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Flavonoid Sulfates: Distribution and Regulation of Biosynthesis in *Flaveria* spp.

Abdelali HANNOUFA

A Thesis

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ABSTRACT

Flavonoid Sulfates:
Distribution and Regulation of Biosynthesis
in Flaveria spp.

Abdelali HANNOUFA

The spatial distribution of sulfated and glucosylated flavonols as well as of the enzymes involved in the later steps of their biosynthesis (flavonol sulfotransferase and glucosyltransferase) was investigated in the shoots of Flaveria bidentis. The highest amounts of both types of flavonoid conjugates (μmol/g F.W.) and the highest activities of their enzymes (pkat/mg) were detected in the terminal bud and the first pair of leaves. Sulfotransferase activity was also highest in the upper stem segments and in the basal section of the leaves. Western blot analysis of protein extracts showed that variations in sulfotransferase activity in different tissues correlate well with the amounts of immunodetected enzyme protein.

Flavonoid sulfates of a number of Flaveria spp. belonging to different photosynthetic traits were determined. Overall, a correlation seemed to exist between the photosynthetic trait and both the level of flavonoid sulfation and the type of aglycones involved.

The effect of UV-B light (313 nm) on sulfotransferase activity was investigated. UV-B was found to cause at least one-fold increase in sulfotransferase activity. Its retardation by actinomycin-D suggests that this activation

takes place at the level of gene transcription.

These results were discussed in relation to the possible involvement of flavonoid sulfates in plant growth and protection against harmfui UV-B radiation, as well as their potential as chemotaxonomic markers.

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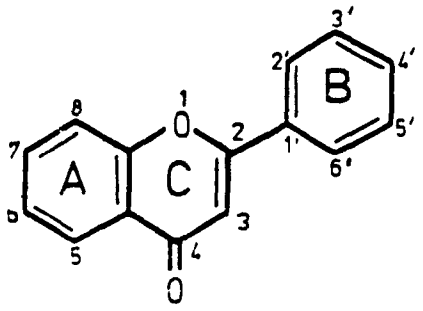
LIST OF ABBREVIATIONS

BAW	n-butanol-acetic acid-water
Bis-Tris	bis(2-hydroxyethyl)imino-tris(hydroxy-methyl)methane
BSA	bovine serum albumin
DIECA	diehtylammonium diethyldithiocarbamate
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EtOAC	ethylacetate
F.W.	fresh weight
GT	glucosyltransferase
HOAc	acetic acid
HPLC	high performance liquid chromatography
MEK	methyl ethyl ketone
MeOH	methanol
PAPS	3'-phosphoadenosine 5'-phosphosulfate
pkat	pkatal
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
ST	sulfotransferase
TBADP	tetrabutylammonium dihydrogen phosphate
TLC	thin layer chromatography
Tris	tris-(hydroxymethyl)aminomethane
UDPG	uridine 5'-diphosphoglucose
UV	ultraviolet

LIST OF GENERIC NAMES

Apigenin	5,7,4'-trihydroxyflavone
Isorhamnetin	3'-methoxy-3,5,7,4'-tetrahydroxyflavone
Kaempferol	3,5,7,4'-tetrahydroxyflavone
Patuletin	6-methoxy-3,5,7,3',4'-pentahydroxyflavone
Quercetin	3,5,7,3',4'-pentahydroxyflavone
Rhamnetin	7-methoxy-3,5,3',4'-tetrahydroxyflavone

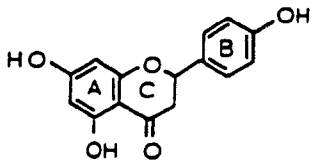


Flavonoid numbering system

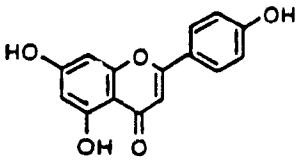
A. INTRODUCTION

In addition to the primary metabolites which are common to all living organisms, higher plants possess the unique ability to synthesize and accumulate a variety of compounds known as "secondary metabolites" (Mann 1987). These include alkaloids, phenolics and terpenoids (Haslam 1985), to mention a few. Flavonoids constitute one of the largest groups of naturally occurring phenolic compounds. Depending on the oxidation level of the heterocyclic ring C, flavonoid compounds are classified to different groups, of which the flavones, isoflavones, flavonols and anthocyanidins (Scheme 1) are the most common. The chemistry and biochemistry of flavonoids are well documented in several books and reviews (Geissman 1962; Harborne 1967; Harborne et al. 1975; Harborne 1988a; Stafford 1990).

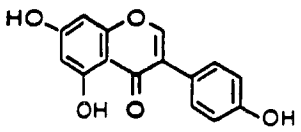
Flavonoids have been shown to fulfil a number of physiological functions, e.g. plant protection against environmental stresses such as microbial invasion (phytoalexins) (Smith et al. 1986), and UV radiation (Beggs et al. 1986). They also contribute to the bright colours of flowers and fruits as anthocyanins (Goodwin 1976). Furthermore, they have been reported to act as chemical signals in plant-microbe interactions (Bolton et al. 1986; Firmin et al. 1986; Peters et al 1986; Sadowsky et al. 1988; Spencer and Towers 1988; Hartwing et al. 1989; Maxwell et al. 1989), and as regulators of polar auxin transport (Jacobs and Rubery 1988). In addition, certain flavonoids have been reported



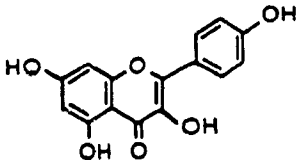
Flavanone



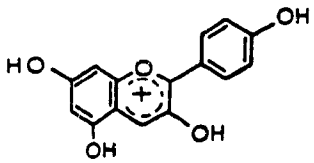
Flavone



Isoflavone



Flavonol



Anthocyanidin

Scheme 1. Different flavonoid classes

to have antiallergic, antiviral, antiinflammatory and anticarcinogenic properties (Cody et al. 1988).

Such diverse functions must require some structural diversity which, in the case of flavonoids, is provided by the presence of different functional groups on these compounds. In fact, within the same flavonoid group, an individual compound may undergo different substitutions, including hydroxylation (Heller and Forkmann 1988), C/O-methylation (Ibrahim et al. 1987; Wollenweber and Jay 1988), C/O-glycosylation (Chopin and Dellamonica 1988; Harborne and Williams 1988), prenylation (Zahringer et al. 1979; 1981), or O-sulfation (Barron et al. 1988).

Sulfation of flavonoids is the most recently discovered conjugation reaction. The first flavonoid sulfate was reported as early as 1937 (Kawaguchi and Kun 1937), but it was not until the first review article in 1975 (Harborne 1975), that these compounds were considered of widespread occurrence. Since then, the increasing number of reports has indicated that flavonoid sulfate esters are present in a large number of plant families including both dicotyledons and monocotyledons (Barron et al. 1988). The genus Flaveria (Asteraceae) is known to be a rich source of these conjugates.

Flaveria spp. belong to different photosynthetic groups and include the C_3, C_4 and C_3-C_4 intermediate types (Edwards and Ku 1987) (Table 1). The two most studied species are F. bidentis and F. chloraefolia. The flavonoid patterns of both species have been well characterized (Barron et al. 1988). To

date, much information is available on the phytochemistry, biosynthesis and enzymology of sulfated flavonoids. However, there is a noticeable lack of knowledge as to the regulation of their biosynthesis, as well as their physiological significance.

Table 1: Examples of *Flaveria* spp. which exhibit different photosynthetic characteristics[1].

C_3	C_3-C_4	C_4
*F. pringlei**	*F. linearis**	*F. bidentis*
	*F. floridana**	*F. trinervia**
	F. chloraefolia	
	*F. brownii**	

[1]Adapted from Edwards and Ku 1987

*The sulfated flavonoid patterns of these species were investigated in this dissertation

B. REVIEW OF LITERATURE

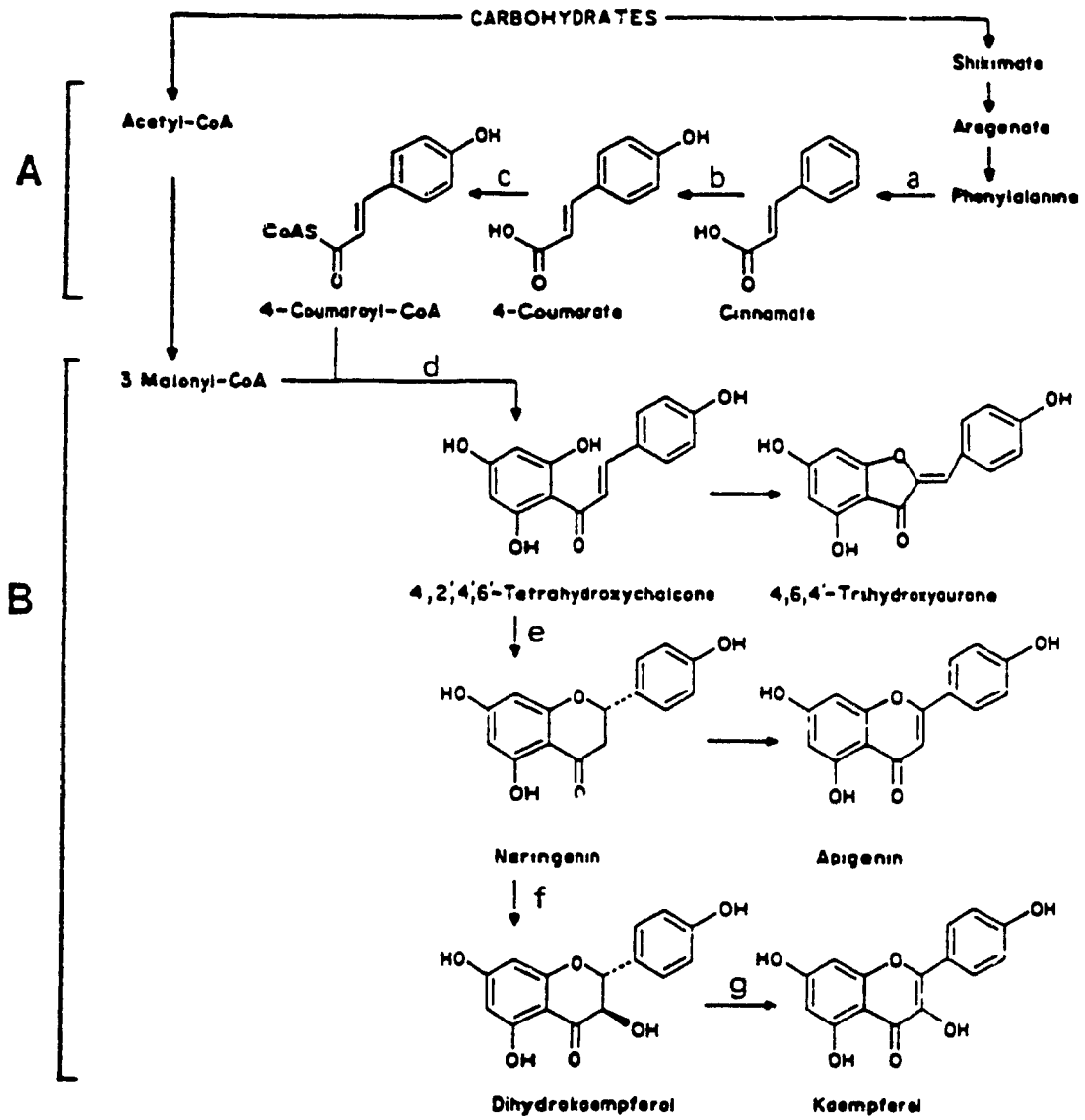
B.1. Flavonoid Biosynthesis Pathway

The C_{15} skeleton of flavonoid aglycones consists of two aromatic rings, A and B, that are joined together by a three-carbon chain forming ring C. Ring B is usually attached to ring C at position 2, and at position 3 in the case of isoflavonoids. Depending on the oxidation state of ring C, different groups of flavonoids may be formed (Scheme 1).

Flavonoids are unique in that the two component aromatic rings A and B are derived from different pathways. Ring B and the three-carbon side chain are derived from the aromatic amino acid L-phenylalanine, which itself is derived from the shikimic acid pathway. Ring A is formed by the head-to-tail condensation of three acetyl-CoA molecules, that are derived from malonyl-CoA. The biosynthetic pathway of flavonoids can be divided into two major parts (Scheme 2).

B.1.1. The phenylpropanoid pathway

The first committed enzyme in this pathway is phenylalanine ammonia-lyase (PAL), which catalyses the deamination of L-phenylalanine to give rise to trans-cinnamic acid. The latter undergoes a hydroxylation step catalysed by cinnamic acid hydroxylase resulting in the formation of 4-coumaric acid. The activation of the latter to 4-coumaroyl-CoA is catalysed by 4-coumaroyl-CoA ligase (Scheme 2A). The details of these enzyme reactions have been reviewed by Heller



Scheme 2. Phenylpropanoid and flavonoid pathways
Adapted from Heller and Forkmann 1988

and Forkmann (1988).

B.1.2. The flavonoid pathway

This pathway includes the reactions starting from the formation of naringenin chalcone to the different classes of flavonoids as well as the last modification steps that flavonoids may undergo (Scheme 2, B). The first and key enzyme in this chain of reactions is the chalcone synthase which catalyses the condensation of three molecules of malonyl-CoA with one molecule of 4-coumaroyl-CoA leading to the formation of naringenin chalcone. The latter is then isomerized to a flavanone, naringenin, a reaction catalysed by chalcone isomerase.

Starting with the flavanone, naringenin, a number of enzymatic steps result in the formation of different flavonoid classes. Isoflavonoids are formed by the oxidative rearrangement of naringenin that involves a 2,3-aryl shift catalysed by isoflavanone synthase. Flavone synthase mediates the introduction of a double bond between C_2 and C_3 of naringenin resulting in the formation of the flavone, apigenin. Hydroxylation of flavanones at the 3-position results in the formation of dihydroflavonols. This hydroxylation is catalysed by a dioxygenase, flavanone 3-hydroxylase. Flavonol synthase catalyses the introduction of a double bond between C_2 and C_3 of dihydroflavonols to give rise to the abundant class of flavonols (Scheme 2B). More information on flavonoid biosynthesis can be found in the excellent reviews by Heller and Forkmann (1988) and Stafford

(1990).

B.1.3. Flavonoid modifications

Except for the hydroxyl groups at positions 5 and 7 which are derived from the malonyl-CoA, and that at position 4' which is introduced at the C_6-C_3 stage, other hydroxylation reactions may take place at positions 3' and 5' of ring B and they are catalysed by position-specific hydroxylases.

In addition to hydroxylation, flavonoids may be C/O methylated, and/or C/O glycosylated and less commonly C-prenylated or O-sulfated. O-Methylation and O-glycosylation have been the most thoroughly studied reactions. A number of O-methyltransferases have been isolated and characterized (for reviews, see Poulton 1981; Heller and Forkmann 1988). In Chrysosplenium americanum, polymethylated flavonols were found to be formed via sequential methylation of the 3, 6, 7, 4' and 2'/5' hydroxyl groups (Ibrahim et al. 1987). O-Glycosylation is another frequent modification reaction. Several O-glycosyltransferases have been isolated from a wide range of plant species. Some of these enzymes were found to exhibit remarkable specificities towards the flavonoid aglycone, the position involved and the sugar transferred (Hösel 1981; Mohle et al. 1985; Ibrahim et al. 1986; Latchinian 1990). However, enzymes catalysing the 3-O-glucosylation of anthocyanins in some plant species were shown to efficiently glucosylate the 3-hydroxyl group of flavonols as well (Teusch et al. 1986a).

The enzymatic sulfation of flavonoids was demonstrated as recently as 1987. Since then, a number of sulfotransferases

exhibiting strict position and flavonol specificities have been isolated from F. chloraefolia and F. bidentis (Varin and Ibrahim 1989; 1991). The biosynthesis of polysulfated flavonols in Flaveria has been shown to proceed by a sequential order of enzymatic sulfations (Varin 1990).

B.2. Cell, Tissue and Organ Distribution of Flavonoids

Flavonoids are accumulated in plants either as lipophilic aglycones associated with the waxy cuticle on the outer surface of the cell wall (Wollenweber and Jay 1988), or as hydrophilic flavonoid glycosides and proanthocyanidins in the large central vacuole (Marty and Leigh 1980; Hrazdina and Wagner 1985). However, there are some exceptions to this general rule. For example, the partially methylated, hydrophobic flavonol glucosides were mainly localized in epidermal and mesophyll walls of Chrysosplenium americanum leaves (Ibrahim et al. 1987).

Tissue distribution of flavonoids has been considered a factor in determining their functions. Flavonoid localization in the epidermal layer is seen as a characteristic of compounds involved in plant protection against UV radiation, whereas those localized in the mesophyll cells may be more suitable for a defensive role. In seedlings of Pseudotsuga menziesii, the light-induced anthocyanins were found in the epidermis only (Stafford 1965), while the constitutive defense compounds, proanthocyanidins, were detected in both the epidermal and the mesophyll cells (Stafford et al. 1989).

Organ specificity of flavonoids has not received enough attention despite the fact that it is of great importance with regard to their possible functions. It is rarely indicated in the literature which parts of the plant were used for flavonoid analysis, and when this is indicated it is not mentioned whether other parts of the plant lack these compounds.

B.3. Regulation of Flavonoid Biosynthesis

Whereas most flavonoids are considered constitutive metabolites, their synthesis is controlled by endogenous factors during plant growth and development (Dangelmayr et al. 1983; Forkmann et al. 1985; Teusch 1986). Furthermore, the synthesis of many flavonoids has been reported to be induced by various external factors (Beggs et al. 1986; Smith and Banks 1986).

B.3.1. Photocontrol of flavonoid biosynthesis

Light is considered to be an important effector of differentiation and development in higher plants. The effect of light on flavonoid biosynthesis was first reported in the late 18th century (Senebier 1799) and was later confirmed in the 19th century (Sorby 1873). More recently, attention was focused on identifying the wavebands responsible for the light effect. UV-B (280-320 nm), UV-A (320-380 nm) and blue light (> 380 nm) in addition to longer wavebands, the red (640 nm) in particular, have been found to be most effective (Eddy and Mapson 1951; Withrow et al. 1953; Piringer and Heinze 1954;

Duell-Plaff and Wellmann 1982).

The capability of different waveband light to induce flavonoid biosynthesis raised the question as to whether only one photoreceptor is responsible for their effect, or that different wavebands have different photoreceptors. Wellmann and his coworkers were able to demonstrate the presence of at least three types of photoreceptors; phytochrome (red), a UV-B receptor and a blue/UV-A receptor (Duell-Plaff and Wellmann 1982). The effect of light on the enzymes involved in flavonoid biosynthesis has been reported in a number of plant species (Table 2).

The effect of light on the enzymes of flavonoid biosynthesis was extensively studied using cell suspension cultures of parsley (Petroselinum hortense). Dark grown cultures were found to have very low, or undetectable, levels of the activities of the enzymes of flavonoid biosynthesis. Therefore, no flavonoids were detected in these cultures. However, when the cultures were exposed to a light source enriched in blue and UV light, a coordinated increase in the activities of the enzymes involved was observed (Hahlbrock et al. 1971, 1976). Enzymes of the general phenylpropanoid Table pathway (Group I enzymes) were found to reach maximal activities five hours after irradiation, whereas the activities of the flavonoid pathway enzymes (Group II enzymes) reached their maxima a few hours later. The increase in the activities of some of these enzymes was found to be due to an increase in the amount and translational activity of their

Table 2. Examples of flavonoid pathway enzymes induced by light[1].

Plant species	Flavonoid Enzymes[2]				
	PAL	C4H	4CL	CHS	CHI
Petroselinum hortense	UV R/FR	UV	UV R/FR	UV R/FR	UV R/FR
Sinapis alba	R/FR			R/FR	R/FR
Anethum graveolens	UV			UV	UV
Fagopyrum esculentum	R/FR	W			
Haplopappus gracilis	UV			UV	UV
Solanum tuberosum	W	W			
Pisum sativum	R/FR	R/FR	W		W
Brassica oleracea	W	W			

[1]UV, UV-B (280-320 nm); R/FR, induced *via* the phytochrome system; W, white light.

[2]PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate-CoA-ligase; CHS, chalcone synthase; CHI, chalcone isomerase (adapted from Beggs *et al.* 1986)

mRNAs (Schröder et al. 1979; Kreuzaler et al. 1983). Using run-off transcription experiments, it was possible to demonstrate that the increase in enzyme activities was correlated with transient increases in the transcription rates of their respective genes (Chappell and Hahlbrock 1984). It was shown, in this system, that a clear relationship exists between the changes in enzyme activities and the concentration of flavonoid glycosides following UV irradiation (Hahlbrock et al. 1976).

Both phytochrome and a blue light photoreceptor, in addition to a UV-B receptor were found to mediate the UV light effect in parsley cell suspension cultures (Duell-Plaff and Wellmann 1982). So far, little is known on how these photoreceptors can affect gene transcription. In parsley, chalcone synthase occurs as only one copy gene (Hermann et al. 1988), which makes it a suitable candidate for "in vivo footprinting" to identify light responsive cis-acting elements, some of which have recently been detected (Schulze-Lefert et al. 1989). Their requirement for UV induction of CHS gene was confirmed by in vitro mutagenesis and transient expression in transformed parsley protoplasts (Schulze-Lefert et al. 1989). One of these elements is conserved in other genes involved in photosynthesis (the small subunit of ribulose bisphosphate carboxylase and the chlorophyll a/b binding protein), as well as other stress responses (Castresana et al. 1988; Guiliano et al. 1988; Schulze-Lefert et al. 1989), which suggests the widespread occurrence of

similar transcription activation mechanisms.

B.3.3. Regulation of flavonoid biosynthesis by stress

Flavonoid biosynthesis can be affected by a variety of stress conditions including both biotic and abiotic elicitors. In this section microbial infection or treatment with biotic elicitors will be briefly reviewed (Table 3).

Cell suspension cultures of parsley (Petroselinum hortense) were thoroughly investigated following treatment with a heat-released elicitor from the cell walls of the fungus Phytophthora megasperma. As shown in Table 3, this treatment caused an increase in the activities of a number of enzymes of flavonoid biosynthesis (Doke et al. 1979; Kuhn et al. 1984). A clear correspondence between the levels of the enzyme activities and the rates of transcription of their respective genes was observed (Chappell and Hahlbrock 1984), which is in accordance with the light effect. Chalcone synthase was further investigated at the gene level. The promoter of CHS was found to contain cis-acting elements responsible for the induction of CHS in response to fungal invasion in bean (Schmid et al. 1990).

B.3.3. Developmental regulation of flavonoid biosynthesis

In addition to light and stress, flavonoid biosynthesis is subject to genetically programmed, developmentally regulated control. The activities of some enzymes of flavonoid biosynthesis have been reported to appear only at certain developmental stages. A number of developmentally regulated

Table 3. Examples of flavonoid biosynthetic enzymes induced by microbial infection or treatment with elicitors.

Enzymes induced[1]	Species and tissue	Pathogen or elicitor[2]	Refs[3].
PAL	Phaseolus vulgaris hypocotyls	Colletotrichum	1, 2, 3
	Phaseolus vulgaris culture	elicitor	4, 5
	Pisum sativum endocarp	elicitor	6
	Glycine max culture	elicitor	7
	Glycine max hypocotyls	Phytophthora	8
	Petroselinum hortense culture	elicitor	9 ,10,11
	Medicago sativa culture	elicitor	12
	Arachis hypogaea culture	elicitor	13
	Arabidopsis thaliana culture	elicitor	14
4CL	Petroselinum hortense culture	elicitor	9, 10, 11
	Medicago sativa culture	elicitor	12
	Arabidopsis thaliana culture	elicitor	14
CAH	Medicago sativa culture	elicitor	12
	Arachis hypogaea culture	elicitor	13
CHS	Phaseolus vulgaris hypocotyls	Colletotrichum	1 ,4
	Phaseolus vulgaris culture	elicitor	1 ,15
	Petroselinum hortense culture	elicitor	9
	Glycine max culture	elicitor	7
	Glycine max hypocotyls	Phytophthora	8
	Medicago sativa culture	elicitor	12
	Sorghum bicolor mesocotyls	Colletotrichum	16
CHI	Phaseolus vulgaris hypocotyls	Colletotrichum	4
	Phaseolus vulgaris culture	elicitor	4
	Midicago sativa culture	elicitor	12
IGT	Glycine max leaves	elicitor	17

[1]PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA-ligase; CHS, chalcone synthase; CHI, chalcone isomerase; IGT, isoflavone 7-O-glucosyltransferase.

[2]Sources and types of elicitors are reported in respective references.

[3]Numbers correspond to the following references.

1. Bell et al. 1984
2. Cramer et al. 1985a
3. Cramer et al. 1985b
4. Bolwell et al. 1985
5. Lawton et al. 1983
6. Loschke et al. 1983
7. Ebel 1984
8. Esnault et al. 1987
9. Chappell and Hahlbrock 1984
10. Doke et al. 1979
11. Kuhn et al. 1984
12. Dalkin et al. 1990
13. Steffens et al. 1989
14. Davis and Asubel 1989
15. Ryder et al. 1984
16. Lue et al. 1989
17. Cosio et al. 1985

enzymes are shown in Table 4.

Most of the early work on developmental regulation was concerned with the effect of growth hormones on flavonoid biosynthesis in plant cell cultures. Gibberellic acid (GA_3) has been reported to inhibit the synthesis of petunidin 3-glucoside (an anthocyanin) and other flavonoids in cultures of intact Spirodela oligorhiza (Furuya and Thimann 1964), as well as anthocyanin synthesis in cell suspension cultures of Daucus carota at the level of chalcone synthase (Hinderer et al. 1984). The auxin, 2,4-D, inhibited anthocyanin accumulation and mRNA synthesis for chalcone synthase in carrot cell suspension cultures (Ozeki and Komamine 1985).

The developmental regulation of flavonoid biosynthesis in intact plants can be exemplified by the biosynthesis of anthocyanins in Matthiola incana buds. The activities of five enzymes involved in anthocyanin biosynthesis reached their highest levels at the early stages of bud development and decreased thereafter. Anthocyanin accumulation increased in the early stages of development and their high levels continued even when the enzyme activities were negligible, which can well be explained by the slow rate of anthocyanin degradation (Dangelmayr et al. 1983).

As in the case with light and stress regulations, CHS was the most studied developmentally regulated enzyme. Analysis of the bean chalcone synthase promoter showed the presence of cis-acting elements involved in genetic control of tissue- and cell type-specific biosynthesis of flavonoids during

Table 4. Developmentally regulated enzymes of flavonoid biosynthesis

Enzymes	Plant species and tissue	Refs[1].
Flavonol synthase	Petunia hybrida flowers and buds	1
	Matthiola incana flowers and buds	2
Flavonol 3-O-glucosyl-transferase	Matthiola incana flowers	3
Isovitexin galactosyl-transferase	Silene pratensis cotyledons and rosette leaves	4
Chalcone synthase	Matthiola incana flowers and buds	
Chalcone isomerase		
Flavanone 3-hydroxylase		
Flavonoid 3'-hydroxylase		
Flavonoid 3-O-glucosyl-transferase		5
Anthocyanidin 3-O-glucosyl-xylosyltransferase	Matthiola incana petals	6
Anthocyanin 5-O-glucosyl-transferase	Matthiola incana flowers	7

[1]Numbers correspond to the following references

1. Forkmann et al. 1985
2. Spribille and Forkmann 1984
3. Teusch et al. 1986a
4. van Brederode and Steyns 1983
5. Dangelmayr et al. 1983
6. Teusch 1986
7. Teusch et al. 1986b

development (Schmid et al. 1990).

B.4. Significance of flavonoid compounds

The significance of flavonoids has long been a subject for speculations. A number of functions has been suggested for flavonoid compounds, but only a few have been demonstrated experimentally. Flavonoids may act internally in the plant as defense compounds against microbial attack (phytoalexins), or externally as attractants of pollinators or as antifeedants. Their endogenous functions have been the most controversial because they are dependent on the intracellular localization of flavonoids, which makes it difficult to assess their functions in vivo. Some of the better documented functions of flavonoids are described below.

B.4.1. Chemical defenses against environmental stresses

B.4.1.1. UV protectants

UV spectral analysis indicates that all flavonoids, except anthocyanins, strongly absorb light in the UV range. This fact, in addition to the finding that flavonoids are most abundant in the epidermal layers (Hrazdina et al. 1982), have supported the view that one of the raisons d'être of flavonoids is to protect the plant against harmful UV radiation. A more direct evidence supporting this hypothesis derives from the fact that enzymes of flavonoid biosynthesis are induced by UV light (see Section B.3.1.). However, it is not clear why anthocyanin biosynthesis is induced by UV light when they do not absorb in the UV range.

B.4.1.2. Antimicrobial defenses

Flavonoids have been shown to fulfil this function either as pre-infectional, constitutively formed metabolites, or as de novo synthesized compounds in response to microbial invasion.

a. Constitutive defense

Certain constitutively accumulated flavonoids have the potential to act as fungitoxic substances. In fact, the germination of Gloeosporium perennans was found to be inhibited by the anthocyanidin, cyanidin, (Hulme and Edney 1960). Studies of white lupin, Lupinus albus, have shown that two fungitoxic compounds, wighteone (6-dimethylallyl 5,7,4'-trihydroxyisoflavone) and its 2'-hydroxy derivative, luteone, were present on the leaf surface and within the leaf tissues. The fungitoxicity and noninducibility of these compounds were demonstrated (Harborne et al. 1976). In addition, some methylated flavonoids have been implicated in the resistance of citrus leaves to fungal attack. These substances were found to be highly fungitoxic and to accumulate in the leaves in amounts sufficient to deter any fungal attack (Piattelli and Impellizzeri 1971). Pre-infectional flavonoid fungitoxins, most probably, act on spore germination and/or germ tube development. Detailed information on flavonoids as pre-infectional agents in disease resistance can be found in a recent review by Harborne (1987).

b. Inducible defense

Compounds formed de novo in response to fungal invasion are called phytoalexins. To date, nearly 200 phytoalexins have been characterized (Ingham 1983; Harborne 1986). About half of these are isoflavones, isoflavanones, pterocarpans and isoflavans. They have been isolated mostly from different species of the Leguminosae. Other less frequent flavonoid phytoalexins include flavans, flavanones and flavonols (Kemp and Burden 1986; Smith and Banks 1986).

In the Leguminosae, an apparent relationship exists between the structure of flavonoid phytoalexins and their fungitoxicity. Different classes of isoflavonoids exhibit an increasing fungitoxicity in the following order: isoflavanes> isoflavanones >pterocarpans >isoflavones (Van Etten 1976). Another structure-activity relationship is that in which O-methylation, isoprenylation or methylenedioxygenation of the isoflavonoid ring system tend to increase toxicity (Harborne 1988b). Flavonoid phytoalexins are generally localized in the necrotic area initiated by and surrounding the site of pathogen contact with the host tissue. This localized cell death tends to isolate the pathogen from the host's nutrients and leads to the release of various toxic compounds, particularly phenolics, from the dying plant cells (Stafford 1990).

B.4.2. Chemical signals in plant-microbe interactions

The symbiosis between bacteria of the genus Rhizobium and their legume host plants results in the formation of nitrogen-

fixing root nodules, a process that requires the induction of nodulation genes. These genes are organized into several transcriptional units: nodABC, nodFE and nodD. NodD is a positively acting regulatory gene. The induction of the other two operons necessitates the nodD gene product and some other compounds exuded from the host plant roots. Interestingly, these compounds have recently been shown to be flavonoids. The latter include flavanones, flavones, isoflavones and chalcones (Firmin et al. 1986; Peters et al. 1986; Sadowsky et al. 1988; Hartwing et al. 1989; Maxwell et al. 1989). However, not all flavonoids induce nodulation. In fact, the isoflavones, daidzein, was reported to inhibit the induction by flavanones and flavones in R. leguminosarum (Firmin et al. 1986), although it induced the nod genes in the slow-growing Bradyrhizobium japonicum (Kosslak et al. 1987; Gottfert et al. 1988). The levels of flavone nodulation signal are considered to be the rate limiting factor in nitrogen fixation and seedling growth in alfalfa (Kapulnik et al. 1987).

B.4.3. Compounds of pharmacological significance

In addition to their roles in plants, flavonoids represent a considerable portion of dietary substances that can have effects upon humans and animals. In a recent review on the pharmacological significance of flavonoids (Cody et al. 1988) certain flavonoids were reported as antiallergic, antiviral, antiinflammatory and anticarcinogenic agents. In fact, flavonoid compounds have been suggested to be behind the value of most medicinal plants.

The most obvious flavonoid acting as a medicinal agent is rutin (quercetin 3-rutinoside, vitamin P), which is still used around the world as a hypotensive and antiinflammatory agent. Recently, quercetin, a common flavonol, was reported to inhibit the cell growth of leukaemia cells and Ehrich ascites tumor cells (Suolinna et al. 1975).

B.5. Flavonoid Sulfates

In the most recent review on flavonoid sulfates (Barron et al. 1988), 101 such compounds have been listed. They are mostly sulfate esters of common hydroxyflavones and hydroxyflavonols or their methyl ethers and, to a lesser extent, of their glycosylated derivatives. Fifty six of the reported flavonoid sulfates were flavonols, and the remaining were flavones. The sulfate group, in these conjugates, was found to be attached directly to a hydroxyl group on the flavonoid aglycone or, in the case of glycosylated flavonoids, either to the flavonoid ring or the glycosyl moiety. In flavonols, sulfation may take place at positions 3, 7 and, to a lesser extent, positions 3' and 4'. Mono-, di-, tri- and tetrasulfated flavonols have been reported. Flavones, too, can be sulfated at position 7 and unlike flavonols, sulfation at positions 3' and 4' is not uncommon. In addition, flavones sulfated at positions 6 and 8 have also been reported. Highly sulfated flavones are very rare.

B.5.1. Enzymatic Synthesis

Varin et al. (1987) were able to demonstrate the long-

time postulated enzymatic sulfation of flavonoids, and speculated on the presence of a family of flavonol-specific sulfotransferases (STs). These enzymes use 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as their natural sulfate donor (co-substrate). The physiological concentraion of PAPS in the plant tissues is not known. However, the four position-specific STs that have been isolated from F. chloraefolia and F. bidentis showed Km values (ca. 0.3 μM) for both the sulfate donor, PAPS, and their respective flavonol acceptors (Varin 1990). The first enzyme in the sequence of polysulfation, the 3-ST, has been purified to apparent homogeneity and antibodies to this enzyme were produced and characterized (Varin 1990).

Sulfation of flavonoids was considered a later step in the biosynthesis of flavonoids based on the observation that cell-free extracts of Flaveria spp. catalysed the sulfation of several flavonols (Varin et al. 1987a).

B.5.2. Distribution

Flavonoid sulfates have so far been reported in 250 species belonging to 17 dicotyledonous and 15 monocotyledonous families (Barron et al. 1988). Overall, these families are herbaceous and/or morphologically advanced. It is very difficult to make any generalization on the systematic distribution of these compounds since not all species within a given family accumulate flavonoid sulfates. Even within the species of the same genus, only some of them have the capability to synthesize such compounds. Moreover, these families are taxonomically distant, which led to the

conclusion that the presence/absence of flavonoid sulfates has no systematic significance.

B.5.3. Significance of flavonoid sulfates

The frequent occurrence of flavonoid sulfates in plant families known to inhabit aquatic, saline and gypseous habitats led to the speculation that these compounds may represent an environmental adaptation (Harborne 1975). However, this view should be regarded with caution since not all plants growing in these habitats accumulate flavonoid sulfates. Sulfation seems to represent a detoxification mechanism by which water solubility is provided to otherwise insoluble compounds. Although not yet investigated, flavonoid sulfates may be driven into the vacuole, so that they do not interfere with essential enzymatic reactions. Sulfation of flavonoids may also play a role in salt uptake and the transfer of inorganic sulfate into organic forms. The presence of higher amounts of flavonoid sulfates in seedlings when compared to mature plants of Flaveria bidentis (Varin et al. 1986) raised the possibility of their involvement in more dynamic roles such as plant growth and development. Further studies on their distribution in plant tissues and cellular localization may contribute to a better understanding of their roles in plants.

Overall, the significance of flavonoid sulfates, as the case with other flavonoids, is still a matter for speculation.

B.5.4. Regulation of the biosynthesis of flavonoid sulfates

Except for two preliminary reports (Nissen and Benson 1964; Varin et al. 1986) no information is available on how the biosynthesis of flavonoid sulfates is regulated. The recent discovery of a number of flavonol sulfotransferases and the cloning of the genes coding for two of these enzymes (Varin 1990) should allow studies of the regulation of the enzymatic synthesis of these conjugates. However, no work has yet been directed towards this area of research.

C. AIM OF THE WORK

In order to determine the raison d'être of flavonoid sulfates, certain aspects of their biosynthesis and accumulation in plant tissues must be considered. Such aspects include the regulation of their biosynthesis, the site of their accumulation within plant tissues and more importantly, their distribution in different species of Flaveria in order to define their potential as taxonomic markers. Therefore, this research project includes three main parts.

1. To study the spatial distribution of flavonoid conjugates, both glucosides and sulfates, and their respective enzymes, glucosyltransferase and sulfotransferase, in the shoots of F. bidentis. The organ specificity of these conjugates is interesting, particularly with regard to their possible functions.
2. The distribution of flavonoid sulfates, with special reference to the level of sulfation was determined in five species of Flaveria which exhibit different photosynthetic characteristics (C_3, C_4 and C_3-C_4 intermediates) in order to find out whether the flavonoid pattern is determined by the photosynthetic behaviour of these plants.
3. Flavonoid sulfation, like most other enzymatic steps of flavonoid biosynthesis, is expected to be regulated by light. Therefore, the effect of light on the biosynthesis of flavonoid sulfates was investigated in F. bidentis.

The choice of F. bidentis, in this study, was dictated by

the fact that the flavonoid pattern of this plant is well characterized (Varin et al. 1986). Moreover, this species accumulates two groups of flavonoid conjugates, sulfated and glucosylated flavonoids. In addition, each group of conjugates involves different flavonoid aglycones. Furthermore, this species accumulates flavonoids of different sulfation levels ranging from mono- to tetrasulfates. These remarkable characteristics make this plant a suitable material for the investigation of the above mentioned aspects.

D. MATERIALS AND METHODS

D.1. Plant Material

Seeds of Flaveria bidentis var angustifolia O.K. (Asteraceae) were kindly provided by Dr. H.R. Juliani, University of Cordoba, Argentina. Seeds of F. chloreafolia A. Gray (Asteraceae) were a generous gift from Dr. A.M. Powell, Sul Ross State University, Alpine, Texas. Seeds of both species were germinated in vermiculite on top of potting soil. Explants of other Flaveria spp. F. brownii, F. floridana, F. pringlei, F. linearis and F. trinervia were obtained from Dr. H. Brown, University of Georgia, Athens, GA. All species were propagated by cuttings and their growth was maintained in the light under greenhouse conditions.

D.2. Chemicals

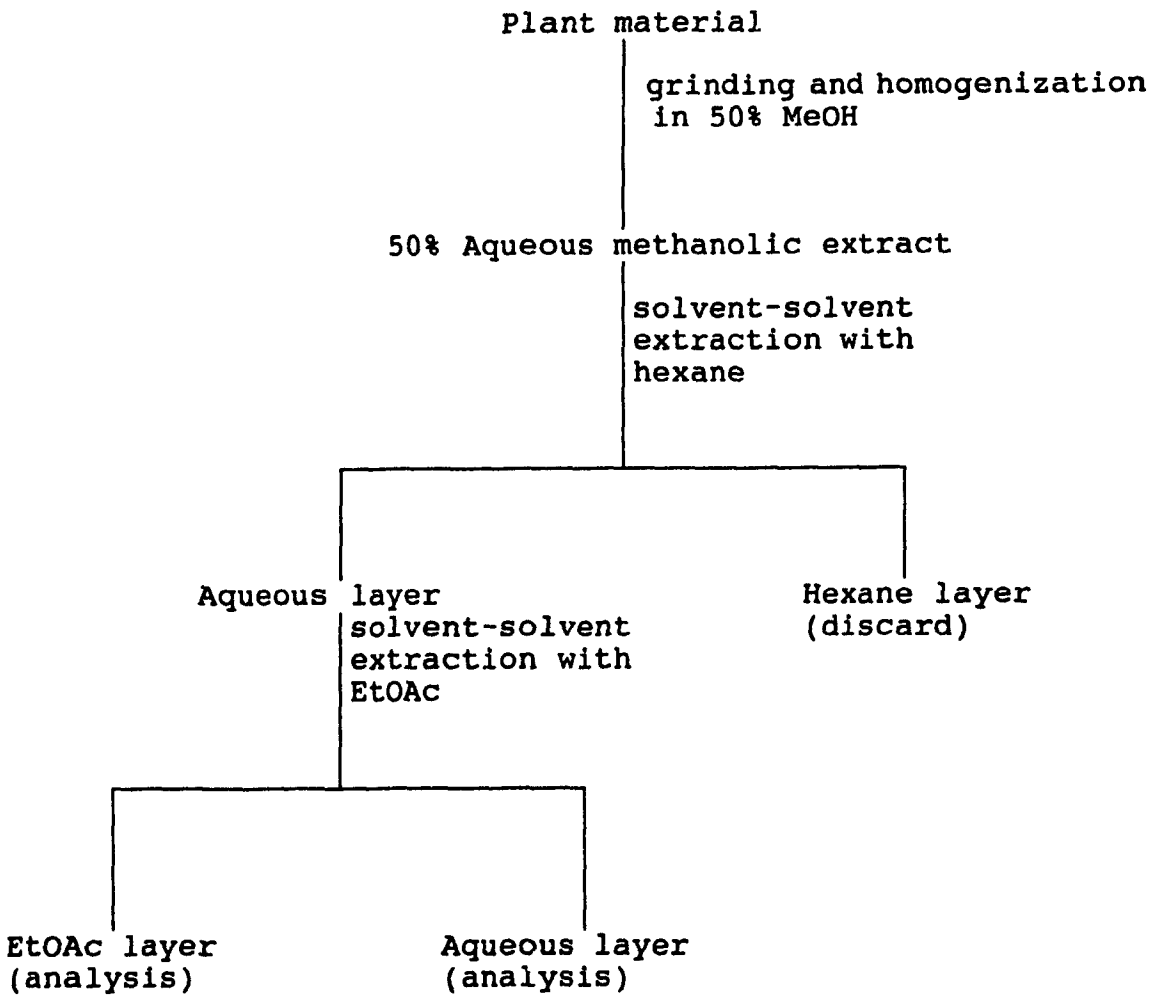
3'-Phosphoadenosine 5'-phospho-[^{35}S]sulfate (PAPS) (1.57 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Uridine 5'-diphospho-[U-^{14}C]glucose (UDPG) (205 mCi/mmol) was from ICN Biomedicals, Inc. (Costa Mesa, CA). TBADP was from Aldrich Chemical Co. (Milwaukee, WI). Sephadex G-25 (PD-10 columns) was purchased from Sigma Chemical Co. (St. Louis, MO). SDS-PAGE, immunoblotting equipment and the protein dye reagent were purchased from Bio-Rad laboratories (Richmond, CA). Flavonoid aglycones were from Roth (Karlsruhe, FRG) or Extrarsynthèse (Bordeaux, France). Flavonoid sulfate esters were synthesized by Barron (1987) and flavonoid glycosides were from our laboratory collection.

D.3. Flavonoid Extraction and Purification

Fresh plant material (ca. 50 g) was frozen in liquid nitrogen, ground to a fine powder and homogenized in 50% MeOH (3/1; v/w) and allowed to stand at room temperature for 24 h, then extracted three times with 50% MeOH. The combined extracts were concentrated under reduced pressure at 30°C, until the volume reached ca. 1/3 of the initial volume. The concentrated extract was then subjected to solvent-solvent extraction with hexane to remove lipids and chlorophyll pigments. After discarding the hexane layer, the aqueous layer was subjected to another solvent-solvent extraction with EtOAc. The organic layer, which contains the flavonoid aglycones, glycosides and monosulfate esters, was evaporated to dryness in a fume hood. The aqueous layer, which contains most flavonoid sulfates, was concentrated to a minimum volume under reduced pressure (Scheme 3). Both the EtOAc and aqueous extracts were redissolved in a minimum volume of 50% MeOH and subjected to TLC analysis using cellulose as a support and BAW (3:1:1; v/v/v) and H_2O as solvent systems. Individual bands were scrapped off the TLC plates and eluted with 50% MeOH, centrifuged and the supernatant was concentrated to a minimum volume. Chromatographically pure compounds were then used for further analysis.

D.4. Flavonoid Identification

Identification of individual flavonoids was carried out by determining their UV spectra, co-chromatography with reference compounds (when available) on cellulose TLC plates



Scheme 3. Extraction of flavonoid sulfates from Flaveria species

using BAW (3:1:1; v/v/v) and H_2O as solvent systems and visualization under UV light (360 nm). Identification of the aglycones was carried out after hydrolysis with 2N HCl at 95°C for 10 min followed by UV spectral analysis and co-chromatography with reference compounds on polyamide DC 6.6 TLC plates, using benzene-MEK-MeOH (3:1:1; v/v/v) as a solvent system. The sulfation level was estimated on the basis of R_f values and by comparing their electrophoretic mobility on Whatman No. 3 chromatographic paper in formic acid-HOAc-H_2O (43:147:1820; v/v/v) mixture, pH 2.2 at 250 Volts and 8 mA for three hours.

D.5. Flavonoid Quantification

Aqueous methanolic extracts (1:1; v/v) of the plant material were analysed for their total flavonoid content by determining their absorbance at 340 nm. Sulfated and glucosylated flavonols were quantified by HPLC. Aliquots of the methanolic extracts were injected into an HPLC using a Microbondapak C_{18} column (particle size, 10 μm). Best separation of the flavonoid conjugates was obtained by isocratic elution with 60% solvent A (10 mM aqueous TBADP) and 40% solvent B (MeOH-HOAc-H_2O, 18:1:1; v/v/v) for 10 min proceeding to 50% A and 50% B in 40 min, and finally to 40% A and 60% B in 10 min, at a flow rate of 1 ml/min. Peaks were integrated and the amount of each group of conjugates was calculated from the total absorbance at 340 nm using an average molar extinction coefficient of 20,000.

D.6. Protein Extraction

Two buffers were used for protein extraction. Buffer A, 0.2 M Tris-HCl, pH 7.5 containing 14 mM 2-mercaptoethanol, 5 mM EDTA and 10 mM DIECA, and buffer B, 50 mM Tris-HCl, pH 7.5 containing 14 mM 2-mercaptoethanol. All extraction procedures were carried out at 4°C unless stated otherwise.

D.6.1. Protein extraction from intact tissues

The plant material was frozen in liquid nitrogen, ground to a fine powder, homogenized in buffer A (1:4; w/v) and filtered through nylon mesh. The filtrate was centrifuged at 27,000 g for 20 min and the supernatant was applied to a G-25 column and the protein was eluted from the column using buffer B. The protein eluates were used as the enzyme source.

D.6.2. Protein extraction from cell cultures

Cultures were harvested at the appropriate time by filtering the cells through filter paper. The following steps were carried out at 4°C. The cells were resuspended in buffer A, and sonicated for three, five-second periods. The homogenate was then centrifuged for two min at 3200 rpm in an Eppendorf centrifuge, and the supernatant was used as the enzyme source.

D.7. Enzyme Assays

D.7.1. Sulfotransferase

Sulfotransferase was assayed as described in (Varin et al. 1987b). The standard assay mixture consisted of 1 μM of

the flavonoid substrate (quercetin), [^{35}S]PAPS (1 μM) containing 220,000 dpm and up to 60 μg protein in buffer B, in a total volume of 100 μl. The reaction was started by the addition of enzyme protein, and was incubated at 30°C for 10 min. The reaction was terminated by the successive addition of 20 μl of 2.5% HOAc and 20 μl of 0.1 M TBADP. Extraction of the reaction products was carried out by the addition of 250 μl of EtOAc, and the mixture was agitated for 2 min on an Eppendorf shaker. The organic layer was separated from the aqueous layer by centrifugation at 3200 rpm for 2 min in an Eppendorf centrifuge. To determine the total sulfating activity of the protein extract, 100 μl of the EtOAc extract was counted for radioactivity in a toluene-based scintillation fluid. The remaining EtOAc extract was concentrated in the fumehood and used for the identification of the reaction products. It was chromatographed on cellulose TLC using BAW (3:1:1; v/v/v) as a solvent. Developed chromatograms were visualized in UV light (360 nm) and then autoradiographed on X-ray film.

D.7.2. Glucosyltransferase

Glucosyltransferase was assayed according to (Bajaj et al. 1983). The assay mixture contained 1.5 μM of the flavonoid substrate (quercetin) and 1.5 μM UDP-[U-C^{14}]glucose and up to 60 μg of protein. The mixture was made up to 100 μl by adding buffer B, and was incubated at 30°C for 30 min. The reaction was terminated by the addition of 10 μl of 6 N HCl and 250 μl of EtOAc. The mixture was shaken for 1 min and centrifuged for

2 min at 3200 rpm. An aliquot of the EtOAc extract (ca. 100 μl) was counted for radioactivity. Product identification was carried out as described for ST (Section C.7.1).

D.8. Protein Estimation

The method of Bradford (Bradford 1976) was used to estimate the protein concentration, using Bio-Rad reagent and bovine serum albumin as the standard protein.

D9. Antibody Production

Polyclonal antibodies to the homogeneous 3-ST of F. chloraefolia were produced in rabbits (Varin 1990) following a standard immunization tec ue (Lamoureux 1986). The serum was separated from red blood cells by clotting and centrifugation at 10,000 xg. The serum titer was verified by ELISA which could detect the 3-ST antigen below levels of 20 ng/microtiter plate.

D.10. SDS-Polyacrylamide Gel Electrophoresis

Protein extract (ca. 50 μg) was taken to dryness in a vacuum speed evaporator. Dry samples were then dissolved in the substrate buffer and analysed by SDS-PAGE using 12% acrylamide gels according to the method of Laemmli (1970). Prestained standard proteins were used as molecular weight markers. These consisted of lysozyme (M_r 16), soybean trypsin inhibitor (M_r 24), carbonic anhydrase (M_r 33), ovalbumin (M_r 47), bovine serum albumin (M_r 84) and phosphorylase B (M_r 110).

D.11. Immunoblotting

After SDS-PAGE, the protocol described in the Bio-Rad Minitransfer blot apparatus was followed in order to electrotransfer the proteins onto nitrocellulose membranes. Briefly, after completion of electrophoresis, the gels were equilibrated for five min in the transfer buffer (25 mM Tris HCl and 192 mM glycine in 20% MeOH, pH 8.3), then the transblotting sandwich was assembled and the proteins were electrotransferred onto the membranes at 90 volt for 60 min. Blots were developed with either anti-3-ST (1:2000), or nonimmune serum (1:2000) as primary antibody, and with alkaline phosphatase conjugated anti-rabbit antibody as secondary antibody, and nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate as substrate. Blotting was carried out using the Bio-Rad immunodetection kit. Blots were stained for total protein by incubation in 0.1% amidoblack in 10% methanol and 2% acetic acid.

D.12. Initiation of Cell Cultures of F. bidentis

Young leaves taken from one month-old seedlings of F. bidentis were surface-sterilized with 10% Clorax for 15-20 min. The leaves were then thoroughly washed with sterile water and blotted on sterile filter paper. The following steps were carried out under sterile conditions in a laminar flowhood. Using a sterile cork borer, disks of 0.5 cm diameter were taken from the leaves and placed on MS semi-solid medium (Murashige and Skoog 1962) containing 0.1 ppm kinetin and 1

ppm 2,4-dichlorophenoxyacetic acid (2,4-D). The medium was solidified with 0.7% agar, and its pH was adjusted to 5.6-5.8. Aliquots were transferred into glass bottles and autoclaved for 20 min at 15 psi. The tissue explants were left on the agar media in the light or in the dark at room temperature until good callus formation had developed. Suspension cultures were initiated by transferring the callus tissue to one-liter nipple flasks containing 250 ml of MS liquid medium. The flasks were allowed to rotate centripetally around an axle connected by a universal joint to a variable-gear motor at 55 rpm in light or dark. About two g of callus were transferred into fresh medium at 15-day intervals, until a homogeneous cell suspension culture was obtained. Subculture was continuously carried out every two weeks.

D.13. Light Irradiation Studies

Light was provided from two 122-cm-long fluorescent light tubes with peak energy at 313 nm (UV-B), each having an irradiance of 0.8 ($W/m^2/nm$) at the indicated wavelength and a total irradiance of 31.62 ($W/m^2/nm$). These tubes were from Q-Panel Company (Cleveland, OH).

D.13.1. Irradiation of plant seedlings

One-month-old seedlings of F. bidentis, grown under greenhouse conditions (Figure 1), were incubated in the dark for 48 h prior to irradiation. During the irradiation period, the light source was positioned 25 cm from the top of seedlings. Irradiation was performed for one h, after which

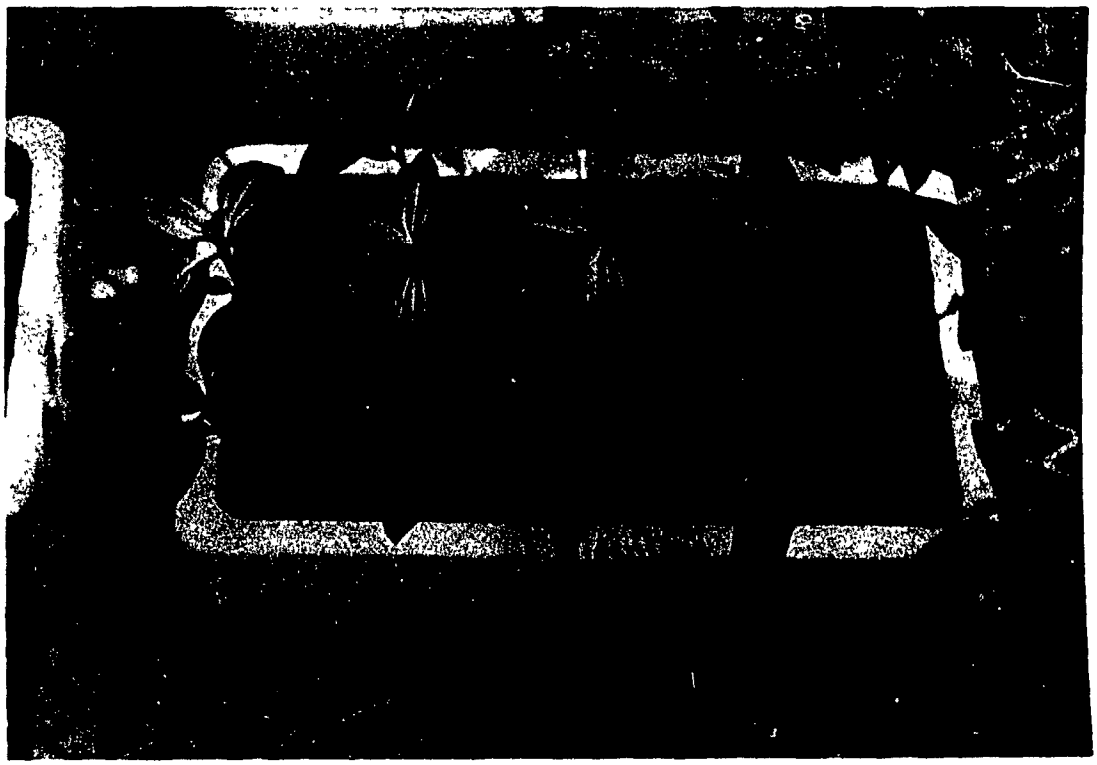


Figure 1. A photograph of one-month-old seedlings of F. bidentis grown under greenhouse conditions

the seedlings were transferred to the dark. Three replicate samples were harvested at the indicated times, frozen in liquid nitrogen and stored at -80°C until they were used for analysis.

D.13.2. Irradiation of cell suspension cultures

Stock cultures were grown in one-litre nipple flasks (Figure 2) in the dark. Three-ml aliquots of two-week-old cultures (ca. 0.5 g F. W.) were transferred to 150-ml flasks containing 20 ml of MS medium. Flasks were agitated on a rotary shaker at 100 rpm in the dark at room temperature. Eight days after inoculation, cells were transferred to glass petri dishes, and irradiated under continuous shaking for one h at a distance of 25 cm. After irradiation, the samples were transferred to the dark. Three replicates were harvested at the appropriate times by filtration through filter paper (Whatman No. 1), quickly frozen in liquid nitrogen and kept at -80°C until they were used for protein extraction.

D.13.3. Treatment with actinomycin-D

Cells were prepared as described above (Section D.13.2). Actinomycin-D was dissolved in 50% DMSO. Cells were treated one h prior to irradiation with 100 µl of 50% DMSO containing actinomycin-D at a final con- -ration of 25 µM. Two controls were used, one consisted of cells treated with 100 µl DMSO during irradiation and the other, of cells treated with 100 µl DMSO in the dark.

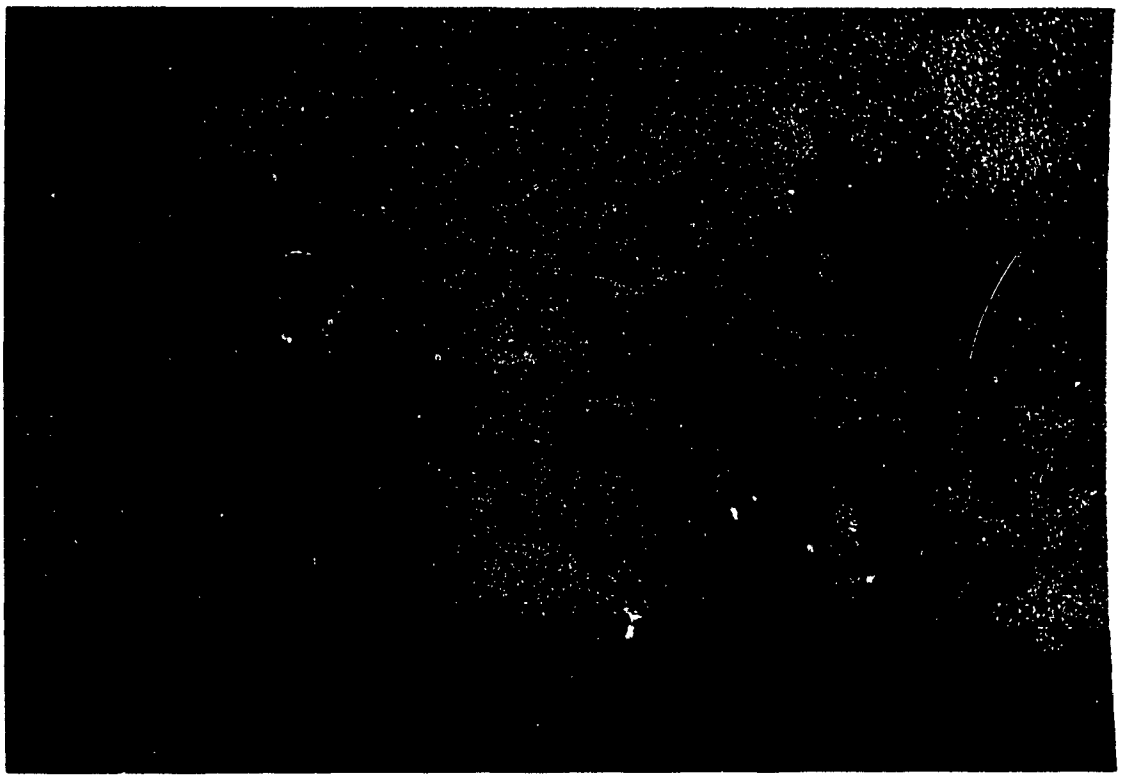


Figure 2. A photograph of cell suspension cultures of F. bidentis in one-liter nipple flasks, fine cells (left) and clumps (right).

E. RESULTS

E.1. Specificity of Anti-3-sulfotransferase Antibody

The specificity of the antibody was verified by Western blot analysis of the partially purified (Fig. 3A, C) and homogeneous (Fig. 3B, D) enzyme preparations of F. chloraefolia, indicating monospecificity of the antibody to the 3-ST protein. However, the appearence of a number of minor immunoreactive bands, other than the 3-ST in protein extracts of F. bidentis (Figs. 6B, 8B and 9B) could represent unrelated proteins which possess antigenic determinants that are recognized by the anti-3-ST antibody.

E.2. Identification of The Reaction Products of Sulfotransferase and Glucosyltransferase of F. bidentis

An aliquot of the ethyl acetate layer of the enzyme reaction product (using quercetin as a substrate) was concentrated in the fumehood and applied to cellulose TLC plates. The plates were developed with BAW (3:1:1; v/v/v) and visualized under UV light (360 nm). Only one spot of R_f value identical to that of quercetin 3-glucoside (0.58) appeared as the GT reaction product. In the case of ST, a compound migrating at the level of quercetin 3-sulfate was detected as the reaction product (Figure 4).

Figure 3. Western blot analysis of partially purified (A,C) and homogeneous (B,D) 3-ST of F. chloraefolia. Protein was electrotransferred to nitrocellulose membrane as described in the Methods section. Lanes A, and B were stained with amido black for protein detection. Lanes C and D were immunodetected with the anti-3-ST antibodies as described in the Methods section. Numbers on the left correspond to molecular weight markers. The immunostained 3-ST migrates at M_r 35.

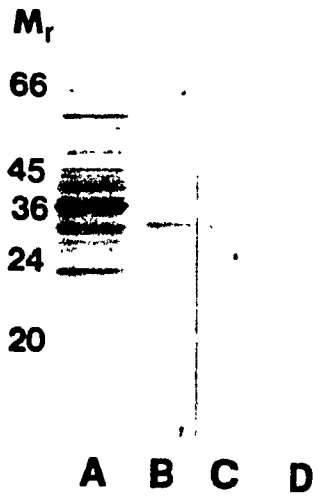


Figure 3.

Figure 4. A photograph of an autoradiogram of the chromatographed reaction products of sulfotransferase and glucosyltransferase using G-25 eluates as the enzyme source. A, reaction product of GT (quercetin 3-glucoside); B, reaction product of ST (quercetin 3-sulfate); O, origin.

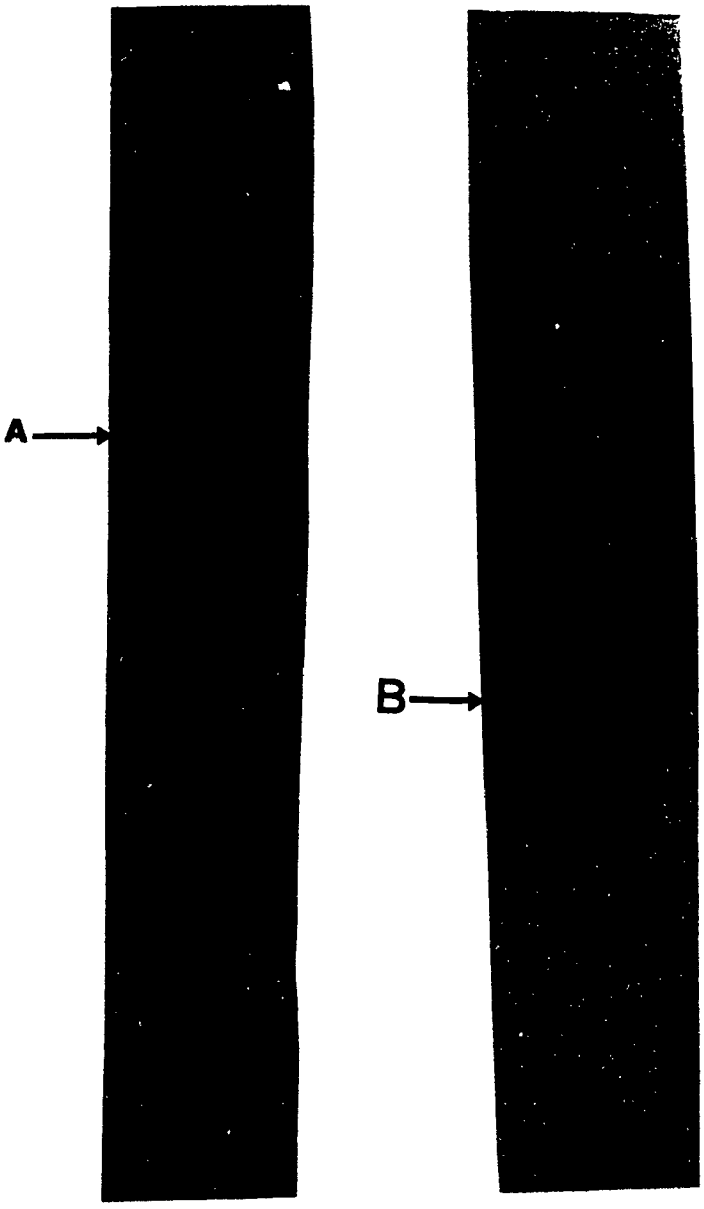


Figure 4.

E.3. Spatial Distribution of The Flavonoid Conjugates and The Latter Enzymes Involved in Their Biosynthesis

E.3.1. Distribution of sulfated and glucosylated flavonoids and their enzymes in leaves along the shoot axis

Leaves taken from different positions along the shoot axis were extracted for enzyme proteins and flavonoids as described in the Methods section. Whereas there were no qualitative variations in the patterns of both groups of flavonoids based on HPLC analysis, there were striking quantitative differences among the different leaves analysed. The highest amounts of flavonoid conjugates (μmol/gram fresh weight) were detected in young tissues (terminal bud and the first pair of leaves), which dropped to almost 10% in the older leaves (Figs. 5, 6A). When either of the flavonoid conjugates was expressed as percentage of total flavonoids, there were marked differences observed between sulfated and glucosylated flavonoids. In contrast to flavonoid sulfates which exhibited a gradual increase with tissue age, the ratio of flavonoid glucosides (as percentage of total flavonoids) was highest in the terminal bud and the first leaf pair, and gradually decreased towards older leaves (Fig. 7).

The proteins extracted from different leaves were assayed for sulfotransferase (ST) and glucosyltransferase (GT) activities. The highest activities of both enzymes were also detected in the terminal bud and declined in expanded leaves (Figs. 5, 6A). The decline in enzyme activity with leaf age

Figure 5. Levels of glucosyltransferase (GT) activity and glucosylated flavonoids (GF) along the shoot axis. GT was assayed as described in the Methods section. For flavonoid quantification, 50% aqueous methanolic extracts of the terminal bud (TB) and lower leaves (A-D) were analysed by HPLC as described in the Methods section. Peaks were integrated and the amounts of flavonoid conjugates were calculated from the total absorbance at 340 nm using an average molar extinction coefficient of 20,000.

Values in figures 5 to 14 are the averages of at least three determinations.

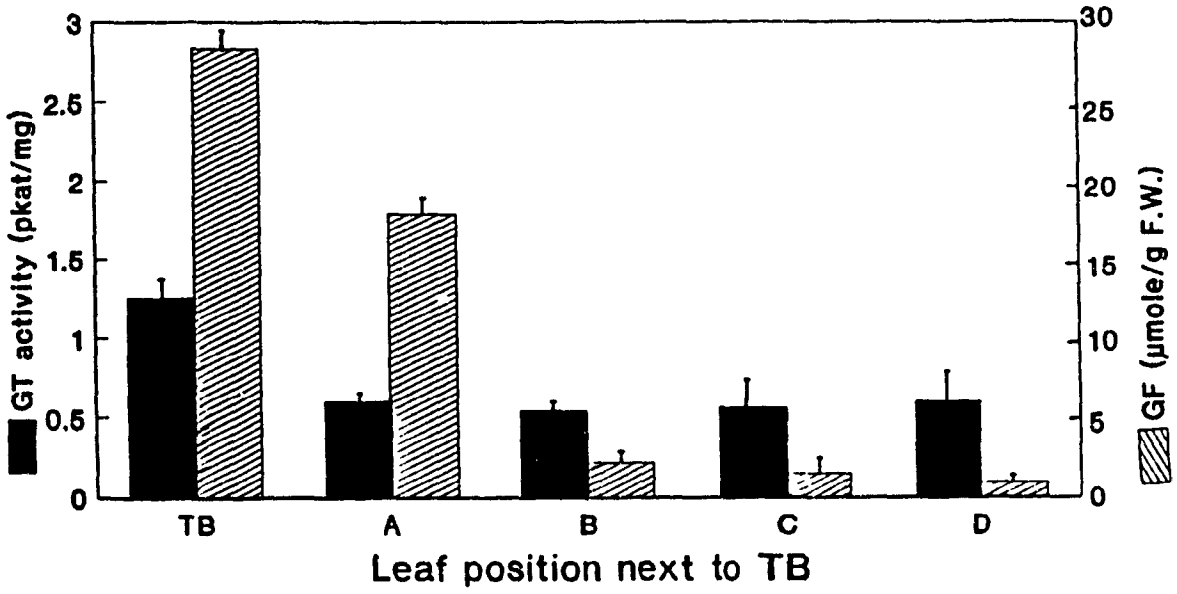
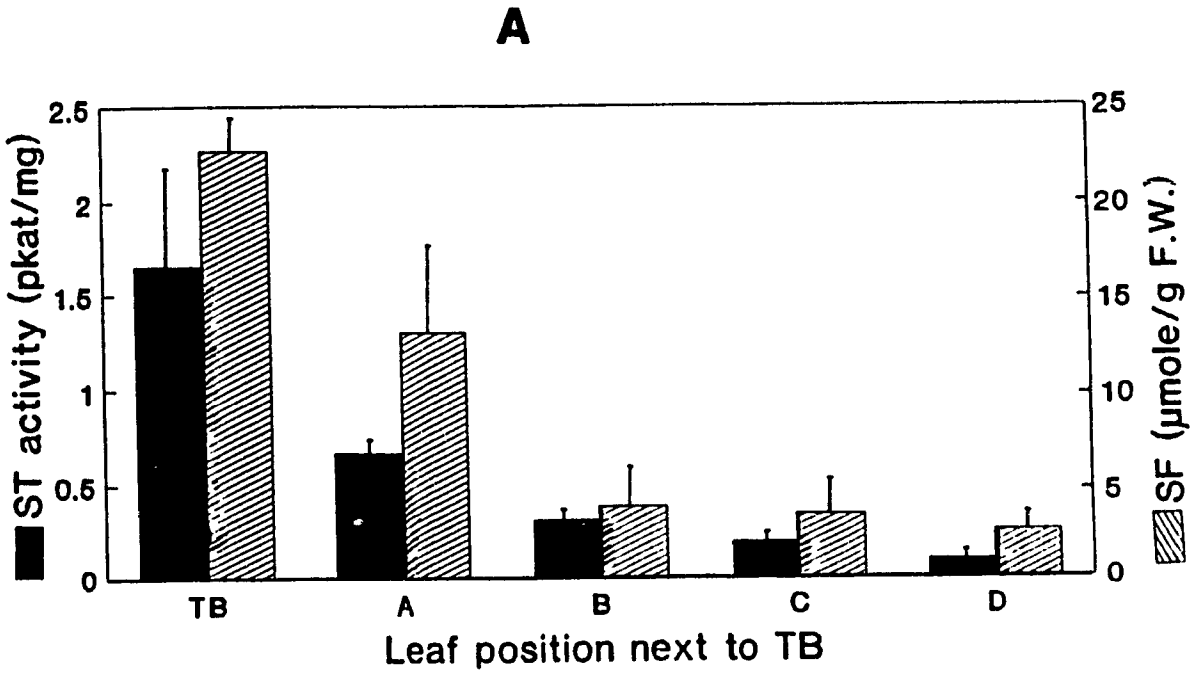

GT activity (pkat/mg)
GF (µmole/g F.W.)
3
2.5
2
1.5
1
0.5
0
30
25
20
15
10
5
0
TB
A
B
C
D
Leaf position next to TB

Figure 5.

Figure 6. (A) Levels of sulfotransferase (ST) activity and sulfated flavonoids (SF) in the terminal bud (TB) and lower leaves (A-D) along the shoot axis. Flavonoid analysis was carried out as described in the legend of Fig. 5, and ST activity was assayed as described in the Methods section. (B) Western blots of ST extracted from terminal bud (TB), different leaves (A-D). The blot was developed with anti-3-ST primary antibody and goat anti-rabbit IgG-alkaline phosphatase conjugated secondary antibody.


A
ST activity (pkat/mg)
SF (µmole/g F.W.)
2.5
2
1.5
1
0.5
0
25
20
15
10
5
0
TB
A
B
C
D
Leaf position next to TB

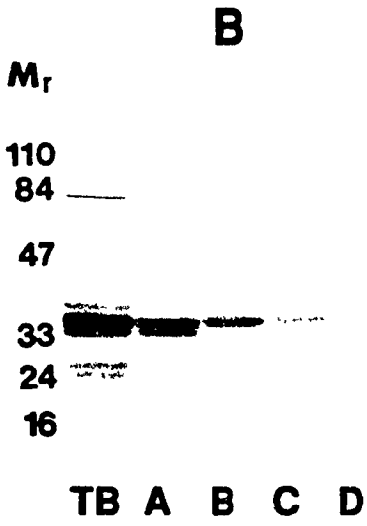

B
Mr
110
84
47
33
24
16
TB A B C D

Figure 6.

Figure 7. Ratios of sulfated (SF) and glucosylated (GF) flavonoids (as percentage of total flavonoids) present in the terminal bud (TB) and leaves (A-D) along the shoot axis of F. bidentis. Flavonoids were quantified as described in the legend of Fig. 5.

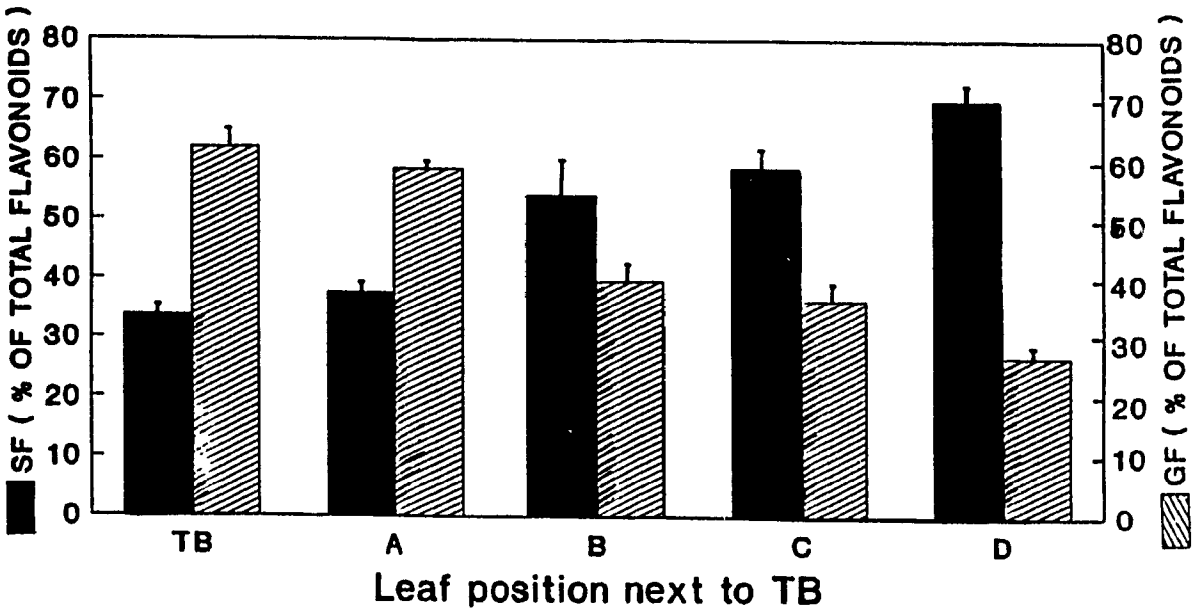

SF (% OF TOTAL FLAVONOIDS)
GF (% OF TOTAL FLAVONOIDS)
0
10
20
30
40
50
60
70
80
TB
A
B
C
D
Leaf position next to TB

Figure 7.

was more pronounced for ST than GT. Western blot analysis of protein extracts using a polyclonal anti-3-ST antibody, showed a positive relationship between the levels of ST activity and the amounts of enzyme protein in the different organs analysed (Fig. 6B). The lack of anti-GT antibodies did not allow for the probing of this enzyme.

E.3.2. Distribution of ST and GT activities within the leaf

The first expanded leaf was divided into cross sections (ca. 0.5 cm wide). Protein was extracted from each section and assayed for both sulfotransferase and glucosyltransferase activities. The highest ST activity was detected in the leaf base section and gradually decreased towards the leaf tip. GT activity, on the other hand, showed no significant variations among the different leaf sections (Fig. 8A). Furthermore, Western blots of the different leaf sections showed a consistently positive correlation between the amount of ST protein and its catalytic activity (Fig. 8B).

E.3.3. Distribution of ST and GT activities along the stem

The deleafed shoot of F. bidentis was divided into internode segments, and the protein was extracted from each segment and assayed for both ST and GT activities. The uppermost internodes exhibited the highest ST and GT activities, which dropped gradually in the lower internodes (Fig. 9A). It is interesting to note that the specific ST and GT activities in stem segments (Fig. 9A) were markedly higher than in the corresponding leaves (Figs. 5, 6A). Furthermore,

Figure 8. (A) Levels of sulfotransferase (ST) and glucosyltransferase (GT) activities in different leaf sections (from apex to base, I-V) and (B) Western blot analysis of ST extracted from these sections (I-V).

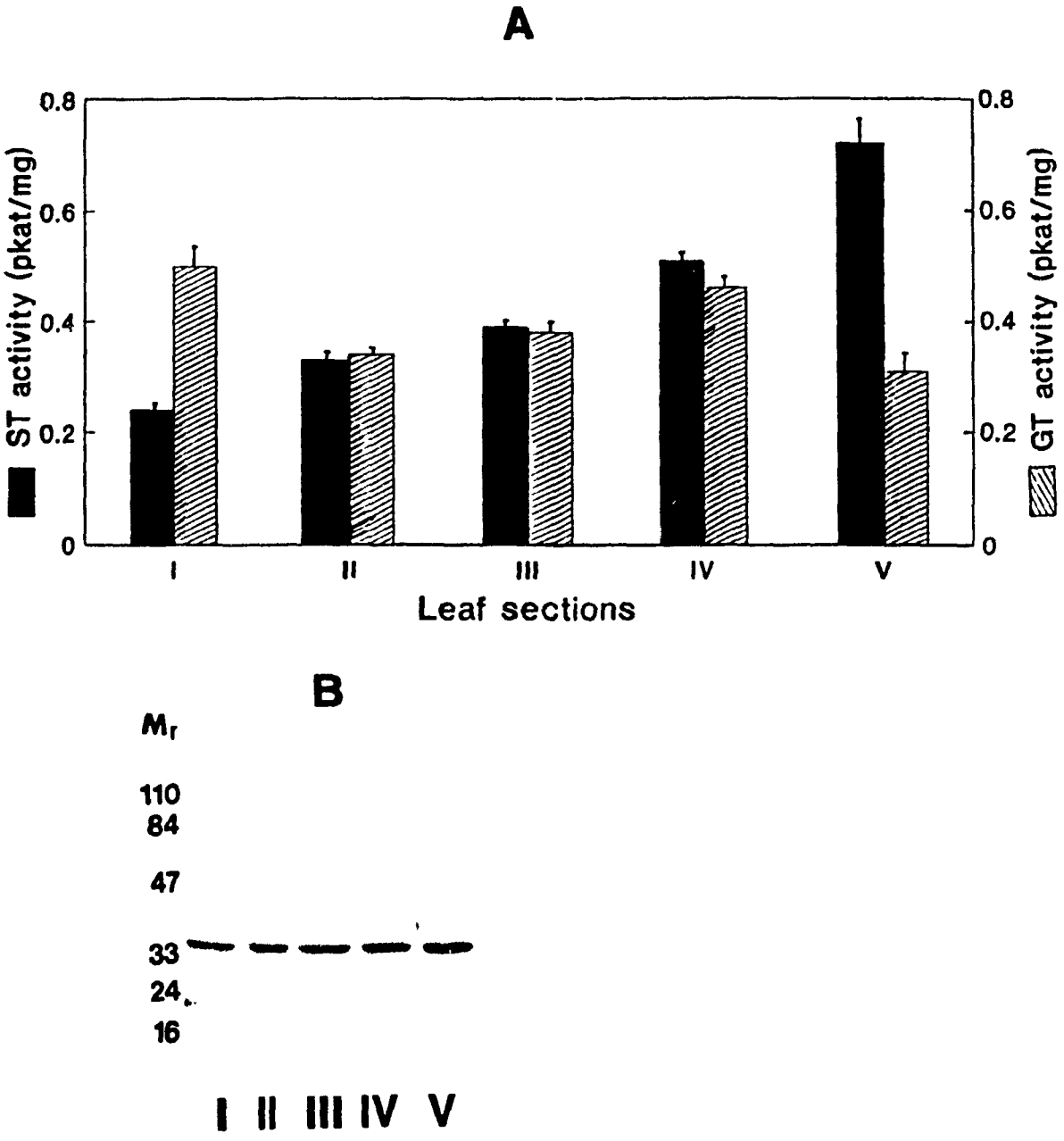
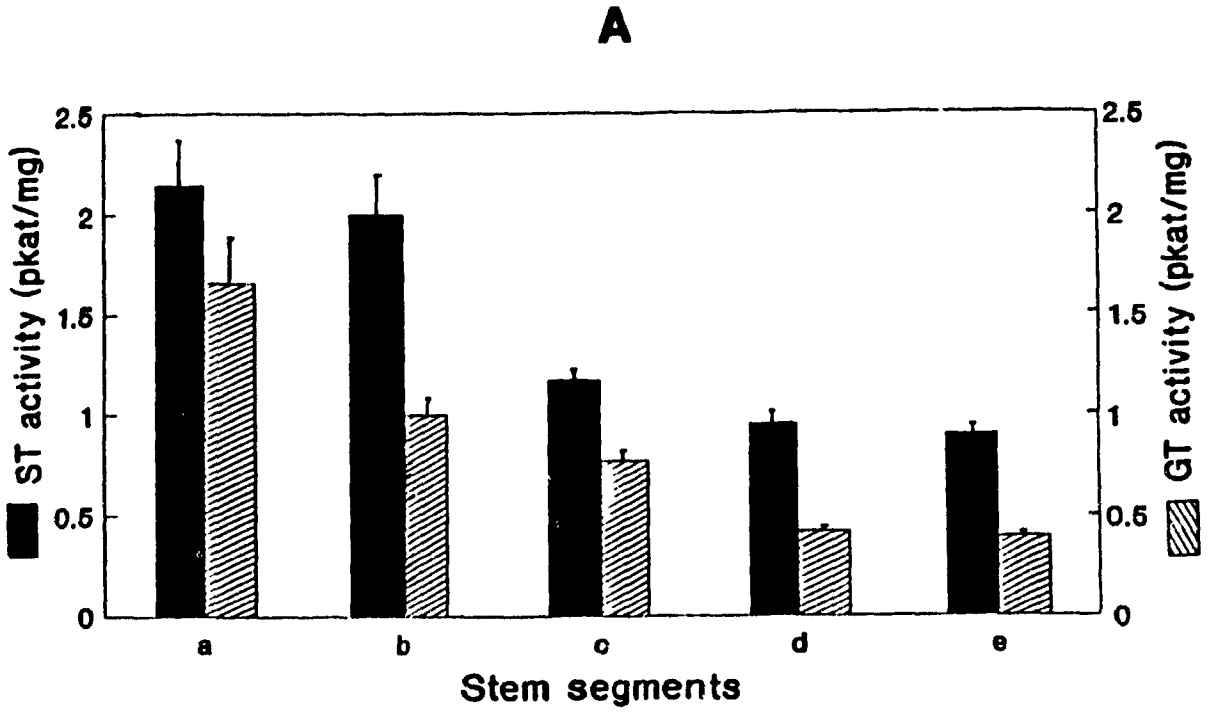

A
0.8
0.6
0.4
0.2
0
ST activity (pkat/mg)
GT activity (pkat/mg)
I
II
III
IV
V
Leaf sections
B
Mr
110
84
47
33
24
16
I II III IV V

Figure 8.

Figure 9. (A) Levels of sulfotransferase (ST) and glucosyltransferase (GT) activities in stem segments (from apex to base, a-e) and (B) Western blot analysis of ST extracted from these segments.


A
2.5
2
1.5
1
0.5
0
ST activity (pkat/mg)
GT activity (pkat/mg)
a
b
c
d
e
Stem segments

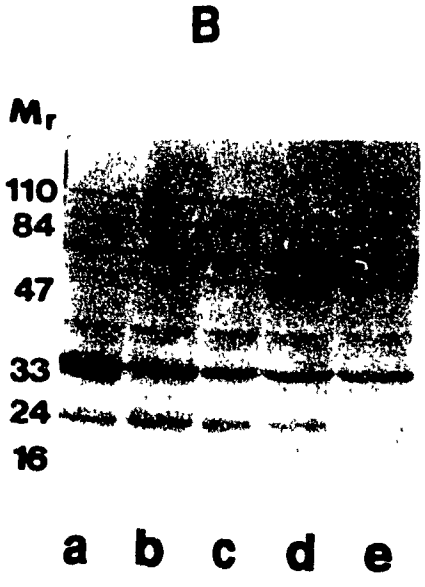

B
Mr
110
84
47
33
24
16
a b c d e

Figure 9.

Western blots of proteins extracted from different stem segments showed that the amounts of ST protein correlate well with their enzyme activities (Fig. 9B). Sulfotransferase activity was detected in mature flowers, but it was absent in the roots.

E.4. Flavonoid Sulfates of Different *Flaveria* spp.

Five *Flaveria* species were analysed for their sulfated flavonoids. These include *F. pringlei* (C_3), *F. linearis* and *F. floridana* (C_3 - C_4), *F. brownii* (C_4-like) and *F. trinervia* (C_4). Shoots of each of these plants were extracted and analysed for their sulfated flavonoid content as described in the Methods section.

Flavonoid sulfates could be detected on TLC plates under UV light (360 nm) as arrow shaped spots. Because of the presence of sulfate groups, they migrate in polar solvents such as H_2O and have low R_f values in nonpolar solvents such as BAW (3:1:1; v/v/v). As described in the Methods section, the extraction procedures resulted in two layers, an organic (EtOAc) layer and an aqueous layer. Both extracts were analysed for their flavonoid sulfate contents. As a general rule, each extract was first analysed by TLC using BAW (3:1:1; v/v/v), and the compounds separated in this step were further subjected to TLC analysis using H_2O as an alternative solvent where highly sulfated compounds exhibit higher R_f values. Total extracts were also subjected to paper electrophoresis as described in the Methods section. This technique is of

particular importance in determining the level of sulfation because the mobility of a given compound increases with the increase in the number of sulfate groups attached to it.

For the identification of the aglycones, flavonoid sulfates were hydrolysed as described in the Methods section. After acid hydrolysis, the flavonoid aglycone and the sulfate were released in the reaction medium. The flavonoid aglycone was then extracted in EtOAc and identified by TLC.

E.4.1. Flaveria pringlei

F. pringlei was found to accumulate a number of flavonoids of different sulfation levels. UV spectra of these compounds were indicative of substituted flavonoid, since acid hydrolysis caused a shift of 10-20 nm in band I (350-370). Identification of aglycones revealed the presence of monosulfates of patuletin and quercetin, as well as quercetin tetrasulfate. The aglycones of compounds 3, 4 and 5 (Table 5) could not be determined either because they were present in very small amounts or they did not migrate with any of the reference compounds. Table 5 summarizes some of the characteristics of flavonoid sulfates of this species.

E.4.1. Flaveria linearis

Analysis of the flavonoid sulfates isolated from F. linearis revealed that this species accumulates the monosulfates of isorhamnetin, patuletin and quercetin. Except for patuletin monosulfate, where a reference compound was not available, the two remaining compounds co-migrated with

Abbreviations Used in Tables (5-9)

[1]UV, NH_3, DPB: colours of compounds visualized under UV light (360 nm), after spraying with ammonia and with 1% diphenylborate, respectively. Dr, dark; Ol, olive; Or, orange; Gr, green; Pr, purple.

[2]Level of sulfation based on R_f values: mono, monosulfated; di, disulfated; tri, trisulfated; tetra, tetrasulfated.

[3]Aglycones identified by TLC after acid hydrolysis: P, patuletin; Q, quercetin; IR, isorhamnetin; R, rhamnetin; K, kaempferol; A, apigenin; N.D., aglycone was not determined; ?, aglycone did not comigrate with any of the reference compounds used.

Table 5. Characteristics of flavonoid sulfates of F. pringlei

compounds	colour[1]			R_f (X100)		UV-spectra λ_{max}(nm)		LS[2]	Ag[3]
	UV	NH_3	DPB	BAW	H_2O	MeOH	MeOH + HCl		
1	Dr	Ol	Or	33	74	348.5, 270	362, 256	mono	P
2	Dr	Ol	Or	33	66	348.5, 268	366, 255	mono	Q
3	Dr	Gr	Or	26	75	357.5, 272	362.5, 256.5	mono	?
4	Dr	Gr	Or	26	63	348.5, 268	366, 254	mono	?
5	Dr	Ol	Yl	05	92	N.D.	N.D.	tri	N.D.
6	Dr	Gr	Pr	02	96	310, 268.5	365.5, 255	tetra	Q

isorhamnetin 3-sulfate and quercetin 3-sulfate, respectively, which further proved their identity. Moreover, this species was found to lack highly sulfated flavonoids. Some of the characteristics of flavonoid sulfates of F. linearis are presented in Table 6.

E.4.3. Flaveria floridana

The pattern of flavonoid sulfates of F. floridana showed a variety of compounds ranging from mono- to trisulfated flavonols, in addition to six other compounds whose aglycones could not be determined. Compounds 3, 5 and 10 co-migrated with isorhamnetin 3-sulfate, quercetin 3-sulfate and quercetin 3,7-disulfate, respectively. After acid hydrolysis, the aglycones were identified except for those compounds which were either present in very small amounts, or whose aglycones did not co-migrate with any of the known aglycones. A summary of some of the characteristics of flavonoid sulfates isolated from F. floridana is presented in Table 7.

E.4.4. Flaveria brownii

This species was found to accumulate mono- to trisulfated flavonoids. Compounds 2, 5 and 8 (Table 8) co-migrated with kaempferol 3-sulfate, quercetin 3,3'-disulfate and quercetin 3,7,4'-trisulfate, respectively. Their aglycones were identified by TLC after acid hydrolysis. Some of the characteristics of flavonoid sulfates isolated from F. brownii are summarized in Table 8.

Table 6. Characteristics of flavonoid sulfates of F. linearis

compounds	colour[1]			R_f (X100)		UV-spectra λ_{max} (nm)		LS[2]	Ag[3]
	UV	NH_3	DPB	BAW	H_2O	MeOH	MeOH + HCl		
1	Dr	Yl	Yl	41	61	347, 254.5	367.5, 254	mono	IR
2	Dr	Pr	Or	34	67	348.5, 257	369, 256.5	mono	P
3	Dr	Pr	Or	34	59	350, 256	372, 299.5	mono	Q

Table 7. Characteristics of flavonoid sulfates of F. floridana

compound	colour[1]			R_f (X100)		UV-spectra λ_{max} (nm)		LS[2]	Ag[3]
	UV	NH_3	DPB	BAW	H_2O	MeOH	MeOH + HCl		
1	Dr	Dr	Dl	50	70	347, 256	365.5, 255	mono	R
2	Dr	Yl	Or	39	70	348.5, 257	366, 255	mono	P
3	Dr	Dr	Yl	39	58	347, 254	366, 253	mono	IR
4	Dr	Yl	Or	30	77	348.5, 268	363, 256	mono	?
5	Dr	Dr	Yl	30	65	348.5, 256	368, 254	mono	Q
6	Dr	Dr	Dl	22	89	331.5, 268	366, 254.5	mono	?
7	Dr	Yl	Or	22	66	357.5, 271	362.5, 256	mono	?
8	Dr	Dr	Dr	22	62	348.5, 257	366.5, 255	mono	?
9	Dr	Yl	Yl	15	89	N.D.	N.D.	di	N.D.
10	Dr	Yl	Yl	15	85	347, 266	364.5, 254	di	Q
11	Dr	Yl	Dr	11	80	N.D.	N.D.	tri	N.D.
12	Dr	Dr	Dl	08	91	320, 268.5	366.5, 25.5	tri	P

Table 8. Characteristics of sulfated flavonoids of F. brownii

compounds	colour[1]			R_f (X100)		UV-spectra λ_{max} (nm)		LS[2]	Ag[3]
	UV	NH_3	DPB	BAW	H_2O	MeOH	MeOH + HCl		
1	Pr	Ol	Yl	58	70	347, 254.5	364, 254.5	mono	?
2	Dr	Yl	Or	47	68	348.5, 256	365, 255.5	mono	K
3	Dr	Ol	Or	34	62	348.5, 256	368.5, 255.5	mono	P
4	Dr	Ol	Yl	26	75	N.D.	N.D.	mono	N.D.
5	Dr	Ol	Ol	18	86	324.5, 266	362, 254	di	Q
6	Dr	Yl	Or	14	85	N.D.	N.D.	di	N.D.
7	Dr	Dr	Yl	09	87	N.D.	N.D.	tri	N.D
8	Dr	Dr	Or	06	88	320, 268	363.5, 252.5	tri	Q

E.4.5. *Flaveria trinervia*

This species was found to accumulate seven flavonoid compounds ranging from mono- to tetrasulfates. These include apigenin, kaempferol and isorhamnetin monosulfates; kaempferol and apigenin disulfates, as well as kaempferol trisulfate and isorhamnetin tetrasulfate. Some characteristics of flavonoid sulfates of *F. trinervia* are summarized in Table 9. It is interesting to note that this is the only species among those investigated that accumulates a large variety of sulfated compounds, including flavone sulfates (apigenin mono- and disulfates).

E.5. Sulfotransferase Activity in Different *Flaveria spp.*

In addition to the identification of flavonoid sulfates of different *Flaveria spp.*, the ST activity was demonstrated in each of them. ST was assayed with three substrates: quercetin, quercetin 3-sulfate and quercetin 3,3'-disulfate. Except for *F. linearis* whose protein extract showed activity with quercetin only, other species exhibited ST activity with the three substrates. To further demonstrate the presence of ST protein in these plants, protein extracts were subjected to Western blot analysis using anti-3-ST antibodies. A band migrating at the level of ST (M_r 35) was observed with all species analysed (Figure 10).

E.6. Effect of Light on Sulfotransferase Activity

The flavonol sulfotransferase would be expected to be regulated by light similar to other enzymes of the pathway. It

Table 9. Characteristics of sulfated flavonoids of F. trinervia

compounds	colour[1]			R_f (X100)		UV-spectra λ_{max} (nm)		LS[2]	Ag[3]
	UV	NH_3	DPB	BAW	H_2O	MeOH	MeOH + HCl		
1	Dr	Yl	Yl	58	63	348.5, 255	367.5, 254	mono	A
2	Dr	Dr	Pr	48	73	348.5, 256	367, 255	mono	K
3	Dr	Pr	Or	38	65	347, 255	365.5, 254.5	mono	IR
4	Dr	Dr	Pr	16	74	347, 269.5	368.5, 256.5	di	K
5	Dr	Yl	Or	16	62	353.5, 256	369.5, 255	di	A
6	Yl	Yl	Yl	13	85	346, 268	364.5, 253	tri	K
7	Dr	Yl	Or	03	92	346, 268.5	365.5, 253	tetra	IR

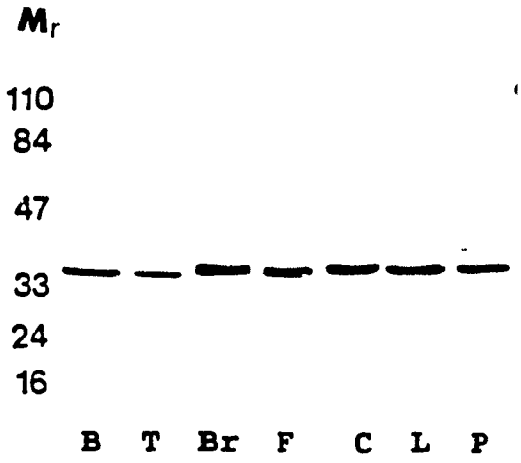


Figure 10. Western blot analysis of protein extracted from different Flaveria spp.

B, F. bidentis
T, F. trinervia
Br, F. brownii
F, F. floridana
C, F. chloraefolia
L, F. linearis
P, F. pringlei

was considered of interest, therefore, to test the effect of light on ST activity. This experiment was performed with young seedlings and cell suspension cultures of F. bidentis. Unlike intact plants, cell suspension cultures of this species were found to accumulate only trace amounts of quercetin 3-sulfate and exhibited very low ST activity.

E.6.1. Changes in ST activity during the growth cycle of cell suspension cultures

Cell suspension cultures of F. bidentis were grown in the dark or in white light, and samples were harvested daily for a period of 10 days. Figure 11 shows the changes in the total amount of protein in dark- and in light-grown cultures during a 10-day growth period. Protein accumulation reached its highest level two days after subculturing either in the dark or in the light. Figure 12 represents the changes in the level of ST activity during the same period of time. ST activity in light-grown cultures reached its maximal level two days after subculture, then decreased gradually to its lowest level by day 10. In the dark-grown cultures, on the other hand, ST activity reached its maximum on the first day after subculture, then slowly decreased to the lowest level by day 9. It is interesting to note that ST activity did not show significant differences under the two conditions, except that it was slightly lower in the dark and reached the peak one day earlier then in the light. The changes in ST activity were in close correspondence to the changes in the total amount of

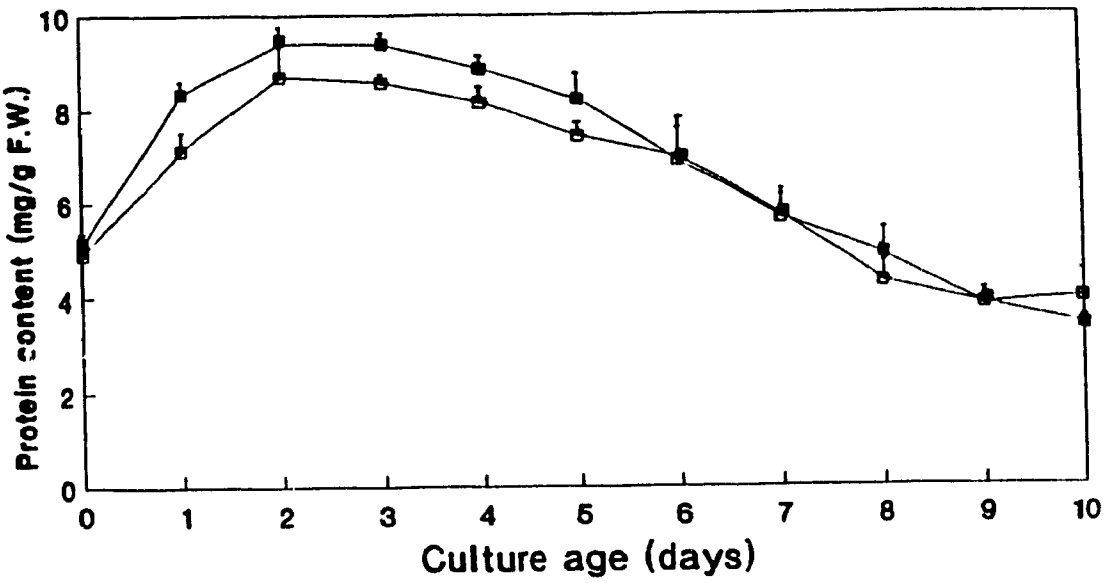


Figure 11. Changes in the total amount of protein in cell suspension cultures of F. bidentis grown either in the light (—■—), or in the dark (—□—) during a 10-day growth cycle.

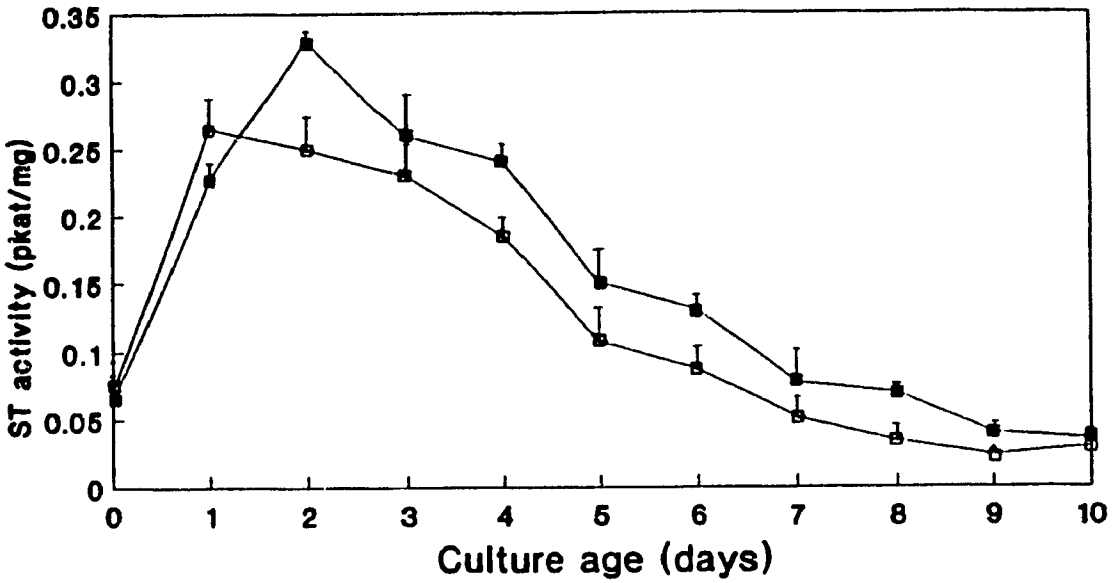


Figure 12. Changes in ST activity in cell suspension cultures of F. bidentis grown either in the light (—■—) or in the dark (—⊟—) during a 10-day growth cycle.

protein under similar conditions. Attempts were made to conduct immunoblotting analysis on ST extracted from different samples, but blots obtained showed very high background and the band corresponding to ST could hardly be seen. However, the finding that the increase in ST activity corresponds to the total amount of protein (Figs. 11, 12) seems to suggest that this increase is the result of de novo protein synthesis.

E.6.2. The effect of UV light

E.6.2.1. Intact plants

One month-old seedlings of F. bidentis (Fig. 1) grown under greenhouse conditions were used and exposed to the light regime described in section D.13.1. Figure 13 shows the effect of UV light on ST activity. Whereas the same level of ST activity was maintained during the experimental period in the control samples, ST activity showed about a three-fold increase after one h of UV irradiation, reaching maximal activity after two h and then decreased to reach the level of the control after eight h.

E.6.2.2. Cell suspension cultures

Cell suspension cultures were prepared as described in Section D.13.2. Eight-day-old cultures, with low ST levels, were used in this investigation. After one h of UV irradiation, under continuous shaking, the cells were transferred to the dark. Whereas ST activity in the dark-grown cultures remained almost constant during the period of the experiment, the level of ST activity increased by about one

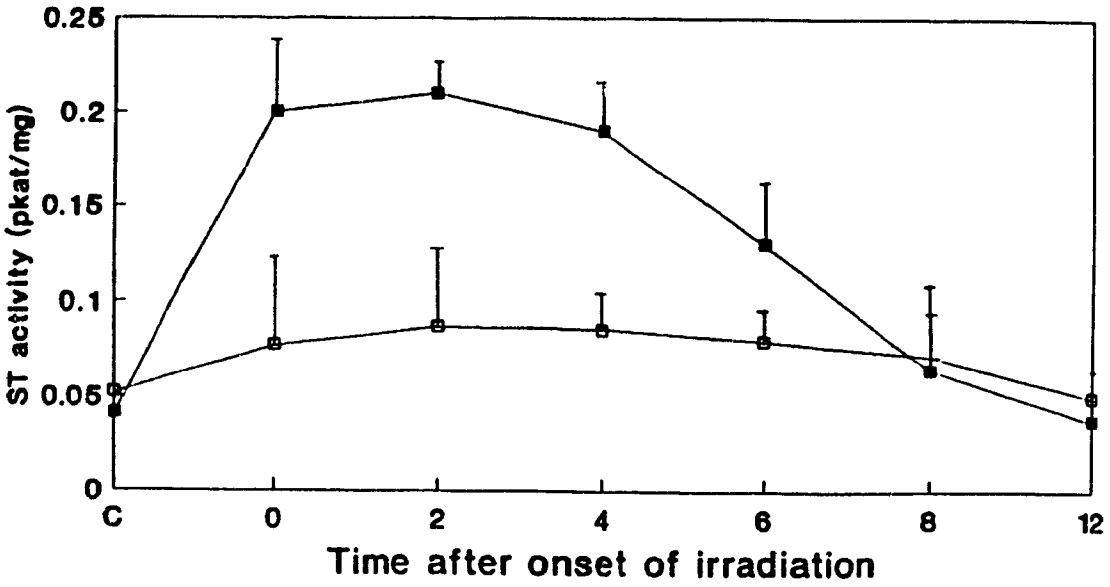


Figure 13. Effect of UV irradiation on ST activity. Changes in ST activity in UV irradiated seedlings (—■—), and in dark control seedlings (—□—). (C - 0) represents one hour of UV irradiation, and time (0) is the beginning of the dark period after UV. Values are the average of three determinations. Experimental procedures were as described in the Methods section.

fold after one h of UV irradiation, then gradually decreased to reach the level of the controls by hour 8. To find out if the increase in ST activity caused by UV light was due to the activation of preformed, inactive protein or to newly synthesized protein (de novo synthesis), another set of cultured cells was treated with actinomycin-D one h prior to UV irradiation. No increase in ST activity was observed in these cells (Fig. 14), indicating that actinomycin-D had prevented ST activation by UV light (Fig. 14).

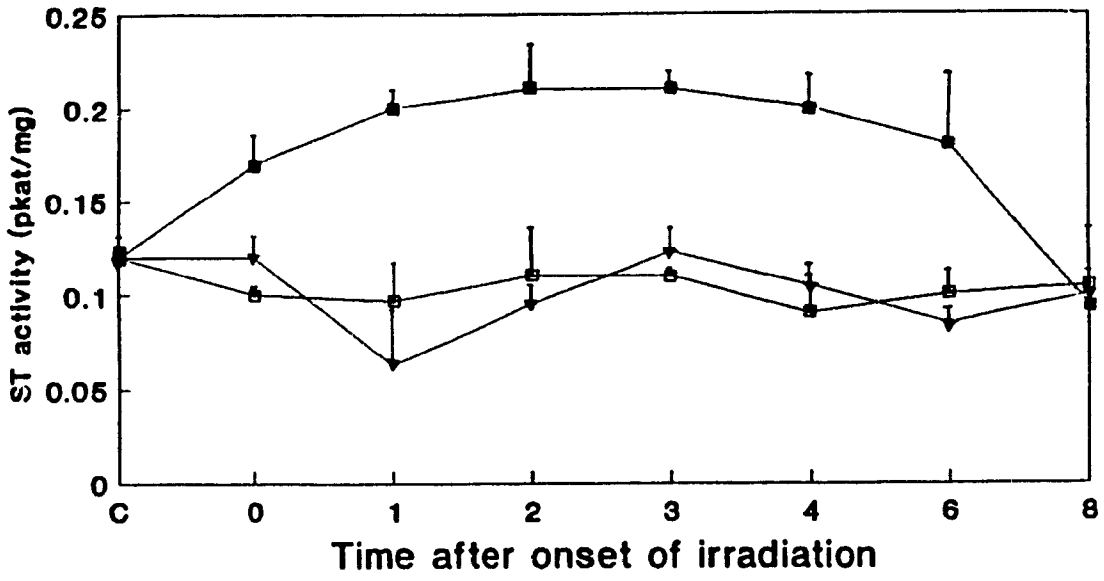


Figure 14. Effect of UV light on ST activity in cell suspension cultures of F. bidentis. Changes in ST activity in cells irradiated with UV light (—■—), in cells irradiated and treated with actinomycin-D (25 μM) (—▼—), and in cells incubated in the dark (—□—). Other descriptions of the legend are as in the legend of Figure 13.

F. DISCUSSION

Despite the increasing number of reports on the phytochemistry, biosynthesis and enzymology of flavonoid sulfates, little is known of the site(s) of their accumulation, the regulation of their biosynthesis or their physiological significance. Furthermore, several classes of flavonoids have been shown to serve as chemotaxonomic markers (Giannasi 1988; Williams and Harborne 1988). In addition, the fact that various species of Flaveria that accumulate flavonoid sulfates belong to different photosynthetic patterns, made it of interest to investigate any relationship between these two characteristics. Therefore, the experiments described in this dissertation were designed to shed some light on some of these aspects.

Flaveria bidentis was chosen for this investigation because of its well characterized flavonoid pattern (Varin et al. 1986; Barron et al. 1988). The latter consists of a number of sulfated and glucosylated flavonols (Table 10). This characteristic feature allowed us to study the relationship between both types of conjugates and the enzymatic steps involved in the later steps of their biosynthesis, namely sulfation and glucosylation.

F.1. Spatial Distribution of The Flavonoid Conjugates and The Later Enzymes Involved in Their Biosynthesis.

The quantitative analysis of flavonol glucosides and sulfates in leaves along the shoot axis revealed some

Table 10. The flavonoid pattern of *Flaveria bidentis*

Flavonoid sulfates	Flavonoid glucosides
Quercetin 3-sulfate	Kaempferol 3-glucoside
Isorhamnetin 3-sulfate	6-Methoxykaempferol 3-glucoside
Quercetin 3,4'-disulfate	Patuletin 3-glucoside
Quercetin 3,7-disulfate	
Isorhamnetin 3,7-disulfate	
Quercetin 3,7,3'-trisulfate	
Quercetin 3,7,4'-trisulfate	
Quercetin 3-acetyl-7,3',4'-trisulfate	
Quercetin 3,7,3',4'-tetrasulfate	

interesting features. The ratios of these conjugates, as percentages of total flavonoids, were found to be differentially distributed (Fig. 7). Young leaves and the terminal bud seem to favour flavonol glucosylation over sulfation. This is in contrast to the older leaves, which suggests that both sulfation and glucosylation of flavonoids may be spatially regulated in F. bidentis. Furthermore, the fact that sulfation and glucosylation involve different types of aglycones (quercetin and isorhamnetin for sulfation and kaempferol, 6-methoxykaempferol and patuletin for glucosylation) seems to support this view.

The presence of high amounts of flavonol glucosides and sulfate esters in the youngest tissues (Figs. 5, 6A) raises the question as to their possible involvement in plant growth and development. It has recently been reported that flavonoid aglycones, and not their conjugates, inhibit the polar transport of auxins by binding to the receptor of the herbicide naphthylphthalamic acid (NPA) and, consequently, block the efflux of auxins (Jacobs and Rubery 1988). It is conceivable, therefore, that flavonoid conjugation, by either glucosylation and/or sulfation, may prevent the binding of free aglycones to the NPA receptor , thus facilitating auxin transport. The predominance of flavonol sulfates in seedlings as compared to mature plants of F. bidentis, as well as their high levels in the terminal bud and the apical stem segment (Varin et al. 1986) tend to support this view. In addition, several reports have indicated that plant secondary

metabolites in general, and flavonoids in particular, are synthesized mainly in young tissues (McClure 1975 and references therein).

The distribution of ST and GT activities was found to correspond with the amount of their respective flavonoid conjugates (Figs. 5, 6). However, the variability in the amounts of flavonoid conjugates may also be due to a dilution factor since the cells expand by taking up water. The instability of these conjugates did not allow for the determination of their amounts on a dry weight basis. Western blot analysis of protein extracts (Fig. 6B) clearly indicates that variations in the levels of ST activity were actually due to differences in the amount of the enzyme protein rather than to changes in its specific activity. However, the absence of an anti-GT antibody precluded the confirmation of this finding in the case of the glucosylation reaction.

The distribution of ST and GT in stem segments (Fig. 9) was found to parallel that in the corresponding leaves. However, both ST and GT activities were higher in stem segments than in the corresponding leaves. This is in agreement with an earlier report (Varin et al. 1986) where flavonol sulfates and flavonol glucosides were found to be more abundant in the stems than in the leaves. The leaf base is known to be a more actively dividing tissue than the leaf tip, since leaf growth continues by the intercalary meristem which originates in the leaf base (Esau 1965). Therefore, ST seems to be associated with the undifferentiated, actively

dividing tissues of the shoot, terminal bud, stem apex and leaf base. In spite of the fact that both the ST and GT are terminal enzymes in flavonoid biosynthesis (Heller and Forkmann 1988), their activities did not follow the same pattern within the leaf. Whereas ST activity was highest in the leaf base and dropped gradually towards the leaf tip, GT activity was distributed equally along the leaf. This finding deserves further experimentation for a better understanding of their role in plant growth.

It is interesting to note that the root system of Flaveria is conspicuous by the absence of both sulfated flavonols or sulfotransferase activity, even though it is considered to be the entry point for sulfate ions into the plant. However, this finding may not be unexpected since both the enzymes involved in the last two steps of PAPS biosynthesis, ATP sulfurylase and APS kinase, have been reported to be chloroplastic enzymes (Anderson 1980).

Therefore, flavonol glucosides and sulfates and their respective enzymes are differentially distributed in F. bidentis. Their distribution seems to suggest that these metabolites have some role to play in plant growth and development. An important aspect that has to be considered in order to correlate with our conclusions is their translocation within the plant. It is still not known whether flavonoid conjugates are synthesized in situ or translocated within the plant.

F.2. Flavonoid Sulfates of *Flaveria* species

The genus *Flaveria* is remarkable in that it includes species belonging to a broad spectrum of photosynthetic traits (Edwards and Ku 1987). These include C_3 and C_4 plants in addition to some C_3-C_4 intermediates. Our work on a number of *Flaveria spp.* of different photosynthetic behaviours indicated that all these species accumulate flavonoid sulfates.

Two *Flaveria spp.* that were thoroughly investigated and their flavonoid patterns were well characterized were *F. chloraefolia* (Barron 1987) and *F. bidentis* (Varin *et al.* 1986). The latter species is a typical C_4 plant, and it was found to accumulate mono- to tetrasulfated flavonols of quercetin and isorhamnetin. *F. chloraefolia*, on the other hand, is a C_3-C_4 species, which was shown to accumulate mono- and disulfate esters of a number of flavonols. These findings led us to speculate that the sulfated flavonoid pattern may be, in some way, related to the photosynthetic behaviour of the plant, particularly with respect to the level of sulfation of these compounds. Therefore, five *Flaveria* species in addition to *F. bidentis* and *F. chloraefolia*, belonging to different photosynthetic traits were analysed for their sulfated flavonoids. As expected, *F. trinervia*, a C_4 species, was found to accumulate mono- to tetrasulfated flavonoids. *F. brownii*, a C_4-like species, and *F. floridana*, a C_3-C_4 species, were found to accumulate mono-, di- and trisulfated flavonoids. However, in another C_3-C_4 species, *F. linearis*, only monosulfate esters of isorhamnetin, quercetin and

patuletin were detected. Therefore, a correlation seems to exist between the photosynthetic character of the species tested and the level of sulfation of constituent flavonoids. However, F. pringlei which is a C_3 species was found to accumulate mono- to tetrasulfated flavonoids. This is a clear deviation from the previous findings, since a C_3 plant was expected to lack highly sulfated flavonoids. It is possible that the pattern of sulfation may be determined not only by the photosynthetic character of the species, but also by some anatomical or physiological aspects, in which case F. pringlei does not fit this general rule.

In spite of the very limited survey of flavonoid sulfates in Flaveria spp., some observations may be drawn as to the nature of the aglycones involved in sulfation. A summary of the data (Table 11) seems to indicate that C_3 species and the intermediate (C_3-C_4 and C_4-like) species utilize quercetin as well as its 6-methoxy derivative (patuletin) as the predominant aglycones for their sulfated conjugates. Sulfated flavonoids of C_4 species, on the other hand, consist of a combination of isorhamnetin and either quercetin or kaempferol. Therefore, there seems to be some correlation between the photosynthetic type and both the level of sulfation and the nature of aglycones involved. However, more species have to be investigated in order to demonstrate the role of these compounds as potential taxonomic markers in the genus Flaveria. Furthermore, it is of interest to note that all flavonoid sulfates identified in these Flaveria species

Table 11. Flavonoid sulfates of *Flaveria* spp. in relation to their photosynthetic trait.

PST[1]	*F.* species	LS[2]	K[3]	Q[3]	IR[3]	R[3]	P[3]	A[3]	UA[4]
C_3	*F. pringlei*								
		mono		+			+		+
		di							
		tri							+
		tetra		+					
C_3-C_4	*F. linearis*								
		mono		+	+		+		
		di							
		tri							
		tetra							
	F. chloraefolia[5]								
		mono		+			+		+[6]
		di		+			+		
		tri							
		tetra							
	F. floridana								
		mono		+	+	+	+		+
		di		+					+
		tri					+		+
		tetra							
C_4-like	*F. brownii*								
		mono	+				+		+
		di		+					+
		tri		+					+
		tetra							
C_4	*F. trinervia*								
		mono	+		+			+	
		di	+					+	
		tri	+						
		tetra			+				
	F. bidentis[5]								
		mono		+	+				
		di		+	+				
		tri		+					
		tetra		+					

Footnotes for Table 11.

[1]PST, photosynthetic trait

[2]LS, level of sulfation

[3]Aglycones: K, kaempferol; Q, quercetin; IR, isorhamnetin; R, rhamnetin; P, patuletin; A, apigenin.

[4]UA, unidentified aglycones.

[5]adapted from Barron et al. 1988.

[6]in addition to those mentioned, this species accumulates monosulfate esters of 6-methoxykaempferol, eupatin, eupalitin, eupatolitin and ombuin.

were sulfate esters of flavonols only, except for the apigenin (flavone) sulfates which were isolated from F. trinervia.

The finding that most Flaveria spp. accumulate polysulfated flavonols and that the enzymatic sulfation has recently been shown to proceed in a sequential order (Varin 1990) indicate that these species must have several flavonol-specific, position-oriented STs. It was found in this study that the substrate specificity of ST in these species corresponds with the pattern of their sulfated flavonoids. Cell-free extracts of F. linearis catalysed the sulfation of the aglycone only, which is not surprising since this plant accumulates flavonol monosulfate esters only. Protein extracts of the four other species catalysed the sulfation of quercetin, quercetin 3-sulfate and quercetin 3,3'-disulfate. This is in agreement with the patterns of their sulfated flavonoids. Similarly, ST activities in F. chloraefolia and F. bidentis have also been shown to reflect their flavonoid sulfate patterns (Varin 1990).

F.3. Effect of Light on Sulfotransferase Activity

Enzymes involved in flavonoid biosynthesis are classified into two groups. Group I includes enzymes of the general phenylpropanoid pathway, and Group II which represents enzymes of the flavonoid pathway (Section B.1.1.). A criterion used to distinguish between the two groups of enzymes is the effect of transferring the cell culture to fresh medium or water in the absence of light. Only the enzymes of Group I are induced by

this process (Schröder et al. 1979 and references therein). Sulfotransferase, an enzyme involved in the later step of flavonoid biosynthesis, should be classified as a Group II enzyme. However, its behaviour upon dilution of the cultures was similar to those enzymes of Group I. Sulfotransferase was induced after transfer of the cell suspension culture to fresh media in the dark as well as in the light (Fig. 12). This is the first instance in which a Group II enzyme is reported to behave as a Group I enzyme.

The positive relationship between the changes in ST activity and the total amount of protein (Figs. 11, 12) seems to indicate that the increase in ST activity was due to de novo synthesis of the enzyme protein, rather than the activation of preformed enzyme. This increase in ST activity was not affected by white light.

UV-B has been reported to have harmful effects on plant growth and flowering as well as their composition and yield (Teramura 1983). Furthermore, such plants respond to this stress by overproducing UV-absorbing flavonoids. Our results showed that sulfotransferase activity is increased by at least one fold after UV irradiation (Figs. 13, 14), which would indicate an increase in the biosynthesis of flavonoid sulfates. Flavonoid sulfates very efficiently absorb light at the UV range. Therefore, it is conceivable that flavonoid sulfates are involved in the protection of plants against UV radiation. If this is true, it would shed some light on the tissue localization of these compounds, since the most

suitable sites for the accumulation of UV-screening compounds are the epidermal and subepidermal tissues. Our laboratory is presently engaged in immunological studies aimed at the localization of these compounds and their enzymes at the tissue and subcellular levels.

The increase in ST activity after UV irradiation was prevented by treating the cells with actinomycin-D (Fig. 14). This antibiotic is known to inhibit DNA transcription by blocking the formation of RNA by DNA-directed RNA polymerase. Thus, UV-B seems to act on sulfotransferase at the level of gene transcription. This is in agreement with earlier reports where some enzymes of the flavonoid pathway were reported to be regulated by UV light at the gene level (for review, see Beggs et al. 1986).

UV light had similar effects on ST activity in both cell cultures and young seedlings of F. bidentis (Figs. 13, 14), but its effect was more pronounced in the seedlings. However, cell cultures are more suitable to conduct these studies since they offer a more homogeneous system with low constitutive levels of ST activity. Nevertheless, totipotent cells of similar age depending on nutrients present in the medium can not be compared to intact plants where highly differentiated cells depend on intracellular communications and active transport of nutrients.

We believe that the induction of ST by UV light in cell cultures could be improved (>1-fold) by altering some conditions of the experiment, such as the growth medium, age

of the culture and the irradiation period.

The experiments described in this dissertation revealed some aspects of the biosynthesis and accumulation of flavonoid sulfates. The consistent pattern of their accumulation and the regulation of their biosynthesis indicate that such compounds must have a more active role in plant growth and development, or possible adaptation to the environment. These findings, as well as the recent discovery of turgorin, 4-O-(6-O-sulfo-ß-D-glucopyranosyl)gallic acid, as regulator of leaf movement in Mimosa pudica (Schildknecht et al. 1990) and a sulfated ß-1,4-tetrasaccharide of D-glucosamine acylated with a C_{16} unsaturated fatty acid as a plant-specific symbiotic signal (Lerouge et al. 1990) represent examples of sulfate esters with important significance to plants. We are convinced that these recent discoveries will stimulate more interest in the study of sulfated metabolites of plants.

G. PERSPECTIVES FOR FUTURE WORK

The work described in this dissertation has indicated the potential significance of flavonoid sulfate esters in plants. However, more experiments will be required in order to be able to assign them specific roles. Such experiments would include:

1. The effect of flavonoid sulfates on the transport of growth hormones, especially the polar transport of auxin.
2. Determination of whether flavonoid sulfates are translocated or synthesized *in situ*.
3. Sulfotransferase was shown to be induced by UV-B light, therefore, it would be interesting to investigate the effect of UV-B on the preceding enzymes of the pathway, starting with chalcone synthase.
4. The localization of these metabolites and the enzymes involved in their biosynthesis at the tissue and organ levels will be very useful in elucidating their function as UV-screening compounds.

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