids found in citrus species are by Horowitz and Gentili (1977) and Kefford and Chandler (1970). Published flavonoid composition surveys in citrus have been published by Kanés et al. (1993) and Nishiura et al. (1969, 1971a, 1971b). Other aspects of flavonoid composition and accumulation in citrus are more fully covered in other chapters in this book.

4. CITRUS CELL CULTURE

The techniques to culture cells derived from citrus species have been around for a number of years (Tisserat et al., 1989; Tisserat and Galletta, 1987). Extensive research was done with explanted tissues from citrus in culture, such as leaf and flower buds, embryo, shoot apex, juice vesicles, stem and root cultures. The development of a defined media by Murashige and Skoog (1962) for culturing of plant cells, refined by Murashige and Tucker (1969) for citrus, promoted further interest in this area. Most of these cultures either produced dedifferentiated cells (callus) or degenerated into callus soon after explanting.

With the emergence of new molecular biology techniques, especially the ability to transform cells derived from woody plant species and regenerate whole plants from these cultures, interest in plant cell culture has expanded greatly in the last 15 years. Many laboratories around the world have established callus and cell culture lines from embryonic tissues of many different citrus species, varieties and relatives (Vardi and Galun, 1988). Techniques have been developed to regenerate whole plants from cell lines that maintain their embryogenic capability (Kobayashi et al., 1985; Moore, 1985; Hidaka and Omura, 1989; Beloualy et al., 1991). These cultures have been mostly used for the production of protoplasts for genetic alteration with the aim of introducing desirable characteristics as either new fruit or rootstock cultivars. Protoplast culture for citrus is discussed by Vardi and Galun (1988), and has been used for the production of somatic hybrids using protoplast fusion (Grosser and Gmitter, 1990; Kobayashi et al., 1991a; Kobayashi et al., 1991b; Ohgawara and Kobayashi, 1991; Tusa et al., 1992; Hidaka and Omura, 1992). These techniques have been used to create interspecies hybrids between sexually incompatible species such as *Citrus* and *Severinia* (Grosser et al., 1988), and *Citrus* and *Atalantia* (Louzada et al., 1993). Foreign DNA fragments have been inserted into cultured citrus cells and whole plants successfully regenerated via *Agrobacterium*-mediated transformation (Hidaka et al., 1990; Feng et al., 1991; Moore et al., 1992; Peña et al., 1995), or direct DNA transfer (Kobayashi and Uchimiya, 1989; Vardi et al., 1990).

Actual published studies of flavonoid biosynthesis and accumulation in citrus species has been rather limited. This is in part due to the observation noted above about secondary metabolism in cell cultures, that cell cultures established from plants are inconsistent and variable in the production of these metabolites. Also, the fairly recent development of techniques to consistently extract active enzymatic preparations from intact plant tissues that have high levels of phenolics and other oxidizing species has only been applied to *Citrus* species since the early 1970s. These two techniques applied to cells and tissues of *Citrus* species would provide the biochemical evidence for the mechanisms of the accumulation of the citrus flavonoids. Though not completely understood even to this day, much of the early part of the *Citrus* phenylpropanoid biosynthetic pathway has been worked out and confirmed by the use of radioactive isotopes and the isolation and characterization of the enzymes involved.

5. CITRUS FLAVONOID BIOSYNTHESIS

5.1. Quantitation of Flavonoids

Early citrus flavonoid biochemistry research was driven by the need to understand the mechanisms of the accumulation of the bitter principles in citrus juices. Hence much of the biosynthetic research on citrus flavonoids has been in grapefruit, pummelo and sour

orange. Though the main bitter principle in grapefruit was known to be the flavanone glycoside naringin, its complete chemical structure was not confirmed until 1963 (Horowitz and Gentili, 1963). Early speculation on the biosynthetic pathway was based on the characterization of the various flavonoids found in the grapefruit, especially in immature tissues (Maier and Metzler, 1967; Maier, 1969).

Definitive biochemical work on flavonoid biosynthesis in citrus species began with the work of Fisher who showed that ^{14}C -labeled phenylalanine fed to detached immature grapefruit leaves resulted in the formation of ^{14}C -labeled naringin, but this conversion did not occur in fully expanded leaves (Fisher, 1968). TLC evaluation of developing grapefruit showed high concentrations of naringin in young, developing fruit (Albach et al., 1969). This demonstrated that flavanone glycoside accumulation was most active in young fruit. Grapefruit tissues at various stages of development were later examined with radioimmunoassays specific for flavanone glycosides (Jourdan et al., 1985). Naringin, which comprises 80% of all the flavanone glycosides found in grapefruit, was found in all tissues examined: seeds, germinating seedlings, 5 month old plants, 1 year old plants, (leaves, stems and roots) flushes from mature trees, developing flowers and fruit, and mature fruit. The highest concentrations were found in young developing leaves, flowers and fruit. The lowest concentrations were found in the roots, stems, and older leaves. Feeding either ^{14}C -phenylalanine or ^{14}C -acetate to detached immature grapefruit resulted in the formation of labeled naringin and prunin (naringenin 7-*O*-glucoside) (Berhow and Vandercook, 1989). Radiolabeled naringin was found in all parts of intact grapefruit seedlings fed labeled acetate—roots, stems and leaves (Berhow and Vandercook, 1991). The highest levels were found in the young developing leaves. If the seedling was taken apart and labeled acetate fed to each part—leaves, stem and roots, labeled naringin was found only in the young expanding leaves. In lemon, hesperidin, the major flavanone glycoside present, is accumulated in a pattern similar to that of the grapefruit. A rapid increase occurred in concentration of hesperidin in developing lemons, followed by a steady decrease in concentration as the fruit expanded to full size and matured (Vandercook and Tisserat, 1989). Interestingly, two other lemon flavonoids, eriocitrin and diosmin, are accumulated later in the course of fruit development and continued to increase in concentration throughout the maturation process. Similar results were found in naringin, neohesperidin and neodiosmin accumulation in developing leaves and fruit of sour orange (Benavente-Garcia et al., 1993; Castillo et al., 1992). Both hesperetin 7-*O*-glucoside and naringenin 7-*O*-glucoside were found in very early stages of the developing sour orange fruit (Castillo et al., 1993) indicating a stepwise addition of sugar molecules as the final steps of biosynthesis. A unique flavanone glycoside, naringin-6"-malonate, was found in immature grapefruit fruit, but little or none could be found in mature fruit (Berhow, 1991). A similar compound, the flavone apigenin 7-*O*-(6-*O*-malonyl) glucoside, was shown to be selectively taken up by isolated vacuoles of parsley, while apigenin 7-*O*-glucoside, was not (Matern et al., 1986). This may be true for citrus flavonoids as well.

These quantitation studies showed that flavonoids are accumulated constitutively in young developing leaves and reproductive tissues of citrus (i.e. during periods of rapid cell division). Citrus species can dedicate a relatively large amount of carbon resource to this synthesis. Immature grapefruit, just after the abscission of the ancillary flower structures, can have up to 40% on a dry weight basis of a single compound, the flavonoid naringin (Maier, 1969). This constitutive accumulation appears to be similar to the pathways in other more studied dicot species, the conversion of phenylalanine into the flavanone aglycone in the cytoplasm, followed by the stepwise addition of sugars, mainly glucose and rhamnose in the case of citrus species, to give the major flavanone glycosides. These are probably earmarked by acylation for transport to the cell vacuole where they are

deacylated and stored. The concentration of the major flavanone glycoside, naringin, hesperidin or neohesperidin in most species, decreases in the fruit tissues as the fruit matures (Albach et al., 1981; Albach and Wutscher, 1988; Jourdan et al., 1985; Vandercook and Tisserat, 1989), but some other minor flavonoids are accumulated throughout the maturation process (Vandercook and Tisserat, 1989).

5.2. Enzymes in Cell-Free Extracts

Enzymatic characterization of flavonoid biosynthesis began in the late 1950s, with the demonstration of the activities of the enzymes chalcone flavanone isomerase (Shimokoriyama, 1957), phenylalanine ammonia lyase (PAL) (Maier and Hasegawa, 1970), and cinnamate 4-hydroxylase (Hasegawa and Maier, 1972) in cell free extracts prepared from young citrus fruits. The presence of PAL in citrus fruit tissue cultured *in vitro* has also been reported (Thorpe et al., 1971). *O*-methylation of flavonoids was demonstrated in cell free extracts prepared from calamondin (Brunet and Ibrahim, 1980). The activities of chalcone synthase, flavanone 7-*O*-glucosyltransferase and flavanone 7-*O*-glucoside-2''-*O*-rhamnotransferase were detected in cell free extracts prepared from several citrus species (Lewinsohn et al., 1989a & 1989b). Flavanone glucosyltransferase activities were reported in cell free extracts of young leaves of grapefruit seedlings (McIntosh and Mansell, 1990) and lemon seedling leaves (Berhow and Smolensky, 1995). A methyltransferase was detected in extracts prepared from young leaves and fruits of sour orange (Benavente-Garcia et al., 1995), and a cytochrome P450-mediated 3'-hydroxylase activity was detected in an extract prepared from sweet orange cell cultures (Doostdar et al., 1995). Purified or partially purified enzymes from citrus sources include PAL from grapefruit (Hasegawa and Maier, 1970), chalcone-flavanone isomerase from sweet orange (Fouche and Dubery, 1994), flavanone 7-*O*-glucoside-2''-*O*-rhamnotransferase from pummelo (Bar-Peled et al., 1991) and a flavanone 7-*O*-glucosyltransferase from grapefruit leaves (McIntosh et al., 1990).

Again, with a few exceptions noted below, all of these activities are found in either young developing leaves or fruit and are part of the constitutive pathway. The rhamnotransferase in pummelo was shown by both activity assays and immunodetection techniques to be specifically located in young leaves, and all flower parts except the anthers and young fruit (Bar-Peled et al., 1993). Both the enzyme activity and the protein molecule itself rapidly disappear as leaves reach full length, and as the fruit approaches about a quarter of its mature weight. Interestingly, there continues to be a net increase of naringin up to the point that the fruit reaches about half of its mature weight. This would seem to point to an import of naringin from the leaves of the plant.

Very little research has been done on induced biosynthetic pathways in citrus. There are two reports on the induction of PAL activity in citrus. It was observed that wounding Valencia orange peel will induce an increase in the activity of PAL (Ismail and Brown, 1979), while fungal infection will suppress the ethylene-induced PAL activity in grapefruit (Lisker et al., 1983). The lack of work on the induced pathway may be due to the fact that there is not a good cell culture model system to study. As will be discussed below, citrus cell cultures generally do not produce and accumulate flavonoids. This is important, as much of the work on the induced pathways have been done with cell cultures in other species of plants.

5.3. Flavonoid Metabolism in Cell Cultures

As noted above, much of the research work in citrus cell culture has been focused on either the culture of fruit tissues or genetic alteration of citrus species. With few excep-

tions, citrus cell cultures generally do not accumulate flavonoids. When they were accumulated, it was usually in either differentiated cultures like fruit and juice vesicle cultures or very young callus cultures recently initiated from fruit tissue. The first report of flavonoid accumulation in cultured citrus tissues came from the laboratory of Kordan (Kordan and Morganstern, 1962; Kordan, 1965). He observed that phenolic-like substances were produced and excreted into the medium from callus cultures derived from juice vesicles of lemon proliferating *in vitro*. Kordan and Morganstern considered these substances to be flavonoids based on thin-layer chromatography evaluation, though they could not confirm this structurally.

In a cultured juice vesicle study, the concentrations of accumulated flavonoids were much lower than those of fruit developing on the tree. Also, the phenolic patterns of callus derived from the cultured vesicles were significantly different than those found in the cultured vesicles or vesicles in fruit from trees (Tisserat et al., 1989; Vandercook and Tisserat, 1989). Del Rio measured the accumulation of naringin and neohesperidin in callus cultures derived from young fruit of sour orange (del Rio et al., 1992). These cultures were eight months old, having been subcultured at four week intervals. One could speculate that these cultures would have continued to produce flavonoids similar to that found in the mature fruit. Low levels of methoxylated flavones occurred in callus cultures of orange and lemon flavedo (Brunet and Ibrahim, 1973), however, these compounds could have been simply be carried over from the original flavedo cells from which the calli were derived.

In general, callus cultures derived from citrus fruit tissues do not accumulate flavonoids, but I have found an exception. In 1991, I examined callus cultures derived from the embryonic fruit tissues of 9 mandarin cultivars, 1 grapefruit cultivar, 1 pummelo hybrid cultivar, 1 sour orange cultivar, 2 tangelo cultivars, 3 papeda hybrids cultivars, 1 kumquat cultivar, 10 sweet orange cultivars, and 2 citrus rootstock cultivars that were maintained by the Japanese Fruit Tree Research Station in Okitsu, Japan. With one exception, which will be discussed in detail below, none of these callus cultures produced measurable levels of methanol-extractable phenolic compounds. Most of these cultures were over 2 years old, and some had been maintained in culture for over 10 years and were still capable of regenerating embryos and ultimately whole plants (Hidaka and Omura, 1989). Other reports have commented on the lack of flavonoid production in callus cultures. Callus derived from sweet orange and grapefruit rapidly lose the ability to produce hesperidin and naringin respectively (Barthe et al., 1987). Regeneration of shoots from cultured cells restored the production and accumulation of these flavonoids. Callus cultures derived from grapefruit were able to glucosylate endogenously added naringenin at the 7 position, but did not accumulate any flavonoids on their own (Lewinsohn et al., 1986). Examination of other cultures by the same research group found that a culture derived from sour orange ovules was able to glucosylate naringenin to prunin and rhamnosylate prunin to form narirutin (Lewinsohn et al., 1989a). Cultures derived from lemon and grapefruit were able to glucosylate exogenously added hesperetin. However, the ability to do this was inconsistent among the cultures examined. The rhamnotransferase protein could not be detected in cell free extracts of grapefruit callus cultures by the antibody prepared against purified protein isolated from young leaves (Bar-Peled et al., 1993). As callus cells were induced to form embryos, the enzymatic activities of both the glucosyltransferase and the rhamnotransferase increased markedly (Gavish et al., 1989). Cytochrome P-450 mediated naringenin 3'-hydroxylase enzyme activity was detected in cell free extracts prepared from sweet orange cultures (Doostdar et al., 1995). This activity is required for the conversion of eriodictyol (5, 7, 3', 4'-tetrahydroxyflavanone) to hesperetin (5, 7, 4'-trihydroxy, 3'-methoxyflavanone) in oranges and lemons. This activity

was also found in young developing leaves and fruit, but not in mature fully expanded tissues. While the presence of flavonoids in the cell cultures was not discussed in this paper, it was noted that the hydroxylase activity was lost when the cells were transferred to a medium that induced the formation of embryos. This is in contrast to other observations noted above.

5.4. Flavonoid Accumulation in Callus Cultures Derived from Mexican Lime

One of the recurring themes in this review is that callus cultures derived from citrus species generally do not accumulate flavonoids. Indeed, the few reported occurrences of this are in either very young callus lines or those in which embryogenesis has been induced. Most callus cultures I have examined are white in color and have no detectable levels of UV-absorbing phenolics.

However, during a 1991 visit to the Okitsu Fruit Tree Research Station citrus genetic research group in Japan, I found one callus line derived from *Citrus aurantifolia* 'Mexican lime' that formed bright yellow cultures. These cells had been in continuous culture for over 8 years. Extraction of freeze-dried cells from these cultures revealed the presence of low levels of UV-absorbing flavonoids. The UV spectra obtained from a diode array detector showed them to be flavonols. We were able to purify the three major peaks and identify them as being kaempferol 3-*O*- β -rutinoside (K-R), kaempferol 3-*O*- β -D-glucopyranoside-6''-(3-hydroxy-3-

Table 1. Major flavonoids found in Mexican lime callus and in Mexican lime leaves

HPLC elut. order	Spectral pattern	Identification	
<i>Callus</i>			
1	flavone	apigenin triglycoside	
2	flavone	diosmetin triglycoside	
3	flavone	diosmetin triglycoside	
4	flavonol	kaempferol diglucoside-HMG ester	
5	cinnamic acid	unknown	
6	cinnamic acid	unknown	
7	flavone	unknown	
8	flavonol	rutin	
9	flavone	isorhoifolin	
10	flavone	diosmin	
11	flavonol	kaempferol 7- <i>O</i> -rutinoside	
12	flavonol	kaempferol 3- <i>O</i> -glucoside-HMG ester	
HPLC elut. order	Spectral pattern	Identification	Conc. mg/g.d.w.
<i>Leaves</i>			
1	flavanone	Narirutin 4'-glucoside	~ 0.1
2	flavone	Apigenin triglycoside	0.1
3	flavanone	Eriocitrin	0.1
4	flavanone	Hesperidin	1.8
5	flavonol	Rutin	0.6
6	flavone	Diosmin	0.5

methylglutarate) (K-HMG-G) and kaempferol 3-O- β -D-glucopyranoside-6''-(3-hydroxy-3-methylglutarate)-7-O- β -D-glucopyranoside (K-HMG-diG) (Berhow et al., 1994) as shown in Table 1.

Acylated flavonoids have not been routinely reported as being present in citrus species. Sugiyama reported an HMG acylated flavonoid found in methanol extracts prepared from the fruit of mandarin oranges (Sugiyama et al., 1993). There are probably other acylated flavonoids in citrus, but their levels are probably normally very low in healthy mature tissues.

These kaempferol compounds are not found in the differentiated tissues of the Mexican lime plants. HPLC examination of extracts from leaf and fruit tissues from mature Mexican lime plants did not detect any of these compounds as indicated in Table 1. Indeed, kaempferol glycosides have not been reported in lime.

It is interesting that for other plant species there are many reports on the accumulation of phenolic compounds in plant cell culture. Three major observations can be made. 1) Production is highly variable and may be different even among cultures prepared from the same plant. Medium composition has an effect on how much of these compounds are accumulated. 2) Higher levels and additional phenolics can be induced by the addition of certain chemicals to the medium, by altering the osmotic potential of the media, by light, etc., these changes in the medium often mimic a "stress," such as adding an elicitor like chitin, a phytohormone such as 2,4-D, heavy metals, and others. 3) Often, the phenolic compounds accumulated in the cell cultures under these conditions are very different from the compounds that are predominately found in mature healthy tissues of the original plant.

A few examples from the literature of the study of phenolic accumulation are given here to compare with what may be going on in the Mexican lime cell cultures. Cell cultures derived from *Vancouveria hexandra* produced large amounts of kaempferol glycosides that were not found in the original plant. The production was influenced by the culture media and required the addition of 2,4-D (Yamamoto et al., 1993). Some cell cultures derived from various *Hypericum* species produced quercetin glycosides. The cultures produced were extremely variable in their ability to produce these flavonoids and other secondary metabolites. A cell culture derived from *H. balearicum* accumulated high levels of quercetin, isoquercitrin and rutin (Kartnig et al., 1996). Several kaempferol and quercetin glycosides were produced in cultures derived from *Dionaea muscipula* plants. Some of the kaempferol glycosides were acylated (Pakulski and Budzianowski, 1996). Regulation studies have shown that light, specifically UV light, is required for anthocyanin accumulation in carrot cell cultures (Takeda, 1990), flavonoid accumulation in parsley cell cultures (Logemann et al., 1995), and that anthocyanin accumulation in grape cell culture (Kakegawa et al., 1995) and flavonoid accumulation in parsley cell culture (Logemann et al., 1995) is related to the cessation of cell division. Addition of elicitors to cell cultures induces the formation of phenolics, including flavonoids. Fungal cells added to tomato cell cultures induce the formation of cinnamic acid esters (Keller et al., 1996; Bernards and Ellis, 1989; Bernards et al., 1991). Addition of chitin to cactus cell cultures induces the formation of several flavonoids. This can also be induced by the addition of yeast extract or autoclaved fungal mycelia (Liu et al., 1993a, b). Flavonoids are also induced by fungal elicitors added to parsley cell cultures (Logemann et al., 1995). These studies generated some ideas as to which chemicals could be added to the medium to affect the flavonoid accumulation in the Mexican lime cultures. Some of the results of studies in my laboratory are shown in Table 2.

Growing the cultures in the dark stops the accumulation, and returning the cultures to light reinitiates the accumulation. The addition of phytohormones has a profound effect

Table 2. Effect of various compounds added to the culture media on flavonoid accumulation in Mexican lime callus cultures

Compound/Classification	Effect
No light	---
Benzyladenine/PGR-cytokinin	—
Gibberellic acid/PGR	+
Abscisic acid/PGR	+
Napthaleneacetic acid/PGR-auxin	+
2,4 Dichlorophenoxyacetic acid/PGR-auxin	NE
Zeatin/PGR-cytokinin	+
Zeatin riboside/PGR-cytokinin	+
Indoleacetic acid/PGR-auxin	NE
Indolebutyric acid/PGR-auxin	NE
Salicylic acid/signal	NE
Acetylsalicylic acid/signal	NE
Arachadonic acid/elicitor	NE
Chitin/elicitor	NE
Fungal extracts/elicitor	NE
Leaf extracts/elicitor	+
Pectin digests/elicitor	+

+: Increase in flavonoid production

—: Decrease in flavonoid production

NE: No effect

on the accumulation of the acylated kaempferol glycosides. The auxins 2,4-D and NAA, gibberellic acid, the cytokinin zeatin, and abscisic acid have a stimulatory effect, while the cytokinin BA had an inhibitory effect. Abscisic acid was stimulatory at all levels examined (Table 2 and Figure 1).

Classical elicitors, such as chitin, arachadonic acid, and autoclaved fungal mycelial preparations, did not seem to have an effect, and neither did the plant "secondary messenger" salicylic acid. Interestingly, carbohydrate fractions prepared from macerated citrus leaves stimulated the formation of additional flavonoids in the Mexican lime cell cultures. This could be mimicked to a lesser degree by the addition of partially digested fractions prepared from polygalacturonic acid and pectin. Addition of these oligosaccharide fractions to Marsh grapefruit callus cultures, which do not normally produce any phenolic compounds, induces them to form low levels of flavonoids including hesperidin. This work will be more fully discussed in an forthcoming paper.

6. CONCLUSION

The use of cell cultures to study secondary metabolism, such as the accumulation of flavonoids, in plants has led to the an understanding of how these pathways are regulated and controlled. Without a doubt, cell cultures are indispensable tools for secondary metabolism research and are, as one reviewer has put it, a pot of gold (Zenk, 1991). Cell cultures are being exploited not only as tools, but as sources for the generation of flavor and colors (Dörnenburg and Knorr, 1996). This approach is being eyed for many other uses including the production of important pharmaceuticals. Though some researchers caution that cells in culture may never live up to its promise as "living cell factories" for pharma-

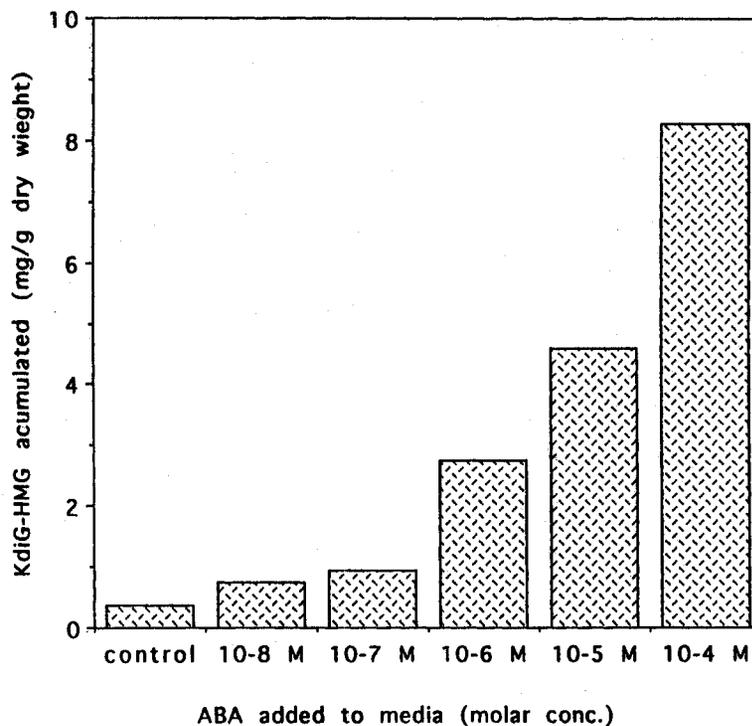


Figure 1. Effect of the addition of abscisic acid to the cell culture medium on the accumulation of kampferol-4,7-*O*-diglucoside HMG ester in Mexican lime callus cultures grown on solid media.

ceuticals and other useful chemicals (Kuc, 1995), new techniques may well overcome some of these obstacles. Flavonoids appear to be universal chemical tools which plants use to deal with their environment. They have been implicated as defenses against other plants, fungi, insects and bacteria, and as regulators of interactions between beneficial fungi, herbivores, and insects, as plant hormones, as important constituents of animal diets, both nutritionally and medicinally. As such, plant flavonoids are the phytochemicals of the future (Dakora, 1995; Shirley, 1996). Citrus flavonoids are unique in that they have qualities, such as taste, that contribute to the overall quality of the juice. Can citrus cell cultures be exploited commercially? The possibility that citrus cell cultures could be used to produce neohesperidin, which can be easily converted into the sweetener neohesperidin dihydrochalcone, has already been broached. The abundance of flavonoids in many agricultural commodities and processing streams allows for the possible exploitation of chemicals like the flavonoids from the processing wastes for agricultural, pesticidal, nutritional, and medicinal purposes looms ahead. To that end, citrus cell culture studies will provide useful information for the bioregulation of the accumulation of these compounds in citrus species. The future can only grow brighter for research in this important field.

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